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Y Chromosome Microdeletions and Partial AZFc Deletions in Infertile Men from South India

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Authors' contributions

This work was carried out in collaboration between all authors. Authors JS and SBK performed the molecular analysis. Authors KS, MSS and GH clinically examined and referred the patients. Authors JS, SBK and CRS drafted the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: Yq microdeletions involving the azoospermia factor (AZF) region are the second most frequent genetic cause of spermatogenic failure next to Klinefelter syndrome. These deletions occur in about 10-15 percent of men with azoospermia and severe oligozoospermia. Molecular screening for AZF deletions has become mandatory in the work-up of infertile men. Further, partial AZFc deletions categorized as gr/gr, b2/b3, b1/b3 and b2/b4 deletions have also been known to affect spermatogenesis. This study aimed to screen for both classical AZF deletions in 250 karyotypically normal infertile men from south India and partial AZFc deletions as a case-control analysis involving 108 fertile men.
Methods: PCR amplification involving two multiplex reactions was carried out using primers for six

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STSs sY84, sY86 (AZFa), sY127, sY134 (AZFb), and sY254, sY255 (AZFc) with two internal controls (*SRY*, *ZFY*). Further, those men who showed deletions with one or both STSs sY1291 and sY1191 were subsequently tested with sY1189 and sY1192 to detect partial AZFc deletions. **Results:** One individual showed deletion of all the three AZF regions while two men had only AZFc deletion. Deletion of partial AZFb (sY127) was seen besides complete AZFc region in the fourth patient. The gr/gr, b2/b3 and b1/b3 deletions were detected in 24 (9.6%), one (0.4%) and nine (3.6%) infertile men in comparison with five, one and two fertile men respectively. The b2/b4 deletion was observed in a single azoospermic individual.
Conclusion: Screening for AZF deletions would help in not only determining the cause for male infertility but also in its management and accurate genetic counselling.

Keywords: Yq microdeletions; AZF region; partial AZFc deletion; male infertility; spermatogenic failure; azoospermia.

1. INTRODUCTION

Infertility is a major health issue of concern causing social stigma, psychological demoralization and additionally the artificial reproductive technologies are expensive and are associated with a risk of adverse pregnancy outcome. It affects one in about seven married couples and is due to the male partner in about 50% of the cases [1,2]. Besides genetic abnormalities other etiological factors underlying male infertility include congenital and acquired urogenital abnormalities, infections of the genital tract, varicocele, endocrine disorders and immunological factors [3]. Chromosomal abnormalities, Yq microdeletions, monogenic defects, multifactorial disorders, mitochondrial DNA mutations and single nucleotide polymorphisms increase the risk for infertility [4,5]. Cytogenetically visible alterations of the Y chromosome in men with idiopathic spermatogenic failure led to the suggestion by Tiepolo and Zuffardi in 1976 of an azoospermia factor (AZF) locus in Yq11.

The non-recombining or the male-specific region which constitutes about 95% of the Y chromosome is flanked on both ends by the pseudoautosomal regions. Using Y-specific probes Vergnaud et al. [6] divided the Y chromosome into 7 intervals in correlation with the naturally occurring deletions. The short arm and centromere represent the 1–4 deletion intervals (distal to proximal). The euchromatic segment of the long arm corresponds to the intervals 5 and 6 (proximal to distal) and the distal heterochromatic region constitutes interval 7. Later, Vollrath et al. [7] with about 200 sequence-tagged sites (STSs) constructed a more precise 43-interval deletion map (1A1A, 1A1B, 1A2, 1B-1E, 2A-2C, 3A-3G, 4A-4B, 5A-5Q, 6A-6F and 7) of the Y chromosome. The male-specific region of the Y chromosome includes the X-transposed, X-degenerate and the ampliconic sequences comprising of eight palindromes, P1 to P8 [8]. These palindromes share a 99.9% arm to arm homology and are believed to have resulted from tandem duplication and inversion during primate evolution [8,9]. Besides homologous recombination between these near-identical sequences, deletions via non-homologous recombination (NHR) have also been reported to occur due to the inherently unstable palindromic structure [10].

Several clinically important microdeletions affecting spermatogenesis were identified in the AZF loci within the intervals 5 and 6, namely AZFa, AZFb (P5/proximal-P1), AZFbc (P5/distal-P1 and P4/distal-P1) and AZFc (b2/b4) [8,9,11,12]. The microdeletions were reported to occur in 10–15% of men with non-obstructive azoospermia and in 5–10% with severe oligozoospermia [13,14]. The prevalence was however, found to differ widely from 1% [15] to 55% [16] largely due to differences in ethnicity, selection criteria and the number of subjects screened [17,18]. A complete AZFa deletion was reported to cause Sertoli cell-only syndrome (SCOS) while AZFb deletion led to pre-meiotic spermatogenic arrest and thus SCOS and azoospermia [11,13,18,19]. The patients with AZFc deletions show diverse phenotypic features ranging from azoospermia to mild/ severe oligozoospermia [1].

Several partial AZFc deletions have also been reported in men with spermatogenic impairment [20,21]. In a study of 20,884 men in five populations from India, Poland, Tunisia, the United States and Vietnam four interstitial deletions were observed to frequently result from nonallelic homologous recombination between

ampliconic sequences [22]. These deletions in decreasing order of prevalence were the most common 1.6Mb gr/gr deletion being detected in one of every 41 men followed by the 1.8Mb b2/b3 deletion in one of 90 men, the 1.6Mb b1/b3 deletion in one of 994 men and lastly the rare variant 3.5Mb b2/b4 deletion in one of every 2,320 men. The b2/b4 deletion was predominantly (145-fold) associated with severe spermatogenic failure (SSF). Further, the gr/gr and b1/b3 deletions increased the risk of SSF by about two-fold, while the b2/b3 deletion was found to be polymorphic and not a risk factor [22]. Conversely, the b2/b3 deletion was reported to be significantly associated with spermatogenic impairment and not the gr/gr deletion in a Han-Chinese population [23]. Furthermore, the b2/b3 partial deletion was more susceptible to complete AZFc deletion than gr/gr subdeletion [23,24]. On the other hand, Giachini et al. [25] observed gr/gr deletion to be a risk factor as it was significantly more frequent in the oligo/azoospermic group than in the normozoospermic men. In another study the gr/gr deletion was associated with reduced sperm motility in 1221 young men of unknown fertility status but not in 791 fertile men from Japan [26]. Repping et al. [20] stated that the gr/gr deletion continues to exist as a polymorphism being maintained by low penetrance and high mutability.

Extended analyses on individuals belonging to different ethnic groups and geographically diverse populations will provide valuable information on the significance of these deletions. Testicular sperm extraction followed by intracytoplasmic sperm injection (ICSI) has allowed many infertile men to beget their own children. However, there exists the likelihood of transmission of the microdeletion to their male offspring [27] and thus screening for the AZF deletions is now included in the diagnostic work-up of infertile men. This paper reports on the classical AZF deletions in infertile south Indian men and on partial AZFc deletions through a case-control analysis.

2. MATERIALS AND METHODS

2.1 Subjects

The study population consisted of 228 infertile men attending the private fertility clinics G.G hospital (n=117), Kanmani Fertility Centre (n=51) and Prashanth Fertility Centre (n=60), and 22 cases referred from government hospitals to the department from September 2012 to June 2015. These men exhibited a normal 46, XY karyotype and their wives were found to be normal upon clinical examination. The study was approved by the Institutional Human Ethics Committee and was carried out according to the Helsinski declaration. After having procured written informed consent, EDTA-coated blood samples were obtained from these infertile men from south India. Data on medical and personal history, laboratory investigations including hormonal profile and spermiogram as per WHO [28] guidelines, and family history were collected. Blood was also drawn from 108 healthy men with proven fertility and from five normal women to be used as positive and negative controls respectively.

2.2 Y Chromosome Microdeletion Analysis

Genomic DNA extracted from blood samples of infertile men by phenol-chloroform-isoamyl alcohol method [29] were screened for deletions in the three classical AZF regions on the Y chromosome adopting the EAA/EMQN guidelines [30]. The two-multiplex PCR method utilized primers for the six STS markers sY84 & sY86 (AZFa), sY127 & sY134 (AZFb), and sY254 & sY255 (AZFc). An initial denaturation at 95^{o}C for 5 min, was followed by 35 cycles of denaturation at 94℃ for 30 sec, annealing at 57℃ for 90 sec and extension at 72℃ for 60 sec, with a final extension at 72℃ for 10 min. The PCR products were resolved in 3% agarose gel and the absence of STSs was confirmed after at least three amplification failures in the presence of successful amplification using the internal controls (*SRY & ZFY*). These markers were also tested using simplex PCR method in the patients with AZF deletions.

2.3 Screening for Partial AZFc Deletions

Men belonging to both the study groups were screened for partial deletions involving AZFc in two stages using plus/minus PCR for four STSs [22]. Cases showing deletion with one or both of the STSs sY1291 and sY1191 were subsequently tested with the markers sY1189 and sY1192 to detect gr/gr and b2/b3 deletions respectively. PCR conditions consisted of an initial denaturation for 5min at 95℃, followed by 35 cycles of 30 sec at 94℃, 45 sec at 62℃ (61℃ for sY1191), and 60 sec at 72℃, with a final extension at 72℃ for 10 min. The amplified products were separated in 2% gel. A deletion involving all four markers indicated b1/b3 or

b2/b4 deletion (with a concomitant deletion of sY254) in the patient. The differences in deletion frequencies between the cases and controls were tested using Fisher's exact test and *P* values of <0.05 were considered as statistically significant.

3. RESULTS

A total of 78 cases of azoospermia, 55 men with oligoasthenoteratozoospermia (OAT), 29 oligoasthenozoospermia (OA), 8 oligoteratozoospermia, 63 asthenozoospermia, 7 asthenoteratozoospermia (AT), 9 cryptozoospermia and a single case of teratozoospermia were screened for AZF deletions. The mean age of the subjects was 35.4±6 years (range 20-53). While a family history of infertility was reported by 68 men, parental consanguinity was noticed in 61 men (Table 1). Life style habits including alcohol consumption and smoking were also observed in 83 and 50 men respectively. One individual chewed tobacco as well. The study group comprised of 45 men with varicocele. While the median values for FSH, LH and testosterone were within normal limits in the different categories of infertile men, the testosterone levels were found to vary widely in the azoospermic, AT, OA and cryptozoospermic groups (Table 2).

Four subjects (1.6 %) showed deletions for one or more AZF regions. A single azoospermic individual, IF116 showed deletions for all the three regions (AZFabc) (Fig. 1). He showed a pericentric inversion of the Y chromosome inv(Yqh) which was short [31]. Testicular biopsy revealed Sertoli cell only syndrome. On follow-up the patient was found to have a male child through ICSI with donor sperm. His father could not be investigated due to non-cooperation. The other three subjects (IF43, IF241 and IF255) had AZFc deletion and one of them (IF43) showed partial AZFb deletion (failed amplification of sY127) as well. While two men had azoospermia, one patient having only AZFc deletion exhibited oligoteratozoospermia (IF241).

Table 1. Clinical features and hormonal levels in infertile men

No. of patients	250
Age (years)	35.4±6 (20-53)[a]
BMI	26±4 (14-41)[a]
Duration of infertility (years)	7.8±4.9 (1-25)[a]
Consanguineous parents	61
Patients with history of:-	
Infertility	68 (27.2)[b]
Alcohol consumption	83 (33.2)[b]
Smoking	50 (20.0)[b]
Varicocele	45 (18.0)[b]
FSH (mIU/ml)	6 (3.4-12.3)[c]
LH (mIU/ml)	5.7 (4.1-8.4)[c]
Total testosterone (ng/dL)	253 (178.5-382.4)[c]

[a] mean ± s.d. (range)
[b] n (%)
[c] median with interquartile range

Evaluation of 250 infertile men without classical deletions revealed four types of partial AZFc deletions. The gr/gr was detected in 24 (9.6%),

Table 2. Summary of the hormonal values recorded in the different categories of infertile men based on their spermiogram

Category	FSH (mIU/ml)	LH (mIU/ml)	Testosterone (ng/ml)
Azoospermia (n = 78)	5.11[a]	5.43[a]	2.31[a]
	(3.2 - 13.7)	(3.8 - 12.1)	(0.18 – 3.81)
Oligoasthenozoospermia (n = 29)	5.8[a]	6.36[a]	2.40[a]
	(4.5 – 11.8)	(5.1 – 10.1)	(0.08 – 3.22)
Oligoasthenoteratozoospermia	7.2[a]	6.8[a]	3.08[a]
(n = 55)	(4.9 – 11.3)	(5.2 – 8.7)	(2.19 – 3.99)
Asthenozoospermia (n = 63)	4.6[a]	5.3[a]	2.70[a]
	(2.9 - 12)	(3.7 – 7.1)	(2.00 – 3.91)
Oligoteratozoospermia (n = 8)	1.78 – 9.77[b]	2.82 – 9.68[b]	2.01 – 5.43[b]
Asthenoteratozoospermia (n = 7)	2.08 – 8.65[b]	1.45 – 11.1[b]	1.24 - 11.84[b]
Teratozoospermia (n = 1)	2.78	NA	2.05
Cryptozoospermia (n = 9)	2.1 - 24.7[b]	1.56 – 9.08[b]	0.025 – 7.60[b]

[a] Median with interquartile range
[b] Range
NA: Not available

b2/b3 deletion in one (0.4%) and b1/b3 deletion in nine (3.6%) infertile men (Fig. 2). The gr/gr, b2/b3 and b1/b3 deletions were noticed in five (4.6%), one (0.9%) and two (1.9%) individuals out of 108 fertile men respectively. The deletion frequencies among infertile men did not differ significantly from those in the control group (Table 3). All these men carrying deletions tested positive for the marker sY1201. It was of interest to observe b2/b4 deletion in an azoospermic individual (IF255) (Fig. 3) which was not detected in the control group.

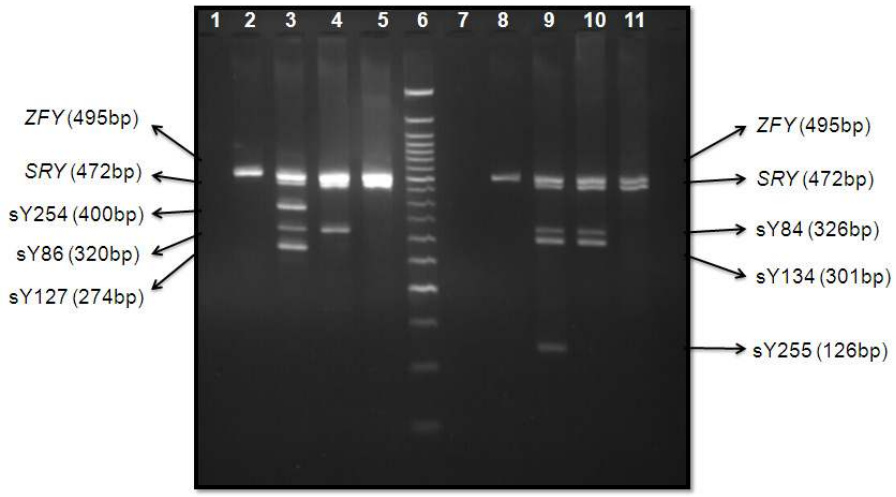

Fig. 1. Gel picture of multiplex PCR using six STSs for AZF deletions in infertile men

Multiplex A: Lane 1- Blank; Lane 2 - Control Female; Lane 3 – No deletion (IF74); Lane 4 – AZFbc deletion (IF43); Lane 5 – AZFabc deletion (IF116); Lane 6 - 50bp ladder

Multiplex B: Lane 7 - Blank; Lane 8 - Control Female; Lane 9 - No deletion (IF74); Lane 10 - AZFc deletion (IF43); Lane 11 – AZFabc deletion (IF116)

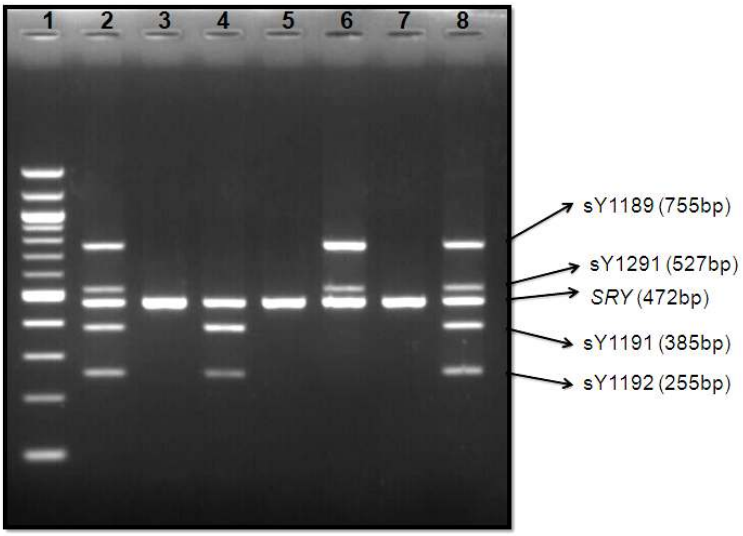

Fig. 2. Gel picture showing partial AZFc deletions in infertile men

Lane 1 - 50bp ladder; Lane 2 - No deletion (IF80); Lane 3 - b1/b3 deletion (If117); Lane 4 - gr/gr deletion (IF135); Lane 5 - b1/b3 deletion (IF146); Lane 6 - b2/b3 deletion (IF167); Lane 7 – b1/b3 deletion (IF169); Lane 8 - No deletion (CF77);

IF – Infertile individual; CF – Fertile man (control)

Table 3. Frequencies of partial AZFc deletions among infertile men and men of proven fertility status

Partial AZFc deletion	Infertile men (n = 250)		Fertile normal men (n = 108)		*P**
	Deletion	No deletion	Deletion	No deletion	
gr/gr	24	226	5	103	0.14
b2/b3	1	249	1	107	0.51
b1/b3	9	241	2	106	0.52

** P value calculated by fisher's exact test*

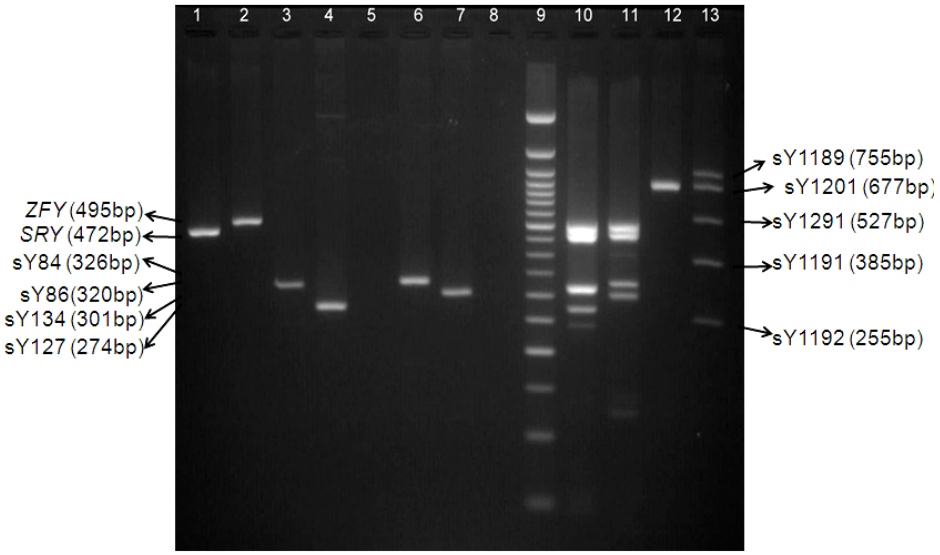

Fig. 3. Gel picture of simplex and multiplex PCR products from the patient IF255 showing b2/b4 deletion

Lane 1 – SRY; Lane 2 - ZFY; Lane 3 – sY86; Lane 4 – sY127; Lane 5 – sY254 deletion; Lane 6 – sY84; Lane 7 – sY134; Lane 8 – sY255 deletion; Lane 9 – 50bp ladder; Lane 10 – sY254 deletion (multiplex A); Lane 11 – sY255 deletion (multiplex B); Lane 12 – deletion for four STSs except sY1201; Lane 13 – Fertile man (CF98) showing all 5 bands

4. DISCUSSION

Microdeletions of the Y chromosome are the second most common cause of spermatogenic failure after Klinefelter syndrome [32]. Deletions involving the three azoospermia factor (AZF) regions, AZFa, AZFb and AZFc on Yq11 have been established to affect spermatogenesis and to cause infertility. In general, these deletions have been reported in about 15% of azoospermic and 5–10% of oligozoospermic men [1,12]. In fact, screening of AZF microdeletions in infertile men has become common in both clinical and research studies. Documented information reveals a wide geographic and ethnic variation in the incidence of Yq microdeletions. Analysis of published data on infertile Indian men also showed a wide variation (0-28%) in the frequency of Yq microdeletions with an average percentage of 7.9% (159/2,011 cases) [33]. However the authors observed a significantly lower frequency (3.4%; 56/1,636) in their study. The present investigation has shown Yq microdeletions in a lower percentage (1.6%; 4/250) of infertile men. These differences could be partly explained due to the selection criteria, the number and genetic background of the patients, and the markers used.

A patient with Klinefelter syndrome was described earlier to have partial AZFb deletion involving sY134 [18]. An azoospermic individual IF43 in the present study showed a partial AZFb deletion through failure of PCR amplification for the sY127 marker. This unusual finding needs to be confirmed using the second choice of markers sY121 for the proximal border and sY143 for the distal border [32]. Although the EAA STS markers are routinely employed for the screening of Yq microdeletions, additional non-EAA

markers have been recommended for characterization of unusual deletions [33]. Further, genes that are ubiquitously expressed or are testis-specific such as *USP9Y*, *DDX3Y*, *RPS4Y2*, *RBMY, PRY, DAZ, BPY, CDY* and *EIF1AY* have to be characterized while assessing Y chromosome integrity [12,21]. The microdeletions mostly arise as *de novo* pre-meiotic germ-line mutations and are observed in DNA obtained from both the peripheral blood leukocytes and the spermatozoa in the proband with an increased likelihood of transmission of infertility to the next generation or as post-zygotic errors and are not seen in the leukocytes. There exists the possibility of fathering a child with an intact Y chromosome in case of the latter [34].

Deletions involving the AZFc region comprised the majority of AZF deletions and were observed in three out of four patients in our study which is in concordance with published data. When the patients were categorized based on their spermiogram it was seen that AZF deletions were present in 3.9% (3 out of 78) of azoospermic men. Deletions of AZFa and AZFb regions are usually associated with azoospermia and failure of testicular sperm retrieval while AZFc deletions can lead to different degrees of spermatogenic failure ranging from oligozoospermia to the absence of germ cells in the testis [1]. The subdeletions of AZFc region namely gr/gr, b2/b3, b1/b3 and b2/b4 have also been found to be associated with male infertility [20,23,35-38]. However, controversial reports do also exist [39,40]. The prevalence of gr/gr deletion studied across five populations ranged from 2% in the United States to 15% in Vietnam and that of the b2/b3 deletion varied from 0.5% in India to 2.2% in Poland. In contrast, there was no significant variation in the prevalence of b1/b3 and b2/b4 deletions [22].

Although the gr/gr deletion was found in an increased proportion of infertile men (9.6%) than in controls (4.6%) in the present study the difference in the frequency was not significant. The b1/b3 deletion was also seen in 3.6% of the patients but only in 1.9% of the fertile men. In contrast, the b2/b3 deletion occurred in one individual each in the two groups. Sathyanarayana and Malini [41] observed only a single case of b2/b3 deletion in a married man with two children in a recent study on 200 Siddi tribal men (104 fertile) living close to Western Ghats forest in the state of Karnataka, South India, thus indicating its non-association with fertility impairment. Interestingly the b2/b4 deletion having a strong impact on the fertility status but reported to be rare was seen in a single case of azoospermia in our patient cohort.

Zhang et al. [24] also did not observe a significant association between both gr/gr and b2/b3 deletion with spermatogenic failure. However, the authors observed a predisposition of the gr/gr deletion to a complete loss of AZFc thus suggesting a two-step process. Stuppia et al. [42] reported the Y chromosome microdeletion in the infertile son to be larger in size than that seen in his father. Further, a significantly increased frequency of complete AZFc deletions was also found in the haplogroups N and Q1 that had a high frequency of partial AZFc deletions. The b2/b3 deletion was associated with the haplogroup N1 [35,39]. In some men with gr/gr deletions, subsequent gene duplication helps to restore the gene copy number [20]. Partial duplications can be analyzed by detecting copy numbers of *DAZ* and *CDY1*. More cases with partial duplications were noted in infertile men (4.0%) than in controls (0.7%) [38]. Future large scale studies should be conducted to identify additional men with deletions/duplications for a better understanding of their pathogenic significance and the effect of genetic background.

5. CONCLUSION

Both classical AZF deletions and partial deletions involving AZFc region which were rare in the tested population can underlie spermatogenic impairment and cause infertility. Further, although retrieval of sperm may be successful for most males with AZFc deletions, the potential transmission of the deletions to their male offspring is to be taken into consideration while contemplating ART. Therefore, screening for Yq microdeletions is mandatory in the management of spermatogenic failure and for accurate counseling.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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