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Chromosomal Abnormalities and Y Chromosome Microdeletions in Infertile Men With Varicocele and Idiopathic Infertility of South Indian Origin

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ABSTRACT: Various factors cause spermatogenesis arrest in men and, in a large number of cases, the underlying reason still remains unknown. Little attention is paid to determining the genetic defects of varicocele-related infertility. The objective of our present study was to investigate the chromosomal abnormalities and Y chromosome microdeletions in infertile men of South Indian origin with varicocele and idiopathic infertility. Metaphase chromosomes of 251 infertile men with varicocele and unexplained infertility were analyzed using Giemsa-Trypsin-Giemsa (GTG) banding and fluorescence in situ hybridization (FISH). The microdeletions in 6 genes and 18 sequence-tagged-sites (STS) in the Yq region were screened using polymerase chain reaction (PCR) techniques. Out of 251 infertile men, 57 (22.7%) men were with varicocele, of which 8.77% were azoospermic, 26.31% were severely oligozoospermic, 21.05% were mildly oligozoospermic, and 43.85% were oligoasthenoteratozoospermic (OAT), and 194 (77.29%), with idiopathic infertility, of which 51% were azoospermic, 13.40% were severely oligozoospermic, 19.07% were mildly oligozoospermic, and 16.4% were with OAT. Genetic defects were observed in 38 (15.13%) infertile individuals, including 14 (24.56%) men with varicocele and 24 (12.37%) men with idiopathic infertility. The frequencies of chromosomal defects in varicocele and idiopathic infertility were 19.3% and 8.76%, respectively, whereas Y chromosome microdeletions were 5.26% and 3.60%, respectively. Overall rate of incidence of chromosomal anomalies and microdeletions in 251 infertile men were 11.5% and 3.98%, respectively, indicating a very significant higher association of genetic defects with varicocele than idiopathic male infertility. Our data also demonstrate that, among infertile men with varicocele, severely oligozoospermic and OAT men with varicocele have higher incidences of genetic defects than mildly oligozoospermic and azoospermic men.

Key words: Genetic defects, male infertility, cytogenetic study, STS markers.

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Approximately 15% of couples are unable to conceive after 1 year of unprotected intercourse. Male factor is solely responsible in about 20% of infertile couples and contributory in another 30–40% (Thonneau et al, 1991). Several factors are proposed to cause infertility in men. These include varicocele, obstruction of spermatic ducts, hypogonadism, cryptorchidism, agglutination of spermatozoa, testicular tumors, presence of antisperm antibodies, low volume of ejaculate, and necrospermia. However, the primary cause of infertility remains unclear. Genetic abnormalities are considered to make an important contribution to these cases of unexplained spermatogenesis failure. It became evident initially from several surveys, which showed up to 15% of numerical and constitutional chromosome anomalies in infertile men. This argument was strengthened further by detection of microscopic deletions in the Yq11 region of infertile men (Tiepolo and Zuffardi, 1976; Ma et al, 1992; Vogt et al, 1992; Reijo et al, 1995). These studies paved the way for the search of a hypothetical azoospermia factor (AZF). Later, using cytogenetic and molecular approaches, a few candidate genes were mapped that qualified as azoospermia factor (Ma et al, 1993; Reijo et al, 1995; Lahn and Page, 1997; Brown et al, 1998; Vogt et al, 1996). Notable among these are RNA binding motif (*RBM*) and deleted in azoospermia (*DAZ*) (Ma et al, 2000). Defects in these genes occurred in the form of microdeletions. Frequencies of *RBM* and *DAZ* deletions have been found to differ among various studies undertaken on diverse populations. Also, microdeletions are not limited to these genes only; they occur all along the long arm of the Y chromosome.

Generally, for estimating prevalence rates of microdeletions, as a rule, anonymous sequence-tagged-sites (STS) markers from the long arm of the Y chromosome are used. Lately, a few studies have focused on gene-based

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screening for the Y chromosome anomalies. Except in one study with a point mutation in the *USP9Y* gene (Sun et al, 1999), in most of the cases gene changes also occur in form of deletions. Taken together, the frequencies of STS and gene deletions among different studies is quite wide and varies between 1–55% (Henegariu et al, 1994; Kobayashi et al, 1994; Reijo et al, 1995, 1996; Najmabadi et al, 1996; Qureshi et al, 1996; Stuppia et al, 1996; Vogt et al, 1996; Foresta et al, 1997, 1998; Girardi et al, 1997; Kremer et al, 1997; Mulhall et al, 1997; Peterlin et al, 1997; Pryor et al, 1997; Simoni et al, 1997; van der Ven et al, 1997; Vereb et al, 1997; Brandell et al, 1998; Grimaldi et al, 1998; Liow et al, 1998; Oliva et al, 1998; Silber et al, 1998; Kent-First et al, 1999; Kim et al, 1999; Kleiman et al, 1999; Krausz et al, 1999; Seifer et al, 1999).

Redmon et al (2002) categorized varicocele as a systemic cause for male infertility and hypothesized that varicocele may not have any association with or effect on male fertility or it may be associated with, but is not the cause of, male subfertility or it may be a direct cause of male subfertility. In this study, we compared the prevalence of chromosomal abnormalities and Y chromosome microdeletions, using cytogenetic and molecular techniques, among men with varicocele-related and idiopathic infertility to determine whether varicocele has any genetic association with male infertility or subfertility.

Materials and Methods

Patient Selection and Clinical Evaluation

A total of 251 men of South Indian origin presenting infertility, seeking help of assisted reproductive techniques (ART) were recruited at the Infertility Institute and Research Centre, Hyderabad, India. Twenty-five normal fertile men of the same origin were considered as controls. All infertile men in age groups ranging from 22 to 46 years (mean, 32.55 years) were referred for evaluations of infertility and technically met the definition of infertility (1 year of unprotected intercourse and not leading to conception) were enrolled in the study regardless of the fertility status of their partners. These men were subjected to comprehensive questionnaires related to their medical, surgical, sexual, and family histories, lifestyle habits (such as smoking, alcohol use, and drug use), exposure to gonadotoxins (such as drugs used in cancer chemotherapy). Further, a thorough physical examination was included as an assessment of secondary sexual characteristics. Every individual provided a minimum of 2 semen specimens, each after sexual abstinence extending from 2 to 5 days. These specimens were evaluated on the basis of the criteria of the World Health Organization and the results were averaged. Informed written consent was obtained from each subject. The Institutional Review Board of the Centre for Cellular and Molecular Biology, Hyderabad, approved this study.

On the basis of the mean sperm count, all the infertile men were categorized accordingly as azoospermia (absence of sperm), severe oligozoospermia (sperm count below 5 million per mL), mild oligozoospermia (sperm count 5–20 million per mL), and oligoasthenoteratozoospermia (abnormal sperm morphology and motility). Decreased sperm motility was determined by using antisperm antibodies. In addition, blood samples were obtained for DNA extraction and serum for measurement of testosterone, prolactin, and follicle-stimulating hormone (FSH) by radioimmunoassay. After a thorough clinical evaluation, a respective diagnosis of infertility was given to each individual accordingly. Those men with obstructive azoospermia, as clinical evidence, were excluded from the study. Semen analysis, hormone profile, and histology of the testicular biopsy performed during testicular sperm extraction were reviewed on charts.

Cytogenetic Analysis

Chromosomal analysis was performed on phytohemagglutinin (PHA)-stimulated peripheral lymphocyte cultures using standard cytogenetic methods (Benn and Perle, 1992; Gosden et al, 1992). Twenty to 30 metaphases were analyzed per individual and, in cases of suspected mosaicism, the numbers of metaphases were increased to a total of 100 for analysis. A resolution of 400-band stage was considered as a minimum; for a more detailed structural analysis, 550–700-band stage was preferred. The routine analysis was based on GTG-banded staining.

Screening for Y-Linked Sequence-Tagged Sites

Genomic DNA was extracted from peripheral venous blood lymphocytes using a standard phenol-chloroform protocol. Screening for Yq microdeletions was carried out in patients using polymerase chain reaction (PCR) techniques by amplifying 24 different STSs corresponding to 3 AZF loci spread over interval 5 and 6. This included sY86, sY87, sY610 (*DBY*), sY620 (*USP9Y*) from *AZFa*; sY127, sY134, sY143, sY634 from *AZFb*; sY153, sY205, sY232, sY254, sY255, sY277, sY283, sY624 from *AZFc*, and sY158, sY160 (heterochromatic distal Yq region). Primers specific for genes *UTY* (*AZFa*), *SMCY*, *EIF1AY*, *CDY2* from the *AZFb* region and *CDY1* (*AZFc*) were also used. Additional STS for *SRY* gene (sY14) was used as a positive control testing Y chromosome specificity. Fertile male DNA and female DNA samples were used as internal controls along with a blank to check any contamination. Briefly, 50–100 ng of genomic DNA was used as template in 25 μL reaction mix, 1× amplification buffer, 1 mmol dNTPs, 10–25 pmol of each primer, and 1.25 IU of *Taq* DNA polymerase. All chemicals were obtained from Roche Diagnostics GmbH (Penzberg, Germany). After an initial denaturation step of 5 min, each PCR reaction was carried at the annealing temperature specific for each primer pair. The PCR products were separated on 2–3% agarose gels stained with ethidium bromide on the basis of the size of the product obtained. Whenever failure of amplification in any sample was detected, 2 additional PCRs were performed to confirm the absence of the unamplified STSs.

Results

Clinical Findings

Semen profiles, hormone levels, and testicular morphology showed large variations in men where genetic defects

Table 1. *Frequencies of chromosomal abnormalities and Y chromosome microdeletions in 251 infertile men with varicocele and idiopathic infertility*

Type of Infertility	No. of Individuals (%)	Chromosomal Abnormalities (%)	Y Chromosome Microdeletions (%)	Total Genetic Defects (%)	*P*-Value (>.05)	Odds ratio	95% Confidence Limits	
							Lower	Upper
Varicocele	57 (22.7)	11 (19.3)	3 (5.26)	14 (24.56)				
Azoospermia	5 (8.77)	2 (3.5)	1 (1.75)	3 (5.26)				
Severe oligozoospermia	15 (26.31)	3 (5.26)	1 (1.75)	4 (7.01)				
Mild oligozoospermia	12 (21.05)	1 (1.75)	1 (1.75)	2 (3.5)				
Oligoasthenoteratozoospermia	25 (43.85)	5 (8.77)	0 (0)	5 (8.77)				
Idiopathic Infertility	194 (77.29)	17 (8.76)	7 (3.60)	24 (12.37)	0.0179*	2.444	1.663	5.123
Azoospermia	99 (51)	7 (3.60)	4 (2.06)	11 (5.67)				
Severe oligozoospermia	26 (13.40)	1 (0.51)	0 (0)	1 (0.51)				
Mild oligozoospermia	37 (19.07)	0 (0)	2 (1.03)	2 (1.03)				
Oligoasthenoteratozoospermia	32 (16.4)	9 (4.63)	1 (0.51)	10 (5.15)				
Total	251	28 (11.5)	10 (3.98)	38 (15.13)				
Azoospermia	104 (41.3)	9 (3.58)	5 (1.99)	14 (5.57)				
Severe oligozoospermia	41 (16.33)	4 (1.59)	1 (0.39)	5 (1.99)				
Mild oligozoospermia	49 (19.52)	1 (0.39)	3 (1.19)	4 (1.59)				
Oligoasthenoteratozoospermia	57 (22.7)	14 (5.57)	1 (0.39)	15 (5.97)				

* Association of genetic defects between varicocele-related and idiopathic male infertility.

were observed. On the basis of semen analysis, 104 (41.3%) individuals were azoospermic, 41 (16.33%) were severely oligozoospermic, 49 (19.52%) were mildly oligozoospermic, and 57 (22.7%) were oligoasthenoteratozoospermic (OAT), as shown in Table 1. The physical and andrological examinations revealed 57 (22.7%) infertile men with varicocele [azoospermic, 5 (8.77%); severely oligozoospermic, 15 (26.31%); mildly oligozoospermic, 12 (21.05%); and OAT, 25 (43.85%)] and 194 (77.29%) men with idiopathic infertility [azoospermic, 99 (51%); severely oligozoospermic, 26 (13.40%); mildly oligozoospermic, 37 (19.07%); and OAT, 32 (16.4%)]. Two individuals (P 3 and P 46) had increased FSH concentrations and Y chromosome microdeletions. No significant differences were detected in the hormone profiles of azoospermic or severely and mildly oligozoospermic patients with or without abnormal karyotype. Elevated testosterone concentrations were seen in 2 individuals, P 46 with 9 STS deleted and P 52 with additional material on chromosome 22. Testicular histology revealed arrest of spermatogenesis at different stages in 3 of 10 patients with microdeletions and in 2 of 28 patients with chromosomal abnormalities.

Cytogenetic Evaluation

The chromosomal abnormalities involving major autosomal aberrations were detected in 28 (11.5%) individuals of 251 infertile men. Chromosomal investigations in 57 infertile men with varicocele showed 11 (19.3%) subjects with chromosomal aberrations confined to autosomes, including inversions, translocation, deletion, insertion, and also unknown additional chromosomal material on 21 and 22 as shown in Table 2. The major anomalies detected in idiopathic infertility were 47,XXY or 47,XYY (Klinefelter syndrome and its variant) in 8 (3.18%) individuals, and extra chromosomal material of unknown origin on chromosome 14 and chromosome 21 were detected in 2 individuals, while others showed autosomal abnormalities, including translocations and deletions. One individual (P 251) with idiopathic infertility and OAT showed duplication of the Yq region (Table 3).

Y Chromosome Microdeletion Screening

Of 251 infertile cases, Y chromosome microdeletions were found in 10 (3.98%) individuals within the *AZFc* region. Two individuals (P 3 and P 46) with azoospermia shared common deletions for 9 STSs broadly covering the *DAZ* region, and 1 individual (P 3) with additional deletion for *CDY1*, gene making the *AZFc* region extremely fragile compared with OAT, with severely and mildly oligozoospermic subjects (Figure; Table 3). These deletions were detected completely within the *AZFc* region in interval 6 of the Y chromosome. No deletions were found in *AZFa* and *AZFb* regions among all infertile men and fertile controls. In addition, 2 more individuals (P 36 and P 39) showed sY254 and sY255 STSs deletions confined to the *DAZ* region. Seven individuals showed sY153 deletions that come from the boundary of the *AZFb* and *AZFc* (*AZFd*) regions. The intactness of the distal region of *AZFc* involving sY158 was not found to be present in 6 individuals. Most frequently deleted STSs among the Y chromosome microdeletions studied were sY153 (70%),

Table 2. *Chromosomal and molecular variations in 57 infertile men with varicocele*

Patient No.	Cause	Genetic Defects/STS Deleted	Scrotal Scan
Chromosomal abnormalities			
P 47	Azoospermia	46,XY inv(9)(p21q13)	Bilateral varicocele
P 53	Azoospermia	46,XY del(4)(p12p14)	Varicocele
P 52	Severe oligozoospermia	46,XY add(22)(p13)	Grade I varicocele
P 185	Severe oligozoospermia	46,XY add(22)(p13)	Varicocele
P 207*	Severe oligozoospermia	46,XY add(22)(p13)	Grade II varicocele
P 165	Mild oligozoospermia	46,XY add(21)(p13)	Bilateral varicocele
P 28	OAT†	46,XY t(4:15)(p33:p11)	Bilateral varicocele
P 175	OAT	46,XY inv(2)(q11.2q13)	Varicocele
P 228	OAT	46,XY add(21)(p13)	Grade I varicocele
P 234	OAT	46,XY ins(9:?)(p13:1)?	Grade II varicocele
P 60	OAT	46,XY add(21)(q13)	Varicocele
Y chromosome microdeletions			
P 200	Azoospermia	sY153, sY158, and sY254	Bilateral varicocele
P 207*	Severe oligozoospermia	sY153, sY158, sY254 and CDY1	Grade II varicocele
P 191	Mild oligozoospermia	sY153, sY158 and sY254	Mild varicocele

* Individual with varicocele having both chromosomal defect and microdeletions.
† OAT indicates oligoasthanoteratozoospermia.

sY254 (70%), sY168 (60%), and sY255 (40%) (Table 4). An interesting observation in 3 individuals (P 191, P 200, and P 207) with varicocele illustrates common deletion pattern for sY153, sY158, sY254, and 1 individual (P 207) with an additional deletion for *CDY1* gene (Figure).

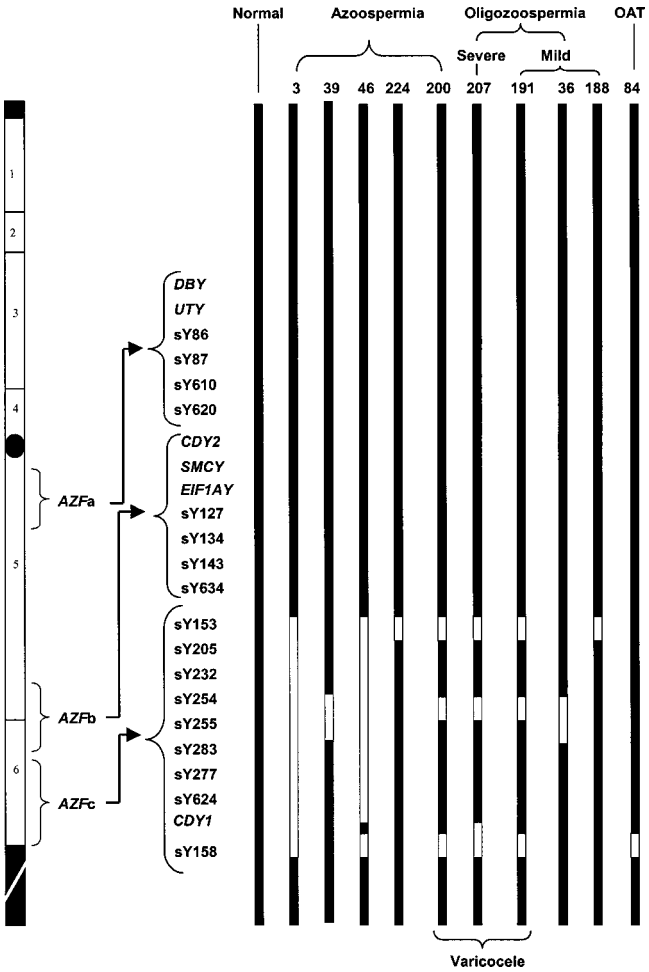
Discussion

Varicocele affects approximately 40% of men attending infertility clinics (Marmar, 2001) and occurs as an abnormal dilation of testicular veins within the pampiniform

Table 3. *Chromosomal and molecular variations in 194 men with idiopathic infertility*

Patient No.	Cause	Genetic Defects/STS Deleted
Chromosomal abnormalities		
P 68	Azoospermia	47,XXY
P 82	Azoospermia	47,XXY
P 95	Azoospermia	46,XY t(X:15)(q28:q22)
P 101	Azoospermia	47,XXY
P 110	Azoospermia	47,XXY
P 174	Azoospermia	47,XXY
P 217	Azoospermia	46,XY add(21)(p13)
P 63	Severe oligozoospermia	46,XY add(14)(p13)
P 23	OAT*	46,XY del(13p)
P 24	OAT	46,XY del(13p)
P 74	OAT	47,XXY
P 116	OAT	46,XY t(1:10)(p32:p15)
P 147	OAT	47,XXY
P 152	OAT	47,XXY
P 232	OAT	46,XY t(15;21)(q22.1:p11.2)
P 250	OAT	46,XY der(13,14)
P 251	OAT	46,XY ?dup(Yq)
Y chromosome microdeletions		
P 3	Azoospermia	sY153, sY158, sY205, sY232, sY254, sY255, sY277, sY283, sY624, and CDY1
P 39	Azoospermia	sY254, sY255
P 46	Azoospermia	sY153, sY158, sY205, sY232, sY254, sY255, sY277, sY283, and sY624
P 224	Azoospermia	sY153, sY254, sY255
P 36	Mild oligozoospermia	sY254, sY255
P 188	Mild oligozoospermia	sY153
P 84	OAT	sY158

* OAT indicates oligoasthanoteratozoospermia.



Polymerase chain reaction (PCR) results of deleted sequence-tagged sites (STSs) in the Yq region detected in individuals with varicocele and idiopathic infertility. A schematic representation of the human Y chromosome, *AZF* regions, deletion intervals, and position of the STSs that are screened are mentioned above. Positions of *AZF* candidate genes are also indicated. The solid filled black bars indicate the presence of STS or genes; empty bars, absence of STS or genes. Patient numbers are listed on the top of the bars.

plexus. Although association of testicular abnormalities with varicocele has been known for centuries (Silber, 2001), its possible role in male infertility is still unclear. The testicular biopsy sections from infertile men with varicocele display variable patterns, ranging from azoospermia to oligozoospermia and normal spermatogenesis (Moro et al, 2000). Because of these distinct clinical manifestations, genetic studies have not paid adequate attention to men with varicocele-related infertility. Despite that varicocele is recognized as one of the most common causes of male infertility, its pathogeneses vis-à-vis genetic defects are poorly understood (Pryor et al, 1997). Obviously, maximum efforts have been focused on the idiopathic cases. Data on Y chromosome microdeletions from 5 studies carried out to date in men with varicocele clearly indicate that genetic defects and varicocele may coexist (Pryor et al, 1997; Moro et al, 2000; Cayan et al, 2001). The overall frequency of genetic defects in the present study is 15.13% (24.56% in varicocele and 12.37% in idiopathic infertility) (Table 1). For the first time, our data clearly show significant high association of genetic defects with varicocele-related infertility (odds ratio = 2.444, 95% CI = 1.663–5.123, $P = .0179$).

Another notable feature was a consistent pattern of Y chromosome noncontiguous STS marker deletions in individuals (P 191, P 200, and P 207) with varicocele and the DNA sequence between these deleted markers was found intact. One individual with severe oligozoospermia and varicocele (P 207) in addition to the above deletions had *CDY1* gene deletion and additional material in the short arm of chromosome 22 (Table 2), presenting both Y chromosome microdeletions and chromosome defects. Deletions in idiopathic cases, however, involved adjacent STS markers (Figure). There is a high incidence of chromosomal defects in severely oligozoospermic men compared with mildly oligozoospermic men among varicocele-related infertile men, idiopathic infertility and overall male infertility that is not the same in the case of Y chromosome microdeletions. There was no significant difference in the cumulative frequencies of Y chromosome microdeletions in individuals with varicocele and idiopathic infertility (5.26% and 3.6%, respectively). No correlation was seen between the sperm concentration and the size or the extent of the microdeletions.

With rapid advances in assisted reproductive technologies, it is gradually becoming a practice to use sper-

Table 4. *Contribution of each deleted STS for male infertility*

S No.	Cause of Infertility	No. of Individuals	Deleted STS									Gene
			sY 153	sY 158	sY 205	sY 232	sY 254	sY 255	sY 277	sY 283	sY 624	CDY1
1	Azoospermia	5	4	3	2	2	4	3	2	2	2	1
2	Severe oligozoospermia	1	1	1	…	…	1	1	…	…	…	1
3	Mild oligozoospermia	3	2	1	…	…	1	…	…	…	…	…
4	OAT*	1	—	1	…	…	…	…	…	…	…	…
	Total	10	7	6	2	2	6	4	2	2	2	2
	Frequency (%)		70	60	20	20	70	40	20	20	20	20

* OAT indicates oligoasthanoteratozoospermia.

matozoa from men with varicocele for intracytoplasmic sperm injection and in vitro fertilization purposes. Because current investigative trends largely emphasize knowing the defects in idiopathic cases, the genetic status of most of the nonidiopathic infertility cases remain untested. The present study is the first report showing strong association of genetic defects with varicocele-related infertility in men. In such cases, if the pregnancy succeeds, a defect has mistakenly been allowed to pass to the next generation. Hence, it is important to consider genetic defects as an etiological factor where Y chromosome mapping and karyotype studies may be essential in the work-up of men with varicocele-related male infertility.

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