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GENETICS

Susceptibility of gr/gr rearrangements to azoospermia or oligozoospermia is dependent on DAZ and CDY1 gene copy deletions

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Abstract
Purpose The purpose of this study was to determine the association of AZFc subdeletions (gr/gr, b1/b3 and b2/b3) and deletion of DAZ and CDY1 gene copies with male infertility
Methods Three hundred twelve controls, 172 azoospermic and 343 oligozoospermic subjects were subjected to AZFc subdeletion typing by STS PCR. Deletion of DAZ and CDY1 gene copies was done using sequence family variant analysis. Sperm concentration and motility were compared between men with and without AZFc subdeletions. Effect of the AZFc subdeletions on ICSI outcome was evaluated.
Results Amongst the three AZFc subdeletions, the frequency of gr/gr was higher in oligozoospermic (10.5 %) and azoospermic (11.6 %) men as compared to controls (5.1 %). In men with AZFc subdeltions, loss of two DAZ and one CDY1 gene copy made them highly susceptible to azoospermia and severe oligozoospermia with OR of 29.7 and 26, respectively. These subdeletions had no effect on ICSI outcome, albeit there were an increased number of poor quality embryos in AZFc subdeleted group.
Conclusion AZFc subdeletions are a major risk factor for male infertility in the Indian population. In the subjects with AZFc subdeletions, the deletion of DAZ and CDY1 gene copies increases its susceptibility to azoospermia or severe oligozoospermia. Since these deletions can be vertically transmitted to the future male offspring by ICSI, it will be essential to counsel the couples for the transmission of the genetic defect in the male offspring born after assisted reproduction and the risk of perpetuating infertility in future generation.

Capsule AZFc subdeletions are a risk factor for male infertility in the Indian population. In men with AZFc subdeletions deletions of both DAZ and CDY1 copies increases the susceptibility to infertility. AZFc subdeletion screening would be clinically relevant for diagnosis of male infertility in Indian popualtion.

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Keywords gr/gr deletions · Male infertility · Sperm concentration · CDY1 · DAZ · ICSI

Introduction

Ten to fifteen percent of couples of reproductive age are infertile and 40 % of these are due to the defects in the male partner [1]. Microdeletions involving the Azoospermia Factor loci (AZF) on the long arm of the Y chromosome (Yq) are a genetic cause of male infertility and the deletions of the AZFa, AZFb or AZFc loci alone or in combination occur in 2–10 % of men with abnormal seminogram [2–6]. Since Yq microdeletions can be vertically transmitted to the male offspring born after assisted reproduction [7, 8], screening for these microdeletions has become a part of the routine diagnostic workup for men with azoospermia or oligozoospermia [5].

Beyond the Yq microdeletions, partial deletions within the AZFc locus are also associated with male infertility. The human AZFc locus is palindromic and repetitive in nature, making it highly susceptible to intrachromosomal rearrangements during meiotic recombination. These intrachromosomal recombinations often lead to deletions/duplications and copy number variations of the eight gene families that are harboured within AZFc [9, 10]. Amongst the various possible rearrangements that can occur, three major types of AZFc subdeletions viz. gr/gr, b1/b3 and b2/b3 have been consistently identified [11–13]. The gr/gr deletions (which is most frequently observed) remove almost half the gene content of AZFc involving two of the four copies of the DAZ (deleted in azoospermia) gene, one of the two copies of CDY1 (chromodomain protein on Y, 1) and BPY2 (basic protein Y-2) genes; the b1/b3 and b2/b3 deletions also remove the similar amount of genetic material and the same genes [14]. Amongst these three, the DAZ and CDY1 are the key genes required for spermatogenesis [15–18]. The DAZ gene has four functional copies in AZFc locus that encode for a RNA binding protein and is essential to promote germ cell progression to meiosis [15, 17]. The CDY1 gene exists in two copies in the AZFc locus and encodes for chromodomain protein 1 which is postulated to be involved in the hyperacetylation of histones in the maturing spermatids [16, 18]. Interestingly, loss of DAZ gene copies lead to a reduction in its expression in the testis of infertile men [19]. Thus it is believed that loss of copies of these genes may be a cause of germ cell loss/degeneration leading to azoospermia or oligozoospermia.

The AZFc subdeletions (gr/gr, b1/b3 and b2/b3) have been detected in men with azoospermia and oligozoospermia. While in some studies the frequency of AZFc subdeletions (mainly the gr/gr) is higher in infertile men as compared to fertile controls [5, 11, 20–22]; others have failed to confirm this association [23, 24]. Based on four meta-analysis studies and a large population study involving >20,000 individuals it appears that the gr/gr deletion is a risk factor for male infertility [13, 20, 21, 25, 26]; the odds ratio is estimated to be 1.4–2.4 [5, 20, 21, 25, 26]. The other AZFc subdeletions (b1/b3 and b2/b3) occur at a lower frequency and their association with male infertility is unclear.

While the association of AZFc subdeletions with male infertility is in general observed, ethnicity has been identified to be a leading determinant for the association. It has been observed that the Europeans and the Han Chinese have a strong association of gr/gr deletions with male infertility [11, 22, 27]; the American and African men with gr/gr deletions do not seem to be susceptible to azoospermia or oligozoospermia [20, 21, 28–30]. In the Asian population, the gr/gr deletions are not associated with male infertility in the Malaysian and Japanese men; a significant association has been observed in the Korean and Chinese men [20, 21, 27, 31–33]. In the context of Indian population, the association of AZFc subdeletions and male infertility is unclear. In one study [34], gr/gr and b1/b3 but not b2/b3 deletions are associated with male infertility. However, in another study, only b2/b3 deletions were found to be associated with male infertility [35]. Thus more studies are required to determine the association of AZFc subdeletions and male infertility in Asian population.

In addition to ethnicity, the heterogeneity in the genes deleted in the AZFc locus due to the rearrangements is thought to influence the fertility outcome in the patients harbouring AZFc subdeletions. It has been observed that not all men harbouring AZFc subdeletions (based on sY1291/sY1191 based screening) have loss of both DAZ and CDY1 gene copies [34, 36]. Corroborating these observations further, copy number variations of DAZ and CDY1 genes were found to be associated with reduction in total motile sperm count in men harbouring AZFc subdeletions [37]. Recently, Lu et al. also reported that loss of individual copies of genes within AZFc also lead to impaired spermatogenesis [38]. Thus it is possible that copy number variations of the DAZ and CDY1 genes may influence the sperm concentration and clinical manifestation of the men with AZFc subdeletions. However, to the best of our knowledge, the involvement of DAZ and CDY1 gene copy deletion with phenotypic manifestations of AZFc subdeletions has not been well explored; there is no data from the Indian subcontinent in this regard.

In the present study, we aimed to determine the frequency of AZFc subdeletions in infertile men from India and to identify whether deletions of DAZ and CDY1 gene copies are determinants of the phenotypic manifestations of AZFc subdeletions. Since men with deletions of entire AZFc have poor outcome after assisted reproduction [5, 39], we also investigated if the AZFc subdeletions have any influence on fertilization rate, embryo transfer rate, embryo quality and pregnancy outcome in male partners of couples undergoing ICSI (intracytoplasmic sperm injection).

Material and methods

Ethics statement

The study was conducted independently at National Institute for Research in Reproductive Health, Mumbai and Mahatma Gandhi Institute of Medical Sciences, Sevagram, India, and was approved independently by the Institutional Ethics Committee for Clinical studies at both the centres. Written informed consent was obtained from all study participants.

Study population

Inclusion criteria

Infertile subjects that had non-obstructive azoospermia (no spermatozoa in the ejaculate) or oligozoospermia (sperm concentration $<15\times10^6$/ml) according to the WHO 2010 [40] guidelines were included in this study. The control group

involved normozoospermic men (sperm concentration ≥20× 10^6/ml) with known fertility status.

Exclusion criteria

All subjects with karyotypic abnormalities, Yq microdeletions, obstructive azoospermia, hypogonadism, hypoandrogenism, chronic diseases, history of pelvic/spinal injuries or those reported to be heavy smokers and/or alcohol intake were excluded from this study.

Determination of sample size

A pilot study was conducted on 100 normozoospermic and 50 azoospermic individuals to determine the frequency of AZFc subdeletions. Based on this, a sample size of 156 normozoospermic and 156 azoospermic men was estimated for a power of 80 % and an alpha cut-off of 5 % (ClinCalc tool, www.clincalc.com). We chose to double the number of control subjects and also enrolled an equivalent number of oligozoospermic subjects. A total of 827 subjects were analysed in this study that included 312 fertile normozoospermic, 172 azoospermic and 343 oligozoospermic individuals.

Detection of AZFc subdeletions

Genomic deoxyribonucleic acid (DNA) was isolated from the whole blood using a commercial kit (Sigma-Aldrich, St Louis., USA) and was subjected to AZFc subdeletion typing. A biplex PCR amplification using the standard sequence tagged site (STS) primer pair for sY1291/sY1191 along with SRY as the endogenous housekeeping control gene was performed. The amplification reactions were carried out using Red Amp (2X) PCR master mix (Sigma-Aldrich, St Louis., USA) for 35 cycles as detailed previously [2, 4, 6]. The primer sequences, optimized annealing temperatures and expected PCR product size are given in Supplementary Table. I. The amplified products were analysed by gel electrophoresis using 2 % agarose gel stained with ethidium bromide and observed under UV transilluminator. The AZFc subdeletions were interpreted depending upon the absence of the STS markers as reported [22]. The absence of sY1291 and presence of sY1191 represents gr/gr deletion; absence of sY1191 and presence of sY1291 represents b2/b3 deletions; absence of both sY1291 and sY1191 represent b1/b3 deletion. To distinguish between b1/b3 and b2/b4 deletions, amplification of sY1161 and sY1206 were carried out. Absence of sY1161 and presence of sY1206 represent b1/b3 deletion whereas presence of sY1161 and absence of sY1206 represent b2/b4 deletion [22].

All the patients with AZFc subdeltions were further characterized using PCR for additional five STS markers that span the entire AZFc locus. The STS markers used for the characterization and their primers have been reported [12, 22] and given in Supplementary Table I.

Detection of DAZ and CDY1 gene copy deletions

Deletions of DAZ and CDY1 gene copies were analysed using sequence family variant (SFV) analysis [41, 42]. Briefly for DAZ gene copy deletions, sY581 and sY587 were amplified and digested overnight using *Sau*3AI and *Dra*I restriction enzyme (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA), respectively. For CDY1 gene copy deletion, a C/A SFV located 7750 bp 5′ of the CDY1 translation start codon was chosen which was amplified and digested overnight using *Pvu*II restriction enzyme (Thermo Fisher). The primer sequences, optimized annealing temperatures and expected PCR product size for sY581, sY587 and CDY1 loci are given in Supplementary Table I.

To validate the DAZ copy deletions, we performed analysis of DAZ2 by SFV and DAZ3 by an STS PCR as described earlier [41, 43]. For DAZ2, PCR amplification was done using sY586 and digested using *Taq*I restriction enzyme (Thermo Fisher). DAZ3/4 deletions were confirmed by STS PCR using DAZ3-specific primers which specifically amplify the 3′ end of DAZ3 gene copy, as described [43].

All PCR-RFLP analysis was independently repeated three times using separate pools of genomic DNA.

Assessment of ART/ICSI outcome

To test the association of AZFc subdeletions with ART outcome, we enrolled 249 subjects that chose to undergo ICSI for biological parenthood. These subjects were carefully chosen to exclude conditions that might affect embryo outcome. All the female partners did not have any endocrine abnormalities, evidence of PCOS or endometriosis. Couples were excluded if there were more than 20 % of poor quality oocytes obtained at retrieval. All the female partners were essentially normo-responders.

The protocol for ovarian hyperstimulation, oocyte retrieval, ICSI and embryo culture has been detailed elsewhere [44, 45]. A successful fertilization was considered with the formation of two polar bodies and two distinct and opposing pronuclei (PN) with evenly distributed nucleoli 16–18 h after ICSI. Fertilization rate was calculated as the number of oocytes fertilized and undergone first cleavage with respect to the number of oocytes microinjected.

Evaluation of embryo cleavage and quality was done 40–44 h after ICSI and defined as grade 1, grade 2 and grade 3 as described earlier [44, 45]. Briefly, grade 1 embryos were defined as blastomeres of equal size without cytoplasmic fragments. Grade 2 embryos were defined as blastomeres of equal size with minor cytoplasmic fragments or blebs. Grade 3 embryos were defined as blastomeres of unequal size with

significant cytoplasmic fragmentation. A maximum of three embryos were transferred per ART/ICSI cycle. Pregnancy was first confirmed by measuring serum β hCG concentration 2 weeks after embryo transfer followed by ultra-sonography done 2 weeks after measuring β hCG levels using commercial assays.

Statistical analysis

Analysis was carried out using the SPSS software for Windows (SPSS Inc., Chicago, IL, USA, version 16). The frequency of AZFc subdeletions and its subtypes were compared between azoospermic/oligozoospermic individuals and controls using the Chi square test/Fishers test with the OpenEpi tool (Dean AG, Sullivan KM, Soe MM, www.openepi.com, version 3.03). Probability (p) values <0.05 were regarded as statistically significant. Differences of sperm concentration and percentage motile sperm between classical, subtype 1, subtype 2, subtype 3 and non-deleted cases were examined by using Kruskall Wallis test. The AZFc subdeletion types and the risk of male infertility were estimated by computing the odds ratio (OR), 95 % Confidence Interval (CI) and probability (p) values using the OpenEpi tool. Fertilization rate was assessed using t test. Pregnancy rate, embryo transfer rate and embryo grade was analysed using Chi square test/Fishers test with the OpenEpi tool.

Results

Frequency of AZFc subdeletions

In this study, a total of 72/827 subjects (8.7 %) had any type of AZFc subdeletion (gr/gr, b1/b3 and b2/b3). 16/312 controls (5.1 %), 20/172 azoospermic subjects (11.6 %) and 36/343 oligozoospermic subjects (10.5 %) had AZFc subdeletions. The frequency of AZFc subdeletions was significantly higher in the infertile men as compared to controls (10.9 vs 5.1 %).

Of the 72 cases with AZFc subdeletions, 59 cases had gr/gr, 5 cases had b1/b3 and 8 cases had b2/b3 deletions (81.9, 6.9 and 11.1 %, respectively). The frequency of gr/gr deletions in azoospermic and oligozoospermic subjects were significantly higher as compared to controls (Fig. 1). There were no significant differences in the frequency of b1/b3 or b2/b3 deletions in azoospermic and oligozoospermic subjects as compared to controls (Fig. 1). The OR, 95 % CI and p values for this group are given in supplementary table II. All the subjects with b1/b3 deletions showed the absence of sY1161 and presence of sY1206. Thus there were no individuals with b2/b4 deletion in this study population. The individuals that were detected to harbour a deletion based on sY1291 and sY1191 screening were further characterized using a panel of five STS markers.

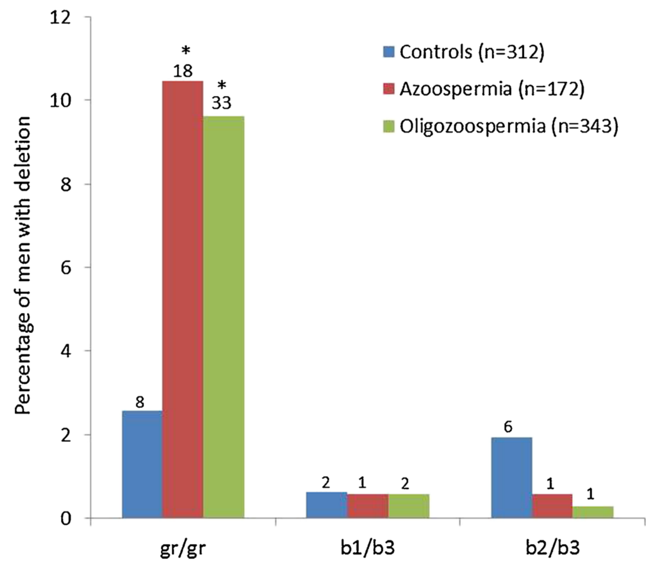


Fig. 1 Frequency of AZFc subdeletions in fertile and infertile men. n are the number of men assessed for each fertility phenotype. *Values above bars* are the number of men with deletion. *Asterisk* indicates values statistically significant ($p<0.05$) as compared to controls

All the 72 individuals showed an expected pattern of deletion (Supplementary Table III).

To strengthen the statistical analysis we pooled the data derived from this study and also reported earlier in the Indian population [34, 35]. The results revealed that the gr/gr deletions were significantly higher in azoospermic and oligozoospermic men. The b1/b3 deletions were significantly higher only in the azoospermic men. The b2/b3 deletions however did not show any significant association with male infertility. The data for this pooled estimate is given in Supplementary Table IV.

Subtypes of AZFc subdeletion and its frequency

All the individuals that were detected to have AZFc subdeletions were further screened for presence of DAZ and CDY1 copies using SFV analysis. All the individuals that had deletions of DAZ1/2 copies were further subjected to analysis of DAZ2 and those that had DAZ3/4 deletions were screened for DAZ3. The results revealed that all the 30 individuals that had DAZ1/2 deletions had DAZ2 deleted; all the 14 subjects with DAZ3/4 deletions had DAZ3 deleted thereby validating the results derived from SFV screening.

Based on the presence or absence of DAZ and CDY1 gene copies deleted (irrespective of gr/gr, b1/b3 and b2/b3), men with AZFc subdeletions could be subclassified in four major categories as shown in Supplementary Table V. Normally there are four copies of DAZ gene and two copies of CDY1 gene in the AZFc locus. A classical deletion was defined as presence of any of the AZFc subdeletions (gr/gr, b1/b3 or b2/b3) with two copies of DAZ and 1 copy of CDY1 gene deleted. Individuals that had all four copies of DAZ but one copy of

CDY1 gene deleted were considered as subtype 1 deletion. Individuals with two copies of DAZ deleted but had both the copies of CDY1 gene present were considered as subtype 2 deletion. Individuals with no deletion of either DAZ or CDY1 gene copies but had AZFc subdeletions (based on sY1291/sY1191 screening) were considered as subtype 3 deletion.

The classical AZFc deletions were significantly higher in azoospermic and oligozoospermic subjects as compared to controls (Fig. 2). However, the frequency of AZFc subtype 1, subtype 2 and subtype 3 deletions were not significantly different in azoospermic and oligozoospermic subjects as compared to controls (Fig. 2). The OR, 95 % CI and p values for this group are given in Supplementary Table VI.

Association of AZFc subdeletion subtypes with sperm count and motility

Information was available for 655 subjects (excluding azoospermia) on sperm concentration and motility where 52 had AZFc subdeletions and the remaining 603 were without any deletions. Amongst the 52 deleted cases, 21 had classical, 11 had subtype 1, 6 had subtype 2 and 14 had subtype 3 deletions. The sperm concentration and percentage motile sperm were significantly lower in cases with classical deletions as compared to non-deleted cases. Subjects with subtype 1, 2 and 3 deletions had sperm concentration and motility comparable to non-deleted subjects (Fig. 3).

Odds ratio of AZFc subdeletion on sperm concentration

To determine if the occurrence of AZFc subdeletions can be predicted based on sperm concentration we calculated the OR for total AZFc subdeletions and classical deletions based on sperm concentration. The OR for any AZFc subdeletion in azoospermic men was 2.4, in men with sperm concentration $0.1–5\times10^6$/ml was 2.8 and in men with sperm concentration $5–20\times10^6$ /ml was 1.6 (Table 1). The OR for classical deletion in azoospermic men was 29.7, in men with sperm concentration $0.1–5\times10^6$/ml was 26 and in men with sperm concentration $5–20\times10^6$ /ml was 13 (Table 1).

Association of AZFc subdeletions with ART/ICSI outcome

Two hundred forty-nine subjects included in this study opted for ICSI for biological parenthood. Of these 10 cases had AZFc subdeletions and 239 did not have any deletions. The data for this group is given in Table 2. There was no difference in mean fertilization rate between the men with or without AZFc subdeletions (77 % vs 78 %). Of the 239 non-deleted cases, embryo transfer was not done in nine cases due to unavailability of good quality embryos or inadequate uterine thickness. Thus the embryo transfer rate was compared for 10 deleted and 230 non-deleted subjects. The embryo transfer rate was comparable between the AZFc subdeleted and non-deleted cases. The numbers of grade III embryos were higher in men with AZFc subdeletions as compared to non-deleted cases; however, this difference was not statistically significant.

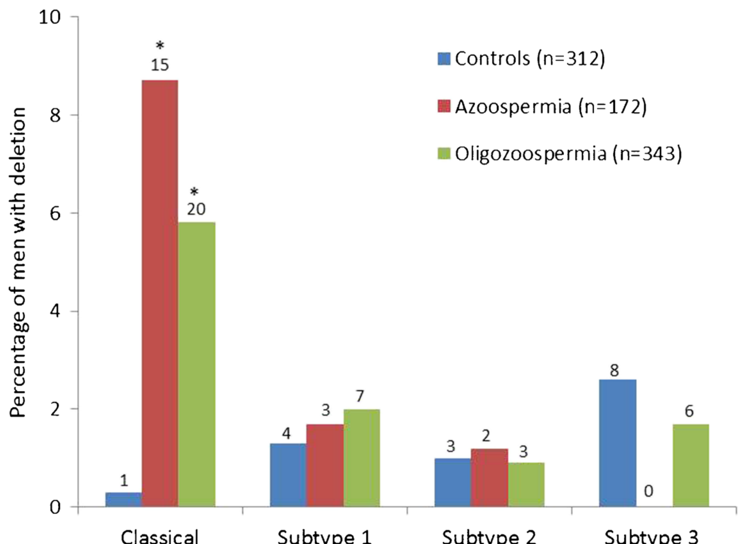
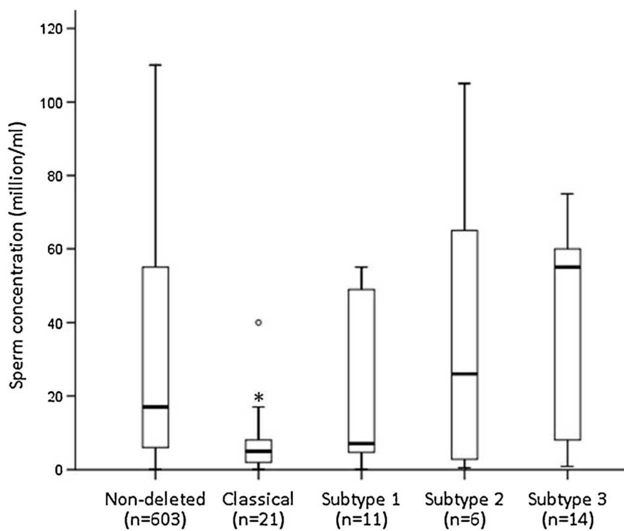


Fig. 2 Frequency of classical, subtype 1, subtype 2 and subtype 3 deletions in fertile and infertile men. *n* are the number of men assessed for each fertility phenotype. *Values above bars* are the number of men with deletion. The STS markers present (*plus sign*) or absent (*minus sign*) and the number of copies of DAZ and CDY1 gene in each category are given below the group. *Asterisk* indicates values statistically significant ($p<0.05$) as compared to controls



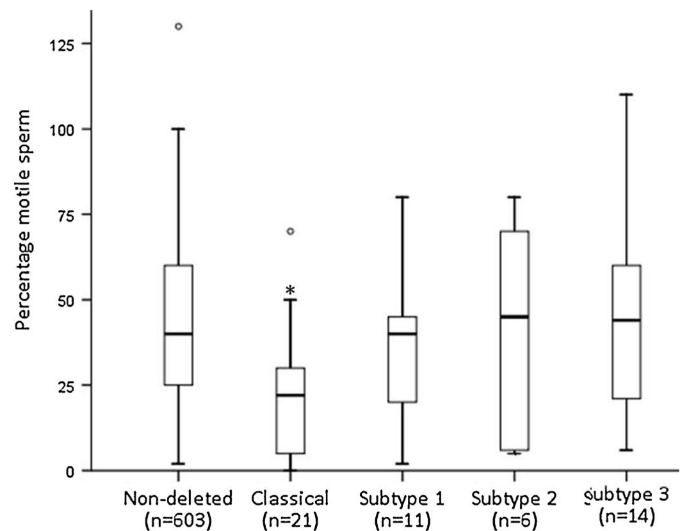


Fig. 3 Sperm concentration (million/milliliter) and percentage motile sperm in men with and without AZFc subdeletions. The men with AZFc subdeletions were classified as described in Supplementary Table V. *n* are the number of men assessed in each group. *Small white circle* indicates the outliers. *Asterisk* indicates values statistically significant ($p<0.05$) as compared to controls

There was no difference in pregnancy rate in the group with AZFc subdeletions as compared to non-deleted group.

Discussion

In the present study, we carried out a systematic analysis of AZFc subdeletions in the Indian population and demonstrate that the gr/gr and b1/b3 deletions are associated with male infertility. We further demonstrate that men with AZFc subdeletions having two copies of DAZ and one copy of CDY1 gene deleted are highly predisposed to azoospermia or severe oligozoospermia. These AZFc subdeletions however do not affect the fertilization rate, embryo transfer rate, embryo quality or pregnancy rate.

In accordance with previous studies [13, 20–22], the gr/gr deletions were found to be most prevalent whereas the b1/b3 and b2/b3 deletions were of rare occurrence in the Indian population. Amongst these, the frequency of gr/gr but not b1/b3 and b2/b3 deletions was significantly higher in men with azoospermia or oligozoospermia as compared to fertile controls, none of the individuals had b2/b4 deletions. This data is in agreement with previous studies in Indian population wherein gr/gr but not b2/b3 was found to be significantly higher in infertile men as compared to controls [34]. The involvement of b1/b3 is yet controversial. In the present study and that reported recently [35], b1/b3 deletions were not found to be associated with male infertility; however, Shahid et al. [34] found a significantly higher numbers of infertile subjects with b1/b3 deletions. At present, the reason for such discrepant findings is unknown; to rule out an effect of sample numbers, we pooled the data from all the three studies and analysed the data statistically. The results of the pooled data revealed that gr/gr and b1/b3 but not b2/b3 deletions are associated with male infertility. The OR for gr/gr deletions is 2.8 and 5.7 for b1/b3 deletion in infertile men. This OR is higher as compared to those reported in meta-analyses and large

Table 1 Odds ratio for any AZFc subdeletion or classical deletion based on sperm concentration as compared to men with normal sperm concentration ($\geq 20\times10^6$/ml)

Sperm concentration (million/ml)	OR	95 % CI	*p*
AZFc subdeletions			
0	2.4	1.2, 4.8	0.02
0.1–5	2.8	1.4, 5.5	3.6×10^{-3}
5–20	1.6	0.8, 3.4	0.3
Classical			
0	29.7	3.9, 227	2.8×10^{-6}
0.1–5	26.0	3.4, 201.2	1.6×10^{-5}
5–20	13.0	1.6, 106.8	7.7×10^{-3}

$p<0.05$ is considered as statistically significant

Table 2 Effect of AZFc subdeletions on embryo quality and pregnancy outcome

	Non deleted (*n*=230)	AZFc subdeleted (*n*=10)
No of embryos transferred (%)	734/1,840 (39.9)	28/60 (46.7)
Embryo grade I (%)	1,436 (84.8)	30 (75)
Embryo grade II (%)	144 (8.5)	4 (9.2)
Embryo grade III (%)	117 (6.9)	7 (15.9)
Pregnancy rate (%)	59 (25.7)	3 (30)

n are the number of men assessed in each group

population-based studies [20, 21, 25, 26] suggesting that both gr/gr and b1/b3 deletions are strong predisposing factors for male infertility in Indian population.

Although gr/gr and b1/b3 deletions seem to be a risk factor for male infertility, there are normozoospermic fertile men with AZFc subdeletions, moreover b2/b3 deletions which remove almost identical amount of genetic material (and the same genes) do not predispose an individual to male infertility. What protects these men from the pathogenic effect of the deletion is presently unknown. In the present study, we observed that not all men with AZFc subdeletions had the expected deletion of DAZ and CDY1 gene copies. As reported previously [36], there were several men who had DAZ and/or CDY1 gene copies intact despite having gr/gr, b1/b3 or b2/b3 deletion. To determine the impact of the DAZ and CDY1 copy deletions, we classified the men with AZFc subdeletions (gr/gr, b1/b3 and b2/b3) based on the DAZ and CDY1 gene copies deleted. Four types of rearrangements viz the classical, subtype 1, subtype 2 and subtype 3 deletions were observed. Amongst these, only the classical deletion (involving removal of two DAZ gene copies and one CDY1 gene copy) was found to be strongly associated with azoospermia and severe oligozoospermia; the sperm concentration and motility were also significantly low in this group as compared to other subtypes. The frequency of subtypes 1, 2 and 3 deletions (where either one of the DAZ or CDY1 gene copies were deleted or both intact) were not significantly different between control and infertile subjects; sperm concentration and motility was comparable to those without deletion. These findings suggest that in men with AZFc subdeletions (irrespective of the type), loss of copies of both DAZ and CDY1 genes predispose an individual to compromised spermatogenesis, retention of copies of either DAZ or CDY1 have a protective effect.

In the present study, we observed that only 50 % (36/72) of men who had AZFc subdeletions had both DAZ and CDY1 gene copies deleted and only in these men the sperm concentration and motility were lower as compared to men without deletions. Thus based on this analysis we could argue that the sY1291/sY1191 based screening strategy might be associated with false detections. Indeed based on a similar strategy, Krausz et al., reported a false deletion rate of 5.3 % [36]; in the present study it was calculated as 19.4 % (14/72). This was not due to PCR failures as a biplex reaction was optimized where the housekeeping SRY was always co-amplified. In addition, all those individuals who reported a deletion were re-verified blindly by a second observer and finally the PCR was performed at a lower annealing temperature to rule out any effect of polymorphism at primer binding site. In all cases reported herein, we always detected the deletion under all the conditions implying that this observation was not due to a technical failure. We assume that the AZFc subdeletions detected in men with intact DAZ and/or CDY1 gene copies could be due to some complex rearrangements. Since these men have sperm concentration and motility identical to non-deleted subjects, we can presume that the deletions observed may be false detection. It is possible that the failure to detect the association of AZFc subdeletions with male infertility in previous studies could be due to such false detection. Indeed, in this study we observed that 5 % of men with normozoospermia had AZFc subdeletions; however upon refining the data based on DAZ and CDY1 gene copies only 0.3 % of them (1/312) had AZFc classical subdeletions, the remaining controls were of other subtypes. These observations prompt us to suggest that clinically it will be essential to evaluate the DAZ and CDY1 status of men with AZFc subdeletion to rule out false detection.

To determine the usefulness of AZFc subdeletion screening in clinical setting, we next asked if sperm concentration can be used a predictive marker for occurrence of AZFc subdeletions. The results demonstrated that the risk of harbouring a classical deletion is extremely high in men with azoospermia (OR 29.7) and severe oligozoospermia (OR 26). Based on this observation we propose that AZFc subdeletion screening followed by DAZ and CDY1 gene copy deletion should be offered in a clinical setup. Since most men with azoospermia and severe oligozoospermia undergo ICSI for biological parenthood, it is obvious that like the microdeletions, the AZFc subdeletions will also be vertically transmitted to their sons. Considering the fact the AZFc subdeletions by themselves confer high risk of azoospermia and oligozoospermia and also the fact that the subdeletions have a propensity to develop into full AZFc deletions in the next generation [46], it may be imperative to offer this testing in clinics and also counsel these men for this risk prior to offering ICSI.

We and others have earlier shown that men with oligozoopsermia have poor outcomes in assisted reproductive technology program despite use of ICSI [45, 47]. Also, men with Yq microdeletions have poor embryo quality and lower success rates of ICSI as compared to non-deleted counterparts [5, 39]. However, the contribution of AZFc subdeletions on outcome of assisted reproduction is unknown. In the present study, we observed that fertilization rate, embryo transfer rate and pregnancy rate were identical in men with and without AZFc subdeletions; however, men with AZFc sudeletions had higher frequency of poor quality embryos as compared to non-deleted controls (albeit this difference failed to reach a statistical significance). Owing to the limited sample size, the stratification of the samples based on the type of deletions was not possible and hence the contribution of the heterogeneity in the gene content to these parameters cannot be probed. However these preliminary observations do indicate that the AZFc subdeletions may affect embryo quality, it will be necessary to take up larger systematic study and determine the consequence of AZFc subdeletions on outcome after ICSI.

Conclusion

The results in the present study demonstrate that AZFc subdeletions are a high-risk factor for impaired spermatogenesis. Refinement of the type of subdeletion based on the DAZ and CDY1 gene copy deletions, demonstrate that deletion of both DAZ and CDY1 gene copies is pathogenic leading to poor semen quality. We propose that a two-step strategy based on sY1291/sY1191 marker followed by DAZ and CDY1 gene dosage analysis should be carried out to rule out the involvement of AZFc subdeletions with male infertility. Therefore our study implies that AZFc subdeletion screening may be considered as a routine practice in a clinical setting.

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Authors' contribution S Sen: Sample collection, experimental standardization and work, data analysis and manuscript preparation

P Ambulkar: Sample collection, experimental standardization and work, data analysis and manuscript preparation

I Hinduja: Study design, recruitment of ICSI candidates and manuscript preparation

K Zaveri: Study design, recruitment of ICSI candidates and manuscript preparation

J Gokral: Initial conceptualization of the study, study design, patient recruitment and manuscript preparation

A Pal: Conceptualization of the project, data collection, data analysis, manuscript preparation and overall coordination

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