

# Somatic cytogenetic and azoospermia factor gene microdeletion studies in infertile men

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## Abstract

The objective of the present study was to determine the frequency of somatic chromosomal anomalies and Y chromosomal microdeletions (azoospermia factor genes, AZF) in infertile males who seek assisted reproduction. These studies are very important because the assisted reproduction techniques (mainly intracytoplasmic sperm injection) bypass the natural selection process and some classical chromosomal abnormalities, microdeletions of AZF genes or some deleterious genic mutations could pass through generations. These genetic abnormalities can cause in the offspring of these patients male infertility, ambiguous external genitalia, mental retardation, and other birth defects. We studied 165 infertile men whose infertility was attributable to testicular problems (60 were azoospermic, 100 were oligospermic and 5 were asthenospermic). We studied 100 metaphases per patient with GTG banding obtained from temporary lymphocyte culture for chromosomal abnormality detection and performed a genomic DNA analysis using 28 Y chromosome-specific sequence-tagged sites for Y AZF microdeletion detection. Karyotyping revealed somatic anomalies in 16 subjects (16/165 = 9.6%). Of these 16, 12 were in the azoospermic group (12/60 = 20%) and 4 were in the oligospermic group (4/100 = 4%). The most common chromosomal anomaly was Klinefelter syndrome (10/165 = 6%). Microdeletions of AZF genes were detected in 12 subjects (12/160 = 7.5%). The frequencies detected are similar to those described previously. These results show the importance of genetic evaluation of infertile males prior to assisted reproduction. Such evaluation can lead to genetic counseling and, consequently, to primary and secondary prevention of mental retardation and birth defects.

## Key words

- Cytogenetics
- Y microdeletions
- Male infertility
- Karyotype
- Chromosome aberrations

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## Introduction

The genetic factors most frequently related to male infertility are somatic chromosomal anomalies and Y chromosomal microdeletions within the Yq11 region, where the genes that control spermatogenesis, known as azoospermia factor genes (AZF), are located (1).

The incidence of somatic chromosomal anomalies in the infertile male population is approximately 10% and this frequency increases as the sperm concentration in ejaculate decreases (2). These anomalies may be numerical or structural and involve sex chromosomes (e.g., 47,XXY) or autosomes (e.g., balanced Robertsonian translocations).

The reported incidence of AZF microdeletions in non-obstructive azoospermia or severe idiopathic oligospermia varies widely due to the selection criteria used. The incidence of such microdeletions is higher in azoospermic than in oligospermic men and, consequently, the frequency of deletion found in different laboratories may vary from 2 to 10% (or higher), reflecting the composition of the study sample (3). After the Klinefelter syndrome, Y chromosomal microdeletions are the most frequent genetic cause of male infertility (3). Analysis of these deletions demonstrates that at least three loci (AZFa, AZFb, and AZFc) are required for normal spermatogenesis (4). Microdeletions of the AZF genes are caused by intrachromosomal recombination events between large homologous repetitive sequence blocks, and it is currently accepted that AZFb contains eight protein-coding genes (CDY2, EIF1AY, HSFY, PRY, RBMYL1, RPS4YS, SMCY, and XKRY) and AZFc contains five such genes (BPY2, CDY1, CSPG4LY, DAZ, and GOLGA2LY), which are all transcribed in testicular tissue and, therefore, are all candidates for some function in human spermatogenesis (5).

In recent years, the importance of genetic studies of male infertility has been high-

lighted by advances in assisted reproduction techniques, particularly intracytoplasmic sperm injection (ICSI). This procedure has raised important questions regarding the potential transmission of genetic abnormalities to the offspring, since the natural selection process of sperm cells is bypassed. Therefore, we evaluated somatic cytogenetic and AZF microdeletions in a sample of 165 Brazilian infertile men whose infertility was related to testicular problems. The aim of the present study was to determine the frequencies and the characteristics of these disorders, in order to indicate appropriate genetic counseling of individuals referred for assisted reproduction.

## Patients and Methods

### Patients

The study involved 165 infertile men whose infertility was related to testicular problems. All subjects were evaluated clinically at the Male Infertility Outpatient Clinic of the University Hospital, Faculdade de Medicina de Ribeirão Preto, Ribeirão Preto, SP, Brazil. Individuals presenting pre- or post-testicular problems were excluded from the study. The sample consisted of 60 patients (60/165 = 36.3%) with azoospermia, 100 patients (100/165 = 60.6%) with oligospermia (sperm count below  $20 \times 10^6$  sperms/mL), and 5 patients (5/165 = 3.0%) with asthenospermia.

This investigation was approved by the Hospital das Clínicas de Ribeirão Preto Ethics Committee (No. 3308/97) and informed written consent was obtained from all patients.

### Somatic cytogenetic analysis

The somatic karyotype of the 165 subjects was determined by temporary lymphocyte culture according to the macroculture technique described by Moorhead et al. (6).

A minimum of 100 metaphases were analyzed per patient, with the aim of detecting chromosomal mosaicism equal to or greater than 3% with a 90% confidence interval or equal to or greater than 5% with a 99% confidence interval, as described by Hook (7). The chromosomal abnormalities were described according to the norms established by the International System for Human Cytogenetic Nomenclature (8).

#### Analysis of the AZF genes (Yq11) by PCR-sequence-tagged sites

Analysis of microdeletions in the AZF (Yq11) region was performed in only 160 patients. In the remaining 5 patients, DNA samples were not available.

Genomic DNA was extracted from the peripheral blood of patients and control subjects using standard techniques. The DNA was amplified by multiplex PCR using 28 Y chromosome-specific sequence-tagged sites (STS) according to the method described by Henegariu et al. (9). Reaction products were separated on 3% agarose gel (Metaphor, FMC Bioproducts, Rocklands, ME, USA) and stained with ethidium bromide. The deletion of one or more PCR fragments was confirmed by analyzing the corresponding patient genomic DNA in single-primer pair PCRs, using the same experimental conditions and primer pairs from the missing amplification products of the multiplex PCR. This analysis was performed at least three times for each microdeleted sample.

## Results

### Somatic cytogenetic analysis

Karyotyping revealed chromosomal anomalies in 16 subjects (16/165 patients = 9.6%). Twelve chromosomal anomalies were detected in the azoospermic patients (12/60 = 20%) and four in the oligospermic patients (4/100 = 4%). The most frequent chromoso-

mal anomaly was Klinefelter syndrome (10/165 = 6%). All of these cases were in the azoospermic group (10/60 = 15.3%), seven in the non-mosaic 47,XXY form and three in the mosaic form - 46,XY (52)/47,XXY (48) - 46,XY (97)/47,XXY (3) - 46,XY (94)/47,XXY (6). The other two patients with chromosomal anomalies in the azoospermic group were one case of 46,XX male and one case of a mosaic 45,X (11)/46,X,del(Y) presenting 13 deleted STS in AZF analysis (Figure 1). In the oligospermic group, 4 cases of chromosomal anomalies were identified - 3 cases of balanced Robertsonian translocations - 45,XY,der (13q,14q); 45,XY,der (13q,14q); 45,XY,der (14q,21q) - and one case of 46,X,del(Y) presenting only one deleted STS in the AZF analysis (Figure 2). Another patient in the oligospermic group presented the 46,XY (90)/47,XY, + i 22 p (10) karyotype. This was considered to be an anomaly with no clinical significance, mainly because the infertility of this patient was attributed to five microdeletions detected in

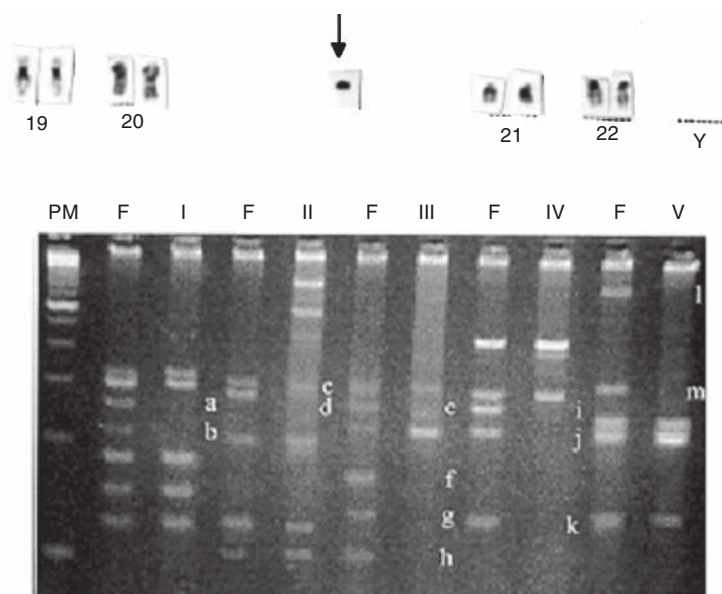


Figure 1. *Top*: Male infertility studies by classical cytogenetics and multiplex PCR for microdeletions of AZF gene detection. Partial karyotype of patient with 46,X,del(Y). The arrow indicates the Y deleted chromosome. *Bottom*: Multiplex PCR revealed deletion of 13 sequence-tagged sites indicated by letters a to m. PM = ladder; F = fertile man; I to V = mix I to V; a = sY117; b = sY102; c = sY143; d = sY157; e = sY105; f = Y6HPc54pr; g = sY153; h = sY97; i = sY127; j = sY109; k = sY149; l = Y6BaH34pr; m = Fr15Hpr.

the AZFc region (case 12; Table 1).

### Microdeletions in the Yq11 region

Microdeletions were detected in 12 patients (12/160 patients = 7.5%) and were most frequently limited to the AZFc region alone (7 patients: cases 1, 2, 4, 8, 9, 10, and 11 = 58.3%), followed by microdeletions in the combined AZFb and AZFc regions (3 patients: cases 5, 6, and 7 = 25%), in the AZFa region alone (1 patient: case 3 = 8.3%) and in the combined AZFa, AZFb, and AZFc regions (1 patient: case 12 = 8.3%). The position, extension and distribution of microdeletions detected in the present sample are shown in Figure 3. The microdeletion frequency was 7% (7/100) in the oligospermic group, 6.6% (4/60) in the azoospermic group and 20% (1/5) in the asthenospermic group. There was an association between the number and position of microdeletions and the sperm counts. When one STS microdeletion was detected in AZFa, oligospermia was mild ( $13 \times 10^6$  sperms/mL), whereas one such microdeletion in the AZFc corresponded to moderate oligospermia ( $6 \times 10^6$  or  $1.3 \times 10^6$  sperms/mL). When there were two such microdeletions (in AZFb and AZFc) we observed only asthenospermia or moderate oligospermia ( $4.5 \times 10^6$  sperms/mL). However, when both microdeletions were in

AZFc, oligospermia was severe ( $1 \times 10^5$  sperms/mL). When there were four or more microdeletions, regardless of the region, oligospermia was always severe (0 to  $2 \times 10^5$  sperms/mL; Table 1). We performed a testicular biopsy and histopathological analysis in 5 of the 12 patients with AZF microdeletions. In 1 of the 5 patients, four microdeletions (one in AZFb and three in AFZc) and severe oligospermia ( $1 \times 10^5$  sperms/mL) were detected and histopathologic evaluation revealed severe hypospermatogenesis. In another patient there were five microdeletions in the AZFc region and azoospermia, and the evaluation revealed tubular atrophy and maturation arrest in spermatocyte I. Another one of the 5 patients presented five microdeletions in the AZFc region and azoospermia, as well as germinal aplasia in 90% of tubules and maturation arrest in 10%. There was also 1 patient with five microdeletions in the AZFc, severe oligospermia ( $2 \times 10^5$  sperms/mL), germinal aplasia in 75% of the tubules and spermatocyte I maturation arrest in 25% of the tubules. Finally, in the 46,X,del(Y) patient with 13 microdeletions and azoospermia, we observed tubular fibrosis, tubular hyalinization and germinal maturation arrest in spermatocytes I (Table 1).

### Discussion

The frequency of somatic chromosomal anomalies detected in our sample was 9.6% (16/165 patients), more prevalent in the azoospermic group (18.4%) than in the oligospermic group (4.0%). In the group of azoospermic patients, sex chromosome anomalies (47,XXY - 46,XY/47,XXY and 46,XX male) predominated. In the oligospermic group, balanced Robertsonian translocations were more common. These findings are very similar to those of previous studies (10-13). In our sample, it was demonstrated that 100 was an appropriate number of metaphases for use in cytogenetic studies since we identified 2 cases of relatively low mosaic

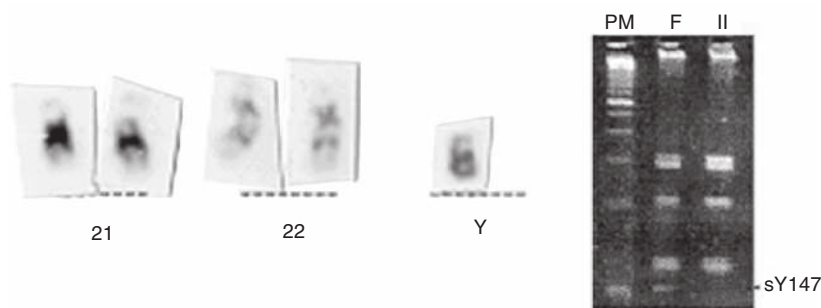


Figure 2. Male infertility studies by classical cytogenetics and multiplex PCR for microdeletions of AZF gene detection. Partial karyotype of 46,XY,del(Y). Multiplex PCR revealed deletion of one sequence-tagged site (sY147). PM = ladder; F = fertile man; II = mix II showing the sY147 deleted.

Klinefelter syndrome (46,XY - 94/47,XXY - 6 and, 46,XY - 97/47,XXY - 3) that would probably not have been detected in an analysis involving fewer metaphases.

The frequency of AZF microdeletions detected in our sample was 7.5% (12/160

patients), a value similar to that previously reported (4,14,15). Rolf et al. (14) for a sample of 3000 infertile males detected a frequency of 7.3% with a higher value (15%) in azoospermic men than in oligospermic men. In contrast, we observed a frequency of

Table 1. Genotype-phenotype association in azoospermia factor gene (AZF) microdeletions.

Case	Microdeletion	Sperms/mL	Clinical diagnosis	Histopathology	
1	sY153	AZFc	1.3 x 10 <sup>6</sup>	Severe oligospermia	Not performed
2	sY147	AZFc	6 x 10 <sup>6</sup>	Moderate oligospermia	Not performed
3	sY151	AZFa	16 x 10 <sup>6</sup>	Mild oligospermia	Not performed
4	sY149	AZFc	1 x 10 <sup>5</sup>	Severe oligospermia	Not performed
	Fr15Hpr	AZFc			
5	sY143	AZFc	4.5 x 10 <sup>6</sup>	Moderate oligospermia	Not performed
	sY147	AZFb			
6	sY143	AZFb	40 x 10 <sup>6</sup>	Asthenospermia	Not performed
	sY147	AZFc			
7	sY143	AZFb	1 x 10 <sup>5</sup>	Severe oligospermia	Severe hypospermatogenesis
	sY147				
	sY157	AZFc			
	Fr15Hpr				
8	sY149	AZFc	1 x 10 <sup>5</sup>	Severe oligospermia	Not performed
	sY153				
	Y6HP52pr				
	sY157				
	Fr15Hpr				
9	sY157	AZFc	0	Azoospermia	Tubular aplasia (90%) Hypospermatogenesis (10%)
	sY147				
	sY153				
	sY149				
	Fr15Hpr				
10	sY157	AZFc	2 x 10 <sup>5</sup>	Severe oligospermia	Tubular aplasia (75%) Maturation arrest (25%)
	sY147				
	sY153				
	sY149				
	Fr15Hpr				
11	sY157	AZFc	0	Azoospermia	Tubular atrophy Maturation arrest
	sY147				
	sY153				
	sY149				
	Fr15Hpr				
12	sY102		0	Azoospermia	Peritubular fibrosis and hyalinization Maturation arrest
	sY105				
	sY97				
	sY109	AZFa			
	sY117				
	sY143				
	sY127				
	Y6HPc54pr				
	Y6BaH34pr	AZFb			
	sY157				
	sY153				
	sY149				
	Fr15Hpr	AZFc			

6.6% in the azoospermic group, comparable to the 7% seen in the oligospermic group. This could be an effect of the small size and slightly unbalanced nature of our sample (oligospermic patients = 100, azoospermic patients = 60). The distribution of microdeletions detected in our sample showed that the AZFc region was the most prevalent (58.3%), followed by the AZFb/AZFc combination (25%), by AZFa (8.3%), and by the AZFa/AZFb/AZFc (also 8.3%) combination. These results differ from those reported by Simoni et al. (3) only in the fact that we detected no microdeletions exclusively in the AZFb region, while they found this to be the second most frequent region in their sample of 34 patients (AZFc = 79%; AZFb = 9%; AZFb/AZFc = 6%; AZFa = 3%, and AZFa/AZFb/AZFc = 3%). The same investigators found that deletions of isolated genes in the AZFa region were related to a variable

testicular phenotype and that complete deletion of the AZFa region makes it virtually impossible to retrieve testicular sperm for ICSI. Similarly, in our sample, the size of the deleted segment was associated with sperm counts, with a clear relationship between the size (number of STS deleted) and the level of sperm count reduction (Table 1). Unfortunately, we only had access to testicular biopsy samples from those patients with 4 or more deleted STS. Nevertheless, in the patient with 4 STS deletions, there was severe hypospermatogenesis, whereas the patient with 5 STS deletions presented more severe testicular involvement with tubular atrophy or aplasia, and the patient with 13 STS deletions presented maturation arrest, as well as peritubular fibrosis and hyalinization. However, the AZFc deletion phenotype is less severe because, in some cases, these deletions are compatible with residual spermatogenesis and, in rare cases, can even be transmitted naturally to the male offspring (14, 16,17). Our case 2, with 1 deleted STS (sY147) in the AZFc, presented a sperm count of  $6 \times 10^6$  sperms/mL, whereas case 1, with a different STS deleted (sY153) in the same AZFc region, had only  $1.3 \times 10^6$  sperms/mL. An explanation for the difference in phenotype between these two patients requires a more complete study of gene expression in these cases.

In view of the genetic risks for the next generation, the importance of careful evaluation of karyotypes and AZF microdeletions in male infertility prior to assisted reproduction by ICSI is evident. In our evaluation, we found that some subjects presented a higher risk to transmit genetic diseases to their offspring. For example, the patient with the balanced Robertsonian translocation 45,XY, der (14q, 21q) has a high risk of fathering a child with Down syndrome, just as the patient with the 46,XX karyotype has a high risk of fathering another male with the same pathology, the patient with the balanced Robertsonian translocation 13q, 14q has a

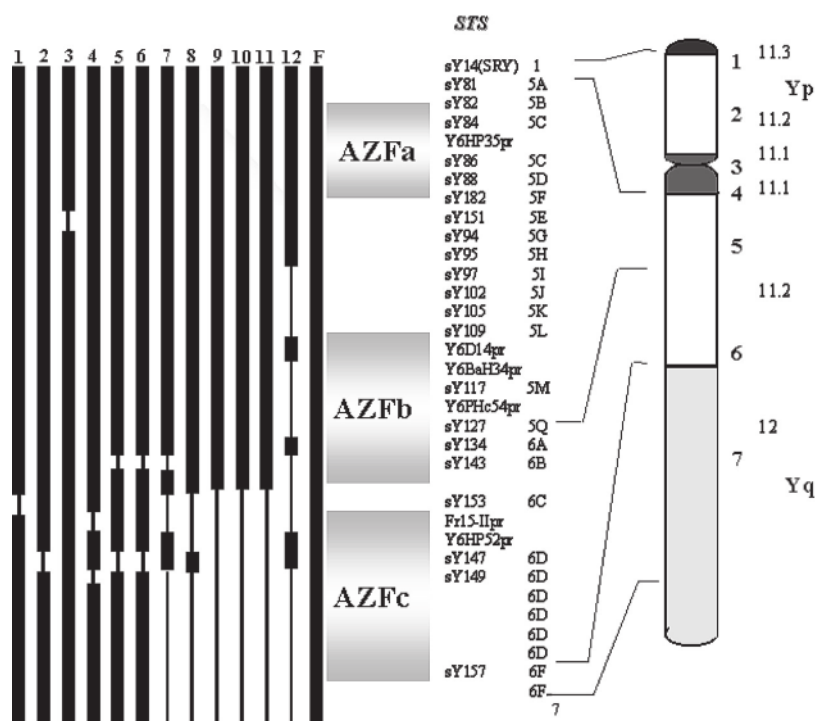


Figure 3. Schematic presentation of the 12 patients with microdeleted segments inside the azoospermia factor gene (AZF) region of the Y chromosome. The patients are positioned by the number of sequence-tagged sites (STS) deleted (in the same order in which they are presented in Table 1). F = fertile man.

high risk of fathering a child with trisomy 13 syndrome, the patients with Klinefelter syndrome have a high risk of fathering children with complete Klinefelter syndrome, and the patients with AZF microdeletions have a high risk of fathering infertile males (16) or offspring with severe clinical consequences other than male infertility, such as the development of sexual ambiguities and Turner stigmata (17). Patsalis et al. (17) screened 12 patients who had a 45,X/46,XY karyotype and presented Turner stigmata or sexual ambiguities, or both, for Y chromosomal microdeletion and detected that one third of these patients had Y chromosome microdeletions. Vogt (5) stated that AZF microdele-

tions can also become pre-mutations for a subsequent complete loss of the Y chromosome in the sperm of AZF-deleted patients, increasing the risk for XO cells and sexual ambiguities since the 45,XO/46,XY mosaic karyotype has been associated with ambiguous genitalia.

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