Screening for AZFc Partial Deletions in Dravidian Men with Nonobstructive Azoospermia and Oligozoospermia

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Context: Dravidians are the predominant population residing in South India with a diverse genetic structure. Considering various genetic discoveries taking place today, it is evident that deletions in the AZFc region are the most common cause of severe spermatogenic failure (SSF) in various populations studied. However, it is significant to note that there is a paucity of scientific literature on AZFc subdeletion screening among the Dravidian population. **Objective:** To investigate the prevalence and association of AZFc subdeletion patterns among Dravidian men with nonobstructive azoospermia (NOA) and oligozoospermia. **Methods:** A population of 354 subjects, including 120 patients with NOA, 109 with oligozoospermia, and 125 normal male controls, were screened using locus-specific sequence tag site markers. **Results:** We found 21 (9.17%) patients with classical AZF deletion, while no deletions were observed in controls. After excluding the samples with AZF deletions, the remaining 208 infertile and 125 control samples were screened for partial AZFc deletions using a standardized multiplex polymerase chain reaction and on analysis revealed that 13 (6.25%) of the infertile samples possessed gr/gr subdeletions and 15 (7.21%) of the infertile samples possessed b2/b3 subdeletions. Six (4.8%) of the normal samples were found to carry gr/gr subdeletions and two (1.6%) had b2/b3 deletions. The b1/b3 deletion was not observed in any of the patient and control samples screened. **Conclusion:** Our finding shows that there is a strong association between b2/b3 subdeletion and SSF in the Dravidian population (odds ratio, 4.78; 95% confidence interval 1.07–21.26) ($p=0.018$). Further studies, including gene copy typing for DAZ and CDY genes and a comprehensive haplogrouping analysis, are recommended in a large and well-selected patient group to elude the genetic mechanism behind this association.

Introduction

THE HUMAN Y CHROMOSOME holds the key to unravel the genetic mysteries behind impaired spermatogenesis. Deletions in the long arm of the Y chromosome (Yq) are considered to be the most frequent cause leading to the quantitative reduction of sperm (Ferlin *et al.*, 2007). To date, most studies were centered on the three spermatogenesis loci located on the male-specific region of the Y chromosome (MSY), namely AZFa, AZFb, and AZFc. Among the three AZF deletion intervals, the AZFc region has been the most frequently deleted locus, which shows capricious phenotypic features, ranging from azoospermia to mild/severe oligozoospermia (Ravel *et al.*, 2009; Krauz *et al.*, 2014).

The peculiar genomic architecture of the AZFc region makes it more susceptible to structural defects. The AZFc locus is made up of a complex of three large palindromes with a structural identity of 99.97% between its arms. The arrangements of the large identical repetitive sequence of the palindromes accelerate the occurrences of deletions through

a homologous recombination (Kuroda-Kawaguchi *et al.*, 2001; Skaletsky *et al.*, 2003). Recently, the existence of several partial AZFc deletions has been identified, including gr/gr, b1/b2, and b2/b3 deletions (Repping *et al.*, 2004). An association between AZFc subdeletions and severe spermatogenic failure (SSF) was observed in several populations, including Australian, European, Han-Chinese, Moroccan, and North Indian populations (Navarro-Costa *et al.*, 2007; Lu *et al.*, 2009; Shahid *et al.*, 2011; Bunyan *et al.*, 2012; Eloualid *et al.*, 2012). However, screening studies in several other populations contradict these findings (Machev *et al.*, 2004; Hucklenbroich *et al.*, 2005; Fernando *et al.*, 2006). Furthermore, the estimated frequency of AZFc subdeletions reported in various population studies is not consistent with each other. It has been proposed that the variations in the deletion frequency and the phenotypic effect may be due to the influence of environmental factors or due to the ethnic background of the Y chromosome (Yang *et al.*, 2008).

The Dravidians are the predominant population found particularly in South India and comprise about 20% of the

Indian population as per the Census of India (2011). Genetic studies on the present day South Indian population revealed that the population itself is an admixture of various racial groups and shows diverse genetic features (Suhasini *et al.*, 2011). Hence, the information received from the genetic screening studies using well-defined case–control samples will serve as useful reference data in relation to the research, diagnosis, and management of male infertility. At this juncture, we made a first-of-its-kind study to assess the association of AZFc subdeletion patterns among Dravidian men with SSF.

Materials and Methods

Study population and patient selection

A total of 354 Dravidian men (28 to 48 years old) born and residing in the South Indian states of Tamil Nadu and Kerala were enrolled for the study. The clinical samples were collected from April 2012 to December 2013 from leading assisted reproduction centers and private infertility clinics across Tamil Nadu and Kerala in South India. Information regarding patient's age, ethnic background, habits, genetic risk factors, medical history, occupational and environmental exposures was extracted using a structured questionnaire. Written informed consent was obtained from all the patients enrolled in the study. Semen analysis was performed according to the guidelines of the World Health Organization (2010). Azoospermia was defined as absence of sperm in the ejaculate following two centrifugations (taken at intervals of 1–3 weeks). Nonobstructive azoospermia (NOA) results from a lack of spermatozoa production in the testes, whereas in obstructive azoospermia (OA), the produced spermatozoa are unable to reach the emitted semen caused by obstruction of the epididymis or vas deferens. In oligozoospermia, the patients had sperm counts of $<20 \times 10^6/\text{mL}$. The control samples are collected from fertile men with normal spermatogenesis. The patients with OA and a medical history of chemotherapy, radiotherapy, genetic disorders, childhood disease, testicular tumor, cryptorchidism, varicocele, and habits concerning smoking, alcohol consumption, and drug intake were excluded from the study.

Serum reproductive hormone analysis

The serum reproductive hormone (including, follicle-stimulating hormone [FSH] and testosterone) (T) levels of patients, as well as male control subjects, were measured using an enzyme-linked fluorescent immunoassay method with a mini-Vidas device (BioMerieux SA). The normal male reference ranges for the serum reproductive hormones used in this study were FSH: 1.5–12.4 mIU/mL and T: 3–10.6 ng/mL.

Molecular investigation

Genomic DNA isolation. Genomic DNA was extracted from peripheral blood lymphocytes using the short spin method (Suganthi *et al.*, 2009). The concentrated DNA was diluted using RNase-free, sterile water (Qiagen) (1:1 ratios) before being analyzed by multiplex polymerase chain reactions (PCR).

Screening for classical AZF deletion. The screening for the classical AZF deletions (AZFa, AZFb, and AZFc) was carried out by means of a sequence tag site (STS)-based

multiplex PCR method using the genomic DNA isolated from all the patient and control samples. Two multiplex PCR set 1 (ZFX/Y, sY14, sY84, sY134, and sY255) and set 2 (ZFX/Y, sY14, sY254, sY86, and sY127) were designed according to the recommendations of the European Academy of Andrology/European Molecular Genetics Quality Network (EAA/EMQN) (Simoni *et al.*, 2004). The multiplex PCR comprised a total volume of 25 μL , of which 14 μL was the master mix (Qiagen), 5 μL of multiplex STS primer mix, 3 μL of diluted DNA, and 3 μL of enzyme-free Millipore water. Amplification was carried out in the thermocycler (Eppendorf AG) using the standard conditions.

Screening for AZFc subdeletions. The patients without classical AZFa, AZFb, and AZFc deletions and all control subjects were assessed for partial AZFc deletions. The STS panel used for the mapping of AZFc subdeletions was selected from previously published literature (Repping *et al.*, 2004). Two multiplex PCR set 3 (sY1191, sY1291, sY1201, and sY1161) and set 4 (sY1206, sY1054, and sY1197) were performed for detecting AZFc partial deletions. The multiplex PCR comprised a total volume of 25 μL , of which 14 μL was the master mix (Qiagen), 5 μL of multiplex STS primer mix, 3 μL of diluted DNA, and 3 μL of enzyme-free Millipore water. Multiplex PCR was carried out using the similar conditions; after an initial denaturation of 94°C for 10 min, 35 cycles were performed as follows: 94°C for 30 s for denaturation, 55°C for 45 s for primer annealing, and 65°C for 4 min for extension. The programs were followed by the final extension step at 67°C for 4 min and then cooling to 4°C to be ready for electrophoretic detection. The PCR products were separated on a 2% agarose gel (Himedia) prepared in 1 $\times$ TBE buffer containing ethidium bromide at a concentration of 0.5 $\mu\text{g}/\text{mL}$ by electrophoresis. An STS marker, which failed to amplify at least three times in a simplex PCR, was considered as deleted.

Statistical analysis

Statistical analysis was carried out by the Statistical Package for Social Science for Windows, version 16.0 (IBM SPSS). The statistical significance of differences was determined using the unpaired t-test (continuous data), chi-square (χ^2) test, and Fisher's exact test (categorical data). A p -value <0.05 was regarded as statistically significant. The odds ratios (ORs) and their confidence intervals (CI, 95%) were also calculated to find the risk of AZFc subdeletions in men with impaired spermatogenesis.

Results

A total of 354 clinical samples were collected during the study period and grouped into two categories: 229 infertile patients (including 120 NOA, 109 oligozoospermic) and 125 normal male controls. The age (mean $\pm$ SD) of men with infertility was 36.33 ± 4.54 (range 28–48 years) and normal fertile control was 35.71 ± 4.33 (range 29–45 years). The serum FSH levels were significantly higher in infertile patients (13.57 ± 7.90 mIU/mL) when compared to control samples (7.10 ± 1.89 mIU/mL) ($p=0.000$), whereas the testosterone (T) (4.55 ± 1.22 and 4.67 ± 1.05 ng/mL) levels of infertile and control groups respectively, came within the normal reference range and showed no statistical significance ($p=0.330$).

Classical AZF deletion screening

The multiplex PCR amplification using STS markers recommended by the EAA was done in all the patient samples and the normal male controls (Fig. 1). Out of the 229 infertile samples, 21 (9.17%) patients were found to carry the deletion of one or more STS markers, whereas no deletions were found in any of the normal male control samples screened. AZFc has been the most frequently deleted locus observed in our study with an estimated frequency of 52.39% (11/21) followed by AZFb/c (5/21, 23.81%), AZFb (3/21, 14.29%), and AZFa (2/21, 9.17%). A significant association was found in the FSH levels in patients with and without Y deletion (16.21 ± 7.96 and 13.31 ± 7.75 mIU/mL) when compared to the control group (7.10 ± 1.89 mIU/mL) ($p=0.000$, $p=0.000$, respectively). However, no statistical association was found between the FSH levels in patients with and without microdeletions ($p=0.104$). The testosterone (T) levels of patients with and without Y deletion (4.28 ± 1.10 and 4.57 ± 1.23 ng/mL) also show no significant statistical association when compared to the control group (4.67 ± 1.05 ng/mL) ($p=0.122$, $p=0.451$, respectively).

Partial AZFc deletion screening

Two sets of multiplex PCR were carried out to identify the gr/gr, b2/b3, and b1/b3 subdeletions (Fig. 1). After excluding 21 patients with classical AZF microdeletions, the rest of the

samples were evaluated for the presence of partial AZFc deletions. Out of 208 infertile patient samples screened, we found 13 (6.25%) gr/gr subdeletions and 15 (47.2%) b2/b3 subdeletions. In contrast, screening of 125 control men revealed 6 (4.8%) gr/gr subdeletions and 2 (1.6%) b2/b3 deletions (Table 1). The b1/b3 deletion was not observed in any of the screened patient and control samples. No statistical significance was observed in the frequency of the gr/gr subdeletions between the infertile patient samples (NOA and oligozoospermic) and normal male controls (Fisher's exact test, $p=0.386$), whereas a strong statistical difference was found in the case of b2/b3 deletions (OR, 4.78; 95% CI 1.07–21.26) (Fisher's exact test, $p=0.018$). The FSH and testosterone (T) levels of patients with and without partial deletions were also estimated and the details are shown in Table 2.

Discussion

The overall incidence of Y chromosome microdeletions in the world population is reported to be around 1% to 55.5%. More specifically, 8.3% of NOA men, 5.5% of men with severe idiopathic oligozoospermia, and 0.98–3.8% of the candidates for intra-cytoplasmic sperm injection (ICSI) are carriers of microdeletions (Van der Ven *et al.*, 1997; Foresta *et al.*, 1998; Ferlin *et al.*, 2007; Salimnejad *et al.*, 2012). It has been postulated that the variations in the frequency of Y chromosome microdeletions in different studies are mainly

FIG. 1. Gel images showing the multiplex polymerase chain reaction (PCR) analysis. **(A)** Results from classical Y chromosome microdeletion screening analysis using multiplex PCR primer set (MP1 and MP2); lane 1 and 15 show 100 bp molecular weight marker (M), lane 2 and 3 show amplified multiplex PCR bands of positive control (normal male), lane 4–11 show patient samples, lane 12 and 13 show negative control of female DNA, and lane 14 water. **(B)** Results from partial AZFc deletion screening using multiplex PCR primer set (MP3 and MP4). The arrow marks indicate the deletion in the corresponding sequence tag site marker.

TABLE 1. FREQUENCIES OF PARTIAL AZFc DELETIONS IN THE STUDY POPULATION AND THE CONTROL GROUP

Study population	No. of subjects screened	gr/gr deletion		b2/b3 deletion	
		Number (%)	OR (95% CI)	Number (%)	OR (95% CI)
Azoospermic	107	8 (7.48)	1.60 (0.54–4.77)	6 (5.61)	3.65 (0.72–18.50)
Oligozoospermic	101	5 (4.95)	1.03 (0.31–3.49)	9 (8.91) ^a	6.02 (1.27–28.51)
Infertile patients ^b	208	13 (6.25)	1.32 (0.49–3.57)	15 (7.21) ^a	4.78 (1.07–21.26)
Normal male controls ^c	125	6 (4.8)	—	2 (1.6)	—

^a $p < 0.05$, significant difference compared with group of normal male controls.

^bThe population of infertile patients is the sum of azoospermic and oligozoospermic subjects.

^cThe normal male controls are fertile men with normal spermatogenesis.

Fisher's exact test (one-sided).

CI, confidence interval; OR, odds ratio.

due to the ethnic background of the study participants or due to the stringency in patient sample selection criteria. In the Indian population, the Yq microdeletion frequency among infertile couples is estimated to be 5.8%, including 6.4% in azoospermia and 5.8% in oligozoospermic patients (Sachdeva *et al.*, 2011; Sen *et al.*, 2013; Suganthi *et al.*, 2014). In the present study, 21 (21/229, 9.17%) patients were affected with Y chromosome microdeletion. Out of the 21 (9.17%) patients, 13 (13/120, 10.83%) were azoospermic and 8 (8/109, 7.34%) were severely oligozoospermic (sperm count $< 5 \times 10^6/\text{mL}$). It is found that there is a statistically significant difference in the frequency of classical AZF deletions ($p = 0.000$) when the infertile men with impaired spermatogenesis were compared with normal male controls. The frequency observed in the present study was higher compared with the average frequency Y deletions seen in other populations, including the Indian population (Simoni *et al.*, 2008; Sen *et al.*, 2013). The variation in the frequency may be due to the stringent patient selection criteria and or due to the genetic background of the study population. Our findings strengthen the assumption that the AZF locus acts at a different phase of spermatogenesis and the deletions of each locus have a deteriorating effect on spermatogenesis and will eventually lead to the quantitative decline of spermatozoa (Kleiman *et al.*, 2007). In addition, the serum reproductive hormonal analysis reveals that the FSH concentration is a good parameter for evaluation of testicular function, but does not have any predictive value in Y deletion screening in our study population. The results are in agreement with the previous findings of Zhang *et al.* (2014).

The clinical correlation between the partial AZFc deletions and male infertility is still a matter of dispute. It has been postulated that the susceptibility and the phenotype effects of AZFc subdeletions may largely depend on the Y chromosome background of the ethnic population studied (Yang *et al.*, 2008). In the present study, we employed an STS-based multiplex PCR for identifying the three types of AZFc subdeletions (gr/gr, b2/b3, and b1/b3) in idiopathic infertile men of Dravidian origin. We found 13 (13/201, 6.47%) infertile patients (including 8 azoospermic and 5 oligozoospermic) and 6 (6/125, 4.8%) normal male controls with gr/gr deletions (Table 1). The frequency of gr/gr deletion observed in our study population (6.47%) was lower than that observed in the North Indian and Han-Chinese populations (Wu *et al.*, 2007; Shahid *et al.*, 2011) and higher compared with the populations from the United States (2.2%) and European countries (5.1%), respectively (Repping *et al.*, 2003; Machev *et al.*, 2004; Hucklenbroich *et al.*, 2005). The variation observed in the frequency of gr/gr subdeletions in our study is because of the genetic background of the study participants. A few studies have reported that gr/gr was more common in infertile men with azoo/oligozoospermia than in men with normozoospermia, suggesting that the deletion may be a significant genetic risk factor for spermatogenesis (Giachini *et al.*, 2005; Lynch *et al.*, 2005; Yang *et al.*, 2008; Shahid *et al.*, 2011). However, other reports failed to show any phenotypic impact of gr/gr deletions on the spermatogenic process. Data from our study indicate that there is no statistically significant difference in the gr/gr deletion frequency between the

TABLE 2. COMPARISON OF CLINICAL CHARACTERISTICS IN STUDY GROUPS WITH AND WITHOUT PARTIAL AZFc MICRODELETIONS

Group	gr/gr subdeletion			b2/b3 subdeletion		
	Age in years	FSH (mIU/mL)	T (ng/mL)	Age in years	FSH (mIU/mL)	T (ng/mL)
Patients with partial AZFc deletions	36.62 ± 4.81	9.74 ± 2.89 ^a	4.91 ± 1.38	35.33 ± 4.25	10.14 ± 3.16 ^a	4.38 ± 1.15
Patients without partial AZFc deletions	36.45 ± 4.46	13.55 ± 7.92 ^a	4.55 ± 1.22	36.54 ± 4.49	13.55 ± 7.95 ^a	4.59 ± 1.24
Normal male controls with partial AZFc deletions	34.67 ± 4.72	7.56 ± 1.96	4.29 ± 0.93	38.00 ± 7.07	7.95 ± 1.05	4.97 ± 0.42
Normal male controls without partial AZFc deletions	35.76 ± 4.33	7.07 ± 1.87	4.69 ± 1.06	35.67 ± 4.31	7.05 ± 1.85	4.67 ± 1.06

Values are presented as mean ± standard deviation.

^a $p < 0.05$, significant difference compared with group of normal male controls.

FSH, follicle-stimulating hormone; T, testosterone.

infertile patients and the normal male controls studied (Fisher's exact test, $p=0.386$). This result is in agreement with the previous studies that have not detected an association between the gr/gr deletion and failure of spermatogenesis (Zhang *et al.*, 2006; Wu *et al.*, 2007; Stahl *et al.*, 2011).

The b2/b3 deletion occurred as a result of the gr/gr or b2/b3 inversion. In the light of previous studies, the b2/b3 deletion was attributed as a potential genetic risk factor for spermatogenesis in different ethnic populations. When compared to the gr/gr deletion, the b2/b3 deletion increases the risk of complete AZFc (b2/b4) deletion. This predisposition may likely be due to the size and distance of the recombination substrate (Lu *et al.*, 2009). According to Repping *et al.* (2004), the b2/b3 partial deletion removes 1.8 Mb of DNA segment and found to be fixed in the Northern Eurasian population with a haplogroup N. However, a few other studies detected the presence of b2/b3 deletion in different Y haplogroups other than the N haplogroup and reported that the b2/b3 subdeletions have no significant impact on spermatogenic failure (Imken *et al.*, 2007; Zhang *et al.*, 2007; Shahid *et al.*, 2011). In contrast to this finding, a significant correlation between the b2/b3 partial deletion and spermatogenic failure was observed in the Moroccan and Han-Chinese populations (Wu *et al.*, 2007; Lu *et al.*, 2009; Eloulid *et al.*, 2012). Till this date, there is no consensus among researchers about the etiopathological role of b2/b3 deletion. In the present study, we found 15 patients with b2/b3 deletion with an estimated frequency of 7.21%. Among the 15 patients, 6 (5.61%) were azoospermic and 9 (8.91%) of them were affected with oligozoospermia. Two (1.6%) of the control samples also showed b2/b3 deletion. A significant correlation between b2/b3 deletion and spermatogenic failure was found in the Dravidian population (OR, 4.78; 95% CI 1.07–21.26) (Fisher's exact test, $p=0.018$). The observed frequency of b2/b3 subdeletion in our study population (7.21%) was lower compared with the Han-Chinese population (9.2%) and higher than the Moroccan (1.34%), Italian (0.5%), and North Indian population (1.44%) (Imken *et al.*, 2007; Wu *et al.*, 2007; Giachini *et al.*, 2008; Shahid *et al.*, 2011). The frequency of b2/b3 subdeletion was found to be higher in oligozoospermic patients (8.91%) compared with azoospermic men (5.61%). A statistically significant difference was found when oligozoospermic men with b2/b3 subdeletions were compared with normal male controls (Fisher's exact test, $p=0.012$) (OR, 6.02; 95% CI 1.27–28.51) (Table 1). The findings strengthen the speculation that the b2/b3 deletion increases the risk of complete AZFc deletion as time passes and thereby leads to SSF (Lu *et al.*, 2009). The serum FSH and T hormone level analysis revealed that these hormones have no predictive value in AZFc partial deletion screening (Table 2).

The Dravidian peoples are genetically divergent from the rest of the Indian population and an accurate distribution pattern of Y haplogroups is still not known. Moreover, the presence of haplogroup N (which is susceptible to partial AZFc deletions) was traced in the Dravidian population with an estimated frequency range of 5% to 42% (Suhasini *et al.*, 2011). In our study, the frequency of b2/b3 subdeletion was higher compared with the related studies (Repping *et al.*, 2004; Eloulid *et al.*, 2012; Rozen *et al.*, 2012). The variation in the frequency and phenotype may be due to several factors, including the experimental design, stringent patient selection

criteria, the impact of environmental factors, and the genetic background of the study population.

Conclusion

To our knowledge, this is the first study in ethnic south Indian males of Dravidian origin. A strong association between b2/b3 subdeletion and patients with SSF was found in the study population, since b2/b3 deletions have increased the risk of complete removal of AZFc region and considering the transmission of these deletions to the male offspring. Further study needs a detailed b2/b3 deletion screening along with the gene copy detection and a comprehensive haplogrouping analysis in a large and well-selected patient group to elicit the genetic mechanism behind this association.

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Author Disclosure Statement

No competing financial interests exist.

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