

Prevalence of Y chromosome deletions in a Brazilian population of nonobstructive azoospermic and severely oligozoospermic men

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Abstract

We determined the prevalence of Y chromosome deletions in a population of 60 Brazilian nonobstructive azoospermic and severely oligozoospermic men. PCR-based screening of microdeletions was performed on lymphocyte DNA for the presence of 14 sequence-tagged sites (STS) located in the azoospermic factor (AZF) on the Yq chromosome. All STS were amplified efficiently in samples from 12 fertile men tested, but failed to be amplified in samples from fertile women, indicating the specificity of PCR conditions for Yq screening. Overall, 4 of the 60 infertile patients tested (6.7%) exhibited deletion of the Y chromosome, 2 of them being severely oligozoospermic patients (P10 and P32) and 2 azoospermic men (patients P47 and P57). Patients P47 and P57 presented larger deletions in the AZFa, AZFb and AZFc subregions, with apparent loss of Yq material evidenced by karyotype analysis. Patients P10 and P32 presented deletions confined to the AZFc region, involving the DAZ locus. Male relatives of patients P10 and P32 had no Y chromosome deletions and presented a normal karyotype, suggesting a *de novo* status of the deletions found. Our data add to the growing literature showing that microdeletions of the Y chromosome can be the cause of male idiopathic infertility.

Key words

- Y chromosome
- Microdeletions
- Severe oligozoospermia
- Azoospermia
- Male infertility
- Azoospermia factor
- AZF region

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Introduction

The existence of a gene, or a gene complex, associated with normal spermatogenesis was first postulated on the basis of a cytogenetic analysis that showed macroscopic deletions of the distal part of the Y chromosome (Yq11) in men with azoospermia (1). This gene or gene cluster was defined as azoospermia factor (AZF). Subsequent PCR-

based screening studies used to amplify sequence-tagged sites (STS) spanning the Y chromosome (2,3) facilitated the detection of small interstitial deletions (i.e., microdeletions) of Yq11 not only in azoospermic (4-6), but also in severely oligozoospermic men (7,8). Since these deletions were variable in both extent and location, the AZF region was divided into three nonoverlapping subregions (AZFa, AZFb and AZFc) located from the

proximal to the distal part of Yq (6). Recent genetic studies have shown that several genes or gene families located in AZF regions are specifically expressed in the testis and could therefore be considered as AZF candidate genes for infertility, such as the RBM (RNA-binding motif) and the DAZ (deleted in azoospermia) gene families (for reviews, see Refs. 9-11). Recently, a fourth AZF subregion (AZFd) between AZFb and AZFc has been proposed (12). Men with deletions located exclusively in the AZFd subregion present mild oligozoospermia or even normal sperm counts associated with normal sperm morphology (12), but a candidate gene has not yet been found in this region.

Over the past few years, screening tests for detecting microdeletions on the long arm of the Y chromosome have established the distribution and characteristics of the deletions among different groups of infertile male patients (for reviews, see Refs. 9-11). Data from PCR studies have demonstrated that the prevalence of Y chromosome microdeletions among men with nonobstructive idiopathic azoospermia or severe oligozoospermia ranges from 3 to 55.5% (5-8,12-22), the higher frequencies being detected in cases of severely impaired spermatogenesis (11). A number of factors have been implicated in the wide variation of Y deletion frequencies reported such as patient selection criteria, experimental designs, environmental influences and ethnic variations (22-27). In the present paper, we report the prevalence of Y chromosome deletions in a Brazilian population of nonobstructive azoospermic and severely oligozoospermic men attending our university-based outpatient clinic.

Material and Methods

Patients

DNA samples from 60 patients referred to the Section of Human Reproduction, Gynecology and Urology Division, Universida-

de Federal de São Paulo-Escola Paulista de Medicina (UNIFESP-EPM) and who gave written informed consent to be submitted to genetic testing were screened for Yq chromosome microdeletions. In addition, 12 fertile men and 2 fertile women were tested as positive and negative controls, respectively. The Institutional Ethics Committee at UNIFESP-EPM approved the study design. Clinical evidence of obstructive azoospermia, or a history of mumps, injury and cryptorchidism were not observed in the selected patients. However, varicocele was not excluded because in this situation the cause of infertility is largely unknown and an associated Y chromosome deletion cannot be excluded (11). The testis volume was measured with a Prader orchidometer. Semen samples were obtained on two different occasions, with each collection being performed after a 3-day period of sexual abstinence and separated by a 3-week interval. A complete semen analysis was performed according to World Health Organization guidelines (28). The diagnosis of azoospermia was established by pellet analysis after semen centrifugation (1,000 g, 20 min). Of the 60 patients included in this study (mean age: 32 years; range 24-49 years), 29 were diagnosed with nonobstructive azoospermia and 31 with severe oligozoospermia ($<5 \times 10^6$ spermatozoa/ml). First-degree male relatives of the men diagnosed with Y chromosome deletions were asked to withdraw blood for DNA analysis in order to determine if the deletion was *de novo*.

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 instructions. Normal reference ranges for men are FSH 1-12 mIU/ml, LH 2-12 mIU/ml and testosterone 262-1593 ng/dl.

DNA isolation and Y chromosome analysis by PCR

To screen for microdeletions in the Y chromosome by PCR, genomic DNA was isolated from lymphocyte nuclei according to standard protocols. Each individual was analyzed for the presence of 12 STS on Yq spread over intervals 5 and 6 in the following subregions: AZFa (sY82, sY84, sY86), AZFb (sY117, sY142, sY143), AZFc (sY147, sY149, sY254, sY255, sY283, sY158). The STS sY160 in the heterochromatin region outside AZF regions was also analyzed. The STS sY14, which is localized in Yp, was used as a positive control for the presence of Y-specific DNA. All STS primers, shown in Table 1, have been previously described (2,3,5,7). PCR analysis was performed on 25 µl of reaction volume containing genomic DNA (100 ng), deoxyribonucleotides (0.1 mmol/l each), *Taq* polymerase (1.25 U), and oligonucleotide primers (1 µmol/l each) at a final concentration of 1 x PCR buffer (20 mmol/l Tris-HCl, pH 8.4, 50 mmol/l KCl, 3 mmol/l MgCl₂). Primers sY117, sY143, sY283, and sY160 were amplified in the presence of 6 mmol/l MgCl₂. Amplifications were obtained with an Idaho Rapidcycler (Idaho Technologies, Idaho Falls, ID, USA) under the following conditions: initial 2 min of denaturation at 94°C followed by 35 sequential cycles, each including 30 s at 94°C, 40 s at 58°C, and 20-60 s at 72°C. The PCR products were separated on 2.5% agarose gels by electrophoresis, stained with ethidium bromide, and photographed. During optimization of the PCR conditions, genomic DNA from fertile men and women served as normal and Y-deleted controls, respectively. The authenticity of the PCR products was confirmed by direct nucleotide sequenc-

ing performed with an ABI PRISM™ 377 automated sequencer (Applied Biosystems, Foster City, CA, USA) and the BigDye™ Terminator Sequencing kit (Applied Biosystems).

Cytogenetic evaluation

Chromosome analysis was carried out on peripheral lymphocytes with G-banding staining by the method of Moorhead et al.

Table 1. Sequence-tagged sites (STS) and primer sequences used for Y chromosome microdeletion analysis.

STS	Primer sequence	Size (base pairs)
sY14	Forward 5' GAATATTCCTCCGCTCTCCGGA 3' Reverse 5' GCTGGTCTCCATTCTTGAG 3'	472
sY82	Forward 5' ATCCTGCCCTTCTGAATCTC 3' Reverse 5' CAGTGCTCACTGATGGATGA 3'	264
sY84	Forward 5' AGAAGGGTCTGAAAGCAGGT 3' Reverse 5' GCCTACTACCTGGAGGCTTC 3'	320
sY86	Forward 5' GTGACACACAGACTATGCTTC 3' Reverse 5' ACACACAGAGGGACAACCCCT 3'	326
sY117	Forward 5' GTTGGTTCCATGCTCCATAC 3' Reverse 5' CAGGGAGAGACCTTTTACC 3'	452
sY142	Forward 5' AGCTTCTATTCGAGGGCTTC 3' Reverse 5' CTCTCTGCAATCCCTGACAT 3'	196
sY143	Forward 5' GCAGGATGAGAAGCAGGTAG 3' Reverse 5' CCGTGTGCTGGAGACTAATC 3'	311
sY147	Forward 5' TTTCTCGTTTGATGATCCTAG 3' Reverse 5' TTAATATGAGAATGAGAACAGATGT 3'	100
sY149	Forward 5' TGTCACTGCCCTAATCCT 3' Reverse 5' TGGTCATGACAAAAGACGAA 3'	132
sY254	Forward 5' GGGTGTTACCAGAAGGCAAA 3' Reverse 5' GAACCGTATCTACCAAAGCAGC 3'	350
sY255	Forward 5' GTTACAGGATTCGGCGTGAT 3' Reverse 5' CTCGTCATGTGCAGCCAC 3'	126
sY283	Forward 5' CAGTGATACTCGCACTTGTGTA 3' Reverse 5' GTTATTTGAAAAGCTACACGGG 3'	497
sY158	Forward 5' CTCAGAAGTCTCTAATAGTTCC 3' Reverse 5' ACAGTGGTTGTAGCGGGTA 3'	231
sY160	Forward 5' TACGGGTCTCGAATGGAATA 3' Reverse 5' TCATTGCATTCCTTTCCATT 3'	236

The size of the expected amplified DNA product is indicated for each STS.

(29). Karyotypes were obtained in the Department of Genetics, UNIFESP-EPM. On average, 20-30 metaphases per patient were analyzed.

Results

Deletion of a specific locus on Yq11 was indicated by the failure of amplification in three independent PCR experiments. The repeated experiments were performed on new samples of DNA extracted from a different blood collection. No microdeletions were observed in any of the fertile men tested. Overall, 4 of the 60 infertile patients tested (6.7%) exhibited microdeletions of the Y chromosome, 2 of them being severely oligozoospermic patients (P10 and P32) and 2 azoospermic men (patients P47 and P57). The severely oligozoospermic patients P10 and P32 failed to amplify STS sY254, sY255 and sY283, which amplify parts of the DAZ gene in the AZFc region. Azoospermic patients presented larger deletions. Deletions

of STS located in the AZFa (sY84, sY86) and AZFc regions (sY147, sY254, sY283, sY158) were found in patient P47, while deletions in the AZFb (sY142) and AZFc regions (sY254, sY255, sY283, sY158) were found in patient P57. Patients P47 and P57 did not amplify sY160, an STS localized in the Y heterochromatin outside the AZF regions. Patients P10 and P32 had microdeletions that could not be detected by classical cytogenetic analysis, both presenting a morphologically normal Y chromosome. Patients P47 and P57, on the other hand, presented a 46,XY (delY)(q11) karyotype. A schematic representation of the deletions detected is given in Figure 1.

Table 1 summarizes the clinical and hormonal characteristics of the 4 patients who presented Yq microdeletions in the PCR-based studies. Testicular volume was normal for patients P10 and P47, but reduced for patients P32 and P57. All 4 patients carrying Y chromosome deletions presented elevated plasma FSH concentrations. Plasma LH and testosterone levels were within the normal reference range, except for azoospermic patient P57 who presented elevated LH and reduced testosterone concentration in plasma.

PCR-based screening and karyotype analysis of the Y chromosome of the father of patient P47 and the brothers of patients P32 and P57 were normal. No relative of patient P10 was available for these tests.

Discussion

In the present study, 4 of the 60 Brazilian infertile patients diagnosed with nonobstructive azoospermia or severe oligozoospermia exhibited deletions of the Y chromosome (6.7%). Of the 4 infertile men detected with deletions of the Y chromosome, 2 were non-obstructive azoospermic men and 2 were severely oligozoospermic men. Although the literature indicates a higher frequency of deletion in azoospermic than in oligozoosper-

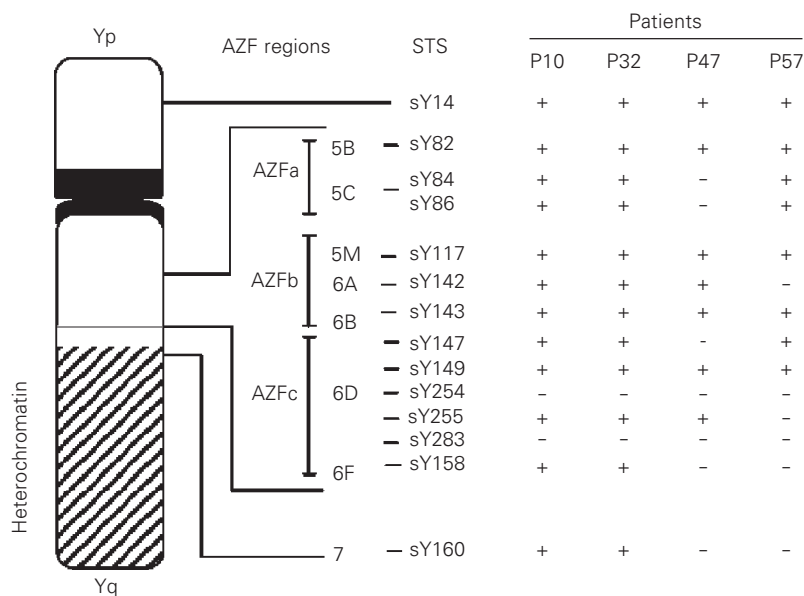


Figure 1. Schematic diagram of the human Y chromosome, including AZF regions (AZFa, AZFb and AZFc) and deletion intervals 5, 6 and 7, as defined by Vogt et al. (6). The list of sequence-tagged sites (STS) screened by PCR and their localization along the Y chromosome is shown. The presence (+) or absence (-) of STS amplification, observed in patients P10, P32, P47 and P57, is also indicated.

mic patients (16,22,24), the incidence of Y chromosome deletions in the present study was similar in the azoospermic (2/29, 6.8%) and the severely oligozoospermic patient groups (2/31, 6.4%). A study on Brazilian infertile men referred from different infertility clinics has been recently reported (30). Among the 65 idiopathic cases of infertility studied, 6 (9.2%) microdeletions were detected, 3 in the AZFb region and 3 in the AZFc region. In another study, Carrara (31) observed Y microdeletions restricted to the AZFc region in 4.3% (3/70) of Brazilian infertile men studied. Thus, taken together, the results indicate that the prevalence of Y chromosome microdeletions in Brazilian infertile men lies at the lower end of the 3-55.5% range reported by the literature in populations of infertile men (5-8,11-22).

Patients P47 and P57, presenting non-obstructive azoospermia, had several non-contiguous microdeletions, as already suggested by karyotype analysis [46,XY (delY) (q11)]. The severely oligozoospermic patients P10 and P32, on the other hand, presented microdeletions confined to the AZFc region, specifically involving the DAZ locus. Since testicular histology was not available, a precise genotype/phenotype correlation was not possible in the present study. It is important to emphasize, however, that all these 4 patients had in common the presence of Y chromosome microdeletions confined to the AZFc regions, specifically in the DAZ locus, where most Y chromosome deletions are reported (5,11). Furthermore, the STS-PCR screening and karyotype analysis of the father of patient P47 and the brothers of patients P32 and P57 did not reveal Y chromosome microdeletions, confirming that Yq

deletions were not paternally inherited, but rather occurred *de novo* during paternal germ cell differentiation or during an early stage of the fertilized egg (7,14,32).

Although all 60 patients analyzed in the present study were diagnosed as idiopathic infertile men, the presence of concomitant varicocele was not excluded during the clinical trial because Y chromosome deletions can coexist with this pathological condition (11,16,25,33) and has been defined as idiopathic by others (34). Both severely oligozoospermic patients (P10 and P32) carrying Y chromosome deletions in the present study had bilateral varicocele. These results are important for the clinical evaluation of these patients since it has been suggested that men with microdeletions and varicocele do not respond to varicocelectomy (35).

Since Y chromosome inheritance is always of paternal origin, men with partial somatic Y chromosome deletions are likely to have sons with similar genetic defects after treatment with assisted reproduction (36-38). Screening for Y deletions would be indicated in patients undergoing intracytoplasmic sperm injection (ICSI) programs and counseling on the risks of transmitting Y microdeletions and other Y chromosomal abnormalities to the offspring should be provided. Routine screening of Y microdeletions in all male patients before ICSI treatment is an important prerequisite for their appropriate counseling.

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