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The Low Prevalence of Y Chromosomal Microdeletions is Observed in the Oligozoospermic Men in the Area of Mato Grosso State and Amazonian Region of Brazilian Patients

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ABSTRACT

OBJECTIVE: To determine the prevalence of chromosomal abnormalities and microdeletions on Y chromosome in infertile patients with oligozoospermia or azoospermia in Mato Grosso state, Brazil.

METHODS: This cross-sectional study enrolled 94 men from infertile couples. Karyotype analysis was performed by lymphocyte culture technique. DNA from each sample was extracted using non-enzymatic method. Microdeletions were investigated by polymerase chain reaction (PCR).

RESULTS: With the use of cytogenetic analysis, five patients (5.3%) had abnormal karyotype, one azoospermic patient (1.1%) had karyotype 46,XY,t(7;1)(qter-p35), one (1.1%) with mild oligozoospermia had karyotype 46,XY,delY(q), and two other azoospermic patients had karyotype 47,XXY, consistent with Klinefelter syndrome (KS). One of them (1.1%) with severe oligozoospermia had karyotype 46,XY,8p+. Microdeletion on Y chromosome was found in the azoospermia factor c (AZFc) region in only one azoospermic patient (1.1%).

CONCLUSIONS: The prevalence of genetic abnormalities in oligo/azoospermic Brazilian men from infertile couple was 5.3%, and microdeletion on Y chromosome was not a common finding in this population (1.1%).

KEYWORDS: infertile male, cytogenetic, Y chromosome, microdeletion

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Introduction

Researchers have demonstrated great interest in estimating correctly a man's fertility potential. The great emphasis on male reproductive health is because of the fact that in 30–50% of subfertile couples a male factor may be involved.[1–3] Severe oligozoospermia (below 5 million spermatozoa/mL) and azoospermia have been reported in 10–15% of the infertile male.[2,4] Infections, traumatisms, cryptorchidism, varicocele, tobacco and/or alcohol use, and genetic factors are all implicated in male infertility.[2,5–7] In addition, obesity, loss or excessive weight gain, eating disorders, malnutrition, or excessive exercise may also be associated with male infertility.[3] The possible presence of a genetic factor must be considered in all couples with infertility, especially in those that will be submitted to complex assisted reproduction techniques due to a male factor.[8–11] Concerning the genetic factors, the most common

abnormal conditions related to male infertility include chromosomal abnormalities, gene mutations, and microdeletions on Y chromosome. Currently, these conditions account for about 15% of all cases of male infertility.[9,11,12]

Chromosomal abnormalities, either numerical or structural, as the major cause of genetic male infertility, may involve one or more autosomal chromosome, sex chromosome, or both. Most of the anomalies are new events, secondary to mutations in parental germ cells; less frequently these etiologies are inherited with a Mendelian transmission. Chromosomal abnormality in infertile men affects about 6–8% of those with non-obstructive azoospermia, is higher than that seen in the general population, and seems to vary inversely with the sperm count.[8,9,12] Monogenic diseases, such as cystic fibrosis, can also be related with male infertility.[13,14] A genetic azoospermia factor (AZF), located at Yq11, is a established and important factor for male germ cell development.[15–18] Microdeletions of the AZF genes are caused by intrachromosomal recombinations among large homologous repetitive sequence blocks, and three loci (AZFa, AZFb, and AZFc) have been identified in this Yq11 cluster. Microdeletions on Yq represent the second most frequent molecular genetic cause of male infertility, translated by severe oligozoospermia or non-obstructive azoospermia.[15,16,19] The deleted in azoospermia (DAZ) gene, located in the AZFc region, was reported to be absent in 10–15% of chromosomally normal men with diagnosis of severe oligozoospermia or non-obstructive azoospermia.[20]

The frequency of microdeletions in AZFc, AZFb, and AZFa regions was reported to be as high as 80%, 1–31% and 0.5–13%, respectively,[21–23] and varies according to the composition of the study population. Prevalence lower than 2% has been found in non-selected patients.[16,20–22] Deletions of the entire AZFa region are more severe and invariably result in sertoli cell-only syndrome (SCOS) and azoospermia.[15,24–26] The diagnosis of a complete deletion of the AZFa region implies the virtual impossibility to retrieve testicular spermatozoa. Microdeletions in AZFb are often associated with azoospermia, whereas AZFc microdeletions are found either in azoospermia or moderate oligozoospermia.[27–29] In azoospermic men with partial or complete deletion of the AZFc region, sperm can be found in the testis in 9–70% of cases.[29,30] However, spermatid or oligozoospermia has been reported to be in association with these delections.[25,27] Complete delections of AZFb and AZFc regions are also characterized by SCOS or azoospermia and, in these cases, no spermatozoa is found using testicular sperm extraction (TESE).[29,31]

Considering that a number of severe infertile men may have chromosome abnormalities or AZF microdeletions on Y chromosome, it is important to establish the prevalence and characteristics of these genetic abnormalities in the clinical setting, in different populations. With the increased use of assisted reproduction techniques, many men, previously considered infertile, can now procreate using these techniques, especially using the intracytoplasmic sperm injection (ICSI). Thus, in addition to the classic semen analysis, patients with severe oligozoospermia or non-obstructive azoospermia should undergo genetic investigation, mainly taking into account that some genetic disorders involve a high risk for transmission to male descendants.[32,33] It is highly recommended that the risk of any genetic disorder transmission is estimated before any treatment is performed in azoospermic or severe oligozoospermic men.[17] Currently, even Y chromosome microdeletions detection is recommended for azoospermic men and man with sperm count less than 5 millions/mL; in most Brazilian clinics of assisted reproduction techniques, this investigation is not routinely performed. The principal aim of this study was to establish the prevalence of chromosomal abnormalities and microdeletions on Y chromosome in men with severe oligozoospermia or azoospermia in this population.

Material and Methods

This cross-sectional study included 94 oligo/azoospermic men seeking clinical assistance in tertiary infertility centers in Mato Grosso State, Brazil, from 2009 to 2013. The frequency of chromosomal microdeletions may vary from 1 to 10%, or even higher, in different populations.[34–37] Basic sperm analysis followed WHO criteria,[38] and only patients with sperm count $<5 \times 10^6$ were included. The sample size of 94 subjects was estimated considering a prevalence of microdeletions on Y chromosome in oligozoospermic and azoospermic men of 7% as found in Brazilian studies on this matter,[9] a confidence interval of 95%, and an inaccuracy of 5%. The equation for sample size determination was $n \times (Z/m)^2\, xp(1 - p)$, where $Z = 1.96$(95% CI), $m = 0.05$ (margin of error), and $p = 0.07$ (previous report on the proportion of Brazilian infertile men with microdeletions).[9,39] All patients participated in a standardized interview to collect the variables of interest: age tobacco or alcohol use, length of marriage, offspring, and a family history of infertility. Information about smoking and alcohol use was obtained during the interview. The number of cigarettes or drinks consumed daily was noted and one drink was 12 g of pure alcohol, 1 beer, 1 glass of table wine, or 3 cl of 40% alcoholic beverage. Users of at least two drinks daily were considered as heavy drinkers. Patients with sperm count higher than 5 millions/mL or with obstructive azoospermia were excluded. The study protocol was approved by the local research ethics committee (CEP/UNIC/2009–no. 2009-9), and all participants gave informed consent for the study before enrollment.

Cytogenetic analysis. Chromosomal analysis was performed using phytohemagglutinin-stimulated peripheral lymphocyte cultures as standard cytogenetic technique.[40] The number of metaphases analyzed followed the criteria recommended by Hook for detecting 8% mosaicism in 40 metaphases, with a confidence interval of 95%.[41] A resolution of 550-band stage was considered satisfactory. The routine analysis was based on G- bands by trypsin using Giemsa GTG-banded staining.

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] Polymerase chain reaction (PCR) was performed to verify the occurrence of microdeletion on Y chromosome, with the aid of a pair of sY14 primers: sex determining region Y protein (SRY) gene (located on the short arm (Yp)) with 472 bp as a control for the testis-determining factor on the short arm of the Y chromosome and for the presence of Y-specific sequences. Three pairs of sequence-tagged site (STS) primers: sY86 (located in the region AZFa) with 320 bp, sY134 (located in the region AZFb) with 301 bp, and sY254 (located in the AZFc sequence) with 400 bp were examined (Invitrogen®, São Paulo, Brazil) (Table 1). The multiplex PCR reaction comprised a total volume of 16 µL, of which 8 µL was the Supermix (Invitrogen®, São Paulo, Brazil), 5 µL of multiplex primer mix and 3 µL of diluted DNA. The cycles of denaturation, annealing, and extension were performed in a thermocycler (GeneAmp PCR System 9600, Perkin Elmer®, MA, USA). The process of amplification was standardized at the local laboratory and consisted of 35 cycles using different programs for each pair of primers (Table 2). A ZFx/ZFy gene was not used for internal PCR control, but in all amplification processes, PCR was monitored using a DNA positive control (normal male individual) and a DNA negative control (normal female individual). PCR products were observed in electrophoresis (1.0% agarose gel stained with ethidium bromide). After processing, the gel was transferred to a transilluminator (Bio-Rad®, CA, USA). Photos were taken under ultraviolet light using Image Capture System L-PIX-STR (Loccus Biotechnology®, São Paulo, Brazil) to register the appearance of bands indicative of microdeletion.

Data with normal distribution are presented as mean (χ) and standard deviation (SD); data with non-Gaussian distribution are shown as median and range. Associations between variables were examined by χ^2 test. All analyses were performed using the SPSS software for Windows (SPSS, version 20, SPSS Inc., Chicago, IL, USA).

Results

The mean age of the patients was 35.3 ± 7.2 years. The medians of the marriage time and number of years of intercourse without any contraceptive method were 8.6 years (range) and 5.7 (1–23) years, respectively. Baseline clinical characteristics of all patients are presented in Table 3. Personal history of mumps infection was present in 49/94 (52%) and genital trauma in 24/94 (25.5%) of patients. Smoking habit was present in 32/94 (34%), alcohol use in 61/94 (64.9%), and other addiction drugs use in 6/94 (6.4%) of patients. Severe oligozoospermia was found in 67% and azoospermia in 33% of the included patients. Cytogenetic analysis was performed in 91 men because in 3 of them cell cytogenetic cultures had poor growth. Abnormal karyotype was found in five patients (5.3%). One patient (1.1%) with severe oligozoospermia had karyotype 46,XY,8p+; another with karyotype 46,XY,t(7;1)(qter-p35) was azoospermic; and the third with karyotype 46,XY,delY(q) had mild oligozoospermia. Other two azoospermic patients had karyotype 47,XXY (2.1%), consistent with Klinefelter syndrome (KS). Molecular analysis was performed in all 94 patients, and microdeletion on Y chromosome was detected in only one azoospermic man (1.1%) (Table 4). This patient had undergone a cytogenetic investigation before the Y-microdeletions screening and was cytogenetically normal. This microdeletion was located at AZFc region and identified using SY254 marker (Fig. 1).

Discussion

Genetic abnormalities involving a complex multigenic disorder can lead to different degrees of spermatogenic failure and account for 15–30% of male infertility.[20,43] In general, patients age and duration of infertility are considered important factors in making decisions regarding how to investigate, treat, and establish a reproductive prognosis. The marriage average time of eight to nine years and exposure time without contraception of five to six years, found in the current study, are in agreement with other studies performed in other countries.[44,45] The percentage of patients (52.1%) reporting mumps infection in childhood in the current study is high. Worldwide, 15–20% of adult men who contract mumps can present orchitis, usually

Table 1. Locus and sequences of the PCR primers used.

PRIMER STS	LOCUS	PRODUCT SIZE (bp)	SEQUENCE (FORWARD/REVERSE)
SY14	SRY	472 bp	F 5′-GAATATTCCCGCTCTCCGGA-3′ R 5′-GCTGGTGCTCCATTCTTGAG-3′
SY86	AZFa	320 bp	F 5′-ACACACAGAGGGACAACCCT-3′ R 5′-GTGACACACAGACTATGCTTC-3′
SY134	AZFb	301 bp	F 5′-GTCTGCCTCACCATAAAACG-3′ R 5′-ACCACTGCCAAAACTTTCAA-3′
SY254	AZFc	400 bp	F 5′-GGGTGTTACCAGAAGGCAAA-3′ R5′-GAACCGTATCTACCAAAGCAGC-3′

Abbreviations: STS, sequence-tagged site; bp, basepair; SRY, sex determining region Y protein; AZF, azoospermia factor.

Table 2. Temperatures' exposure times used in the cycles of gene amplification in PCR.

PRIMERS STS	DENATURATION	ANNEALING	EXTENSION
SRY	94°C–1 min	64°C–1 min	72°C–2 h and 30 min
SY86	72°C–1 min	72°C–10 min	4°C–5 min
SY134	94°C–5 min	94°C–1 min	56°C–30 sec
SY254	72°C–1 min	72°C–10 min	4°C–5 min

Abbreviations: STS, sequence-tagged site; SRY, sex determining region Y protein.

unilateral.[46–48] The prevalence of alcohol consumption, in any amount, in the current study was 64.9%; alcohol consumption in infertile men worldwide is reported as high as 88%.[49] Tobacco is known to affect sperm quality and decrease its ability to fertilize the egg. In addition, tobacco may increase the probability of early spontaneous abortion and reduce the implantation rate.[50–53] One-third of the patients included in the present study reported tobacco use, but no quantification was performed and the variable was considered as binomial. Worldwide, the decline in semen quality has been associated with cigarette smoking.[54–56]

Concerning the causes of male infertility, there is a consensus that alcohol, tobacco, and illicit drugs abuse must be investigated. Once many compounds in tobacco smoke are mutagens, the cigarette smoke may affect male reproduction leading to little reduction in sperm concentration, motility, and morphology.[55–58] At present, smoking is reported in 34%

Table 3. Baseline clinical conditions found in infertile men enrolled in the study.

BASELINE CHARACTERISTICS	FREQUENCY	%
Genital disease		
Criptorquidia	1/94	1.1
STD	1/94	1.1
Prostatitis	2/94	2.1
Varicocele	10/94	10.6
Varicocelectomy	8/94	8.5
Linfoma	2/94	2.1
Other	3/94	3.2
Heat exposure or radiation	18/94	3.2
Mumps in infection	49/94	52.1
Genital trauma	24/94	25.5
Autoimmune disease	3/94	3.2
Family infertility history	17/94	18.1
Genetic disease history	14/94	14.7
Tobacco	32/94	34.0
Addiction drugs use	6/94	6.4
Alcohol use	61/94	64.9

Abbreviation: STD, sexually transmitted disease.

Table 4. Cytogenetic and Y-microdeletion findings in Brazilian oligozoospermic and azoospermic men.

PARAMETER*		FREQUENCY	(%)
Karyotype			
Normal	46,XY	86/91	94.5
Abnormal	46,XY,delY(qter)	1/91	1.1
	46,XY,t(7;1)	1/91	1.1
	47,XXY	2/91	2.2
	46,XY,8p+	1/91	1.1
AZF microdeletion	AZFc	1/94	1.1

Abbreviation: AZF, azoospermia factor.

of infertile men in the current study, and this prevalence is similar to those found in other populations.[59–61] Also currently, sperm mutagenicity capacity of smoking is debatable, and a few investigators have reported on possible relationship between smoking habit and Y chromosome microdeletion; most authors reported no association.[62] A significant association between AZFa microdeletion and smoking, using SY254 microsatellite as marker, was described.[58] The only patient with Y microdeletion using this marker found in the current study was not smoker. Very large population studies are still required to determine whether smoking in fact may induce Y chromosome deletions. The use of at least two drinks of alcoholic beverage a week seen in 65% of patients in the current study is in agreement with the findings of other publications. Worldwide, alcohol consumption in infertile men is as high as 88%.[49,50] Heavy alcohol consumptions, defined as more than 15 drinks a week, reported in 32% of patients in the present study are also found in other populations. The patient with AZFc deletion in the present study used to drink alcohol beverage only at weekend days. Despite quick benefits in the semen analysis can be obtained after alcohol withdrawal,[49] the relationship between alcohol use and Y chromosome deletions remains elusive and further studies are needed.

In an open review including infertile male, chromosome abnormalities were reported on 46% of oligozoospermia and 13.7% of azoospermia men.[8] In the current study, chromosomal abnormalities were found in five patients, and the finding of karyotype 47,XXY in two azoospermic men is consistent with studies performed in other populations.[44,45] Autosomal translocation 46,XY,t(7:1)(p35-qter), found in one azoospermic patient, was already reported in azoospermic or severe oligozoospermic infertile men.[8] The hypothesis for this occurrence is that balanced translocations may interfere with normal chromosome pairing and segregation in meiosis I, leading to the potential formation of unbalanced gametes and subsequent unbalanced abnormal offspring.[19,20] Another possibility is that some autosomal genes involved in male gametogenesis may not be regulated by breakpoint.[45,63] However, the relationship between chromosomal breakpoints

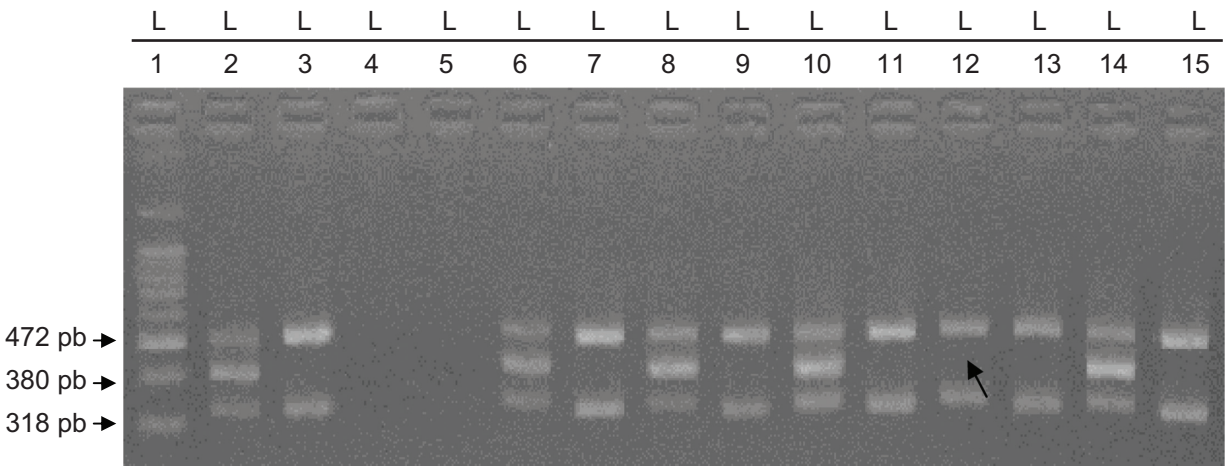

Figure 1. Gel electrophoresis analysis showing the regions AZFa, AZFb, and AZFc of Y chromosome*. **Lane 1:** standard size markers; **Lane 2:** PCR products for normal male positive control plus SRY, AZFa, AZFc products; **Lane 3:** normal male positive control plus SRY, AZFb products; **Lane 4:** normal female negative control plus SRY, AZFa, AZFc products; **Lane 5:** normal female negative control plus SRY, AZFb products; **Lanes 6, 8, 10, 12, and 14:** patients plus SRY, AZFa, AZFc products; and **Lanes 7, 9, 11, 13, and 14:** patients plus SRY, AZFb products.
Notes: *Two multiplex reactions were made for each sample. ↑ The expect band for SY254 was absent in this patient (L12).

and male infertility has been observed with a non-random distribution, and more research is needed in this direction.[44]

The oligozoospermic patient with karyotype 46,XY, delY(q12-qter) seen in the present study had no microdeletion in any of the three regions analyzed, perhaps because the region Yq12-qter, in which had occurred this deletion, is a region of the Y chromosome rich in heterochromatin without any active gene.[64] Although it seems not to be clinically relevant for male infertility, the severe oligozoospermia found in a patient with karyotype 46,XY(8p+) without any other cause identifiable indicates that some genes added to this autosome chromosome may affect spermatogenesis. This finding was not reported yet; as far as we know, only duplication of 8p23.1–p23.3 was already shown to be related to oligoasthenozoospermia.[65]

The finding that cytogenetic changes were more frequent in patients with lower sperm concentration is consistent with other studies.[8,11,16,26] Search for microdeletions on Y chromosome (regions AZFa, AZFb, and AZFc) revealed microdeletions in the AZFc region in only one azoospermic patient with normal karyotype. Globally, the frequency of microdeletions varies between 0.7 and 34.5%, with an average of 8.2%.[10,13,15] It is known that microdeletions at AZFc region are in fact the most frequent (60–80%); less frequently, microdeletions are detected at AZFb (16%) and AZFa (5%) regions. Large microdeletions involving two or three AZF regions have also been reported in 14% of infertile male.[16,24] In about 15% of cases, microdeletions are located at regions not overlapping AZFa, AZFb, or AZFc.[64–67] There is no clear association between the size and location of the deletion, and the histological phenotype of the testicles. However, it is known that the larger the deletion, the more severe is the testicular damage. The difficulty found by many researchers in associating genotype with testicular phenotype is that it is not completely clear and may arise from the selection criteria used for different patients included in different studies.

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.[23,65,67] Moreover, low prevalence of Y microdeletions has been reported in other population.[15,68] Because patients with a Y microdeletion are at least olizoospermic (less than 5 millions spz/mL), it is rare to have enough number of spermatozoa to perform a classic in vitro fertilization (IVF) instead of ICSI. In addition, the knowledge of the existence of microdeletions is useful to provide a correct diagnosis of male infertility. Moreover, it allows the physician to refer the patient to adequate assisted reproduction technique and examine the value of a testicular biopsy pertinence.[20,23] It must be considered that the son of a man with microdeletion may inherit this abnormality.[67] The low prevalence of Y chromosome microdeletion found in the current study could be because of the characteristics of the population or the use of only one primer; despite this procedure is acceptable and may be adopted. The authors recognize that the studied population described here is not representative of the total Brazilian population of infertile men. However, to our knowledge, this is the first study reporting on this subject in Mato Grosso state and Amazonian region.

Author Contributions

Conceived and design the experiments: SFM. Analyzed the data: GCSG, BBG, CA, JSB, MFP, MFG. Wrote the first draft of the manuscript: GCSG, SFM. Contributed to the writing of the manuscript: JSB, BBG. Agree with manuscript results and conclusions: GCSG, BBG, CA, JSB, MFP, MFG,

SFM. Jointly developed the structure and arguments for the paper: GCSG, BBG, SFM. Made critical revisions and approved final version: JSB, SFM. All authors reviewed and approved of the final manuscript.

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