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Insight into Genetic × Epidemiological factors in male infertility: synergistic effect of AZFc partial deletions and habits of smokeless-chewing tobacco

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Abstract

The Y chromosome AZF partial deletions exhibit variations in its association with male infertility across the population divides, and intriguing. Here we have analysed distinct partial deletions (gr/gr, b1/b3 and b2/b3) of the AZFc region among the 728 Bengali-speaking men and compared them with 264 age-matched proven-fertile control subjects. The gr/gr deletion was found to be frequent among azoospermic (P = 0.001) and oligozoospermic (P = 0.03) subjects, and b1/b3 deletions were detected to be significant among severe-oligozoospermic men (P = 0.0405). Furthermore, we analysed the interactions of these deletions with the habits of smokeless chewing tobacco among the participating subjects, taking opportunity of large epidemiological data of the participating subjects. The logistic regression model revealed that the infertile subjects bearing any type of microdeletion and also SCT users had an elevated risk of infertility (P = 0.002). Our work helps to get more insight into the cause of male infertility in the light of gene-environment interaction (G × E) and brings us a significant step closer towards understanding the aetiology of spermatogenesis failure in men.

Introduction

Infertility is one of the major health challenges across the globe, and ~ 15% of couples suffer from the issue of infertility. The underpinning cause of infertility includes both male and female factors, and in nearly 50% of cases, the cause of male infertility is of genetic origin [1, 2]. Among all the genetic factors, Y chromosomal microdeletions (YCMDs) are the most prevalent structural anomaly [3, 4]. Azoospermia factor (AZF) region, located on the distal arm of the Y chromosome (Yq), acts as a hotspot for microdeletion [5]. It carries functionally active genes that control testicular maturation and differentiation related to spermatogenesis [6, 7]. Among all the Y chromosomal AZF microdeletions so far recorded, the AZFc deletion was found to be the most prevalent (about 80%) among azoospermic and severe oligozoospermic men, followed by AZFb (1–5%) and AZFa (0.5–4%) deletions [7–9].

The AZFc region, spans approximately 4.5 Mb of distal part of the Yq, is one of the most comprehensively studied AZF loci due to its high deletion frequency and it harbours multicopy genes associated with male infertility, resulting in variable outcomes from mild to severe spermatogenic dysfunction [10–13]. In addition to classical AZFc complete (b2/b4; ~3.5Mb) deletions, three major partial deletions within the AZFc sub-region have been identified using STS genotyping. These are gr/gr, b1/b3, and b2/b3 deletions, which eliminate about 1.6 Mb, 1.6 Mb, and 1.8 Mb segments from the AZFc locus, respectively (Fig. 1) [14–16]. Among the AZFc partial deletions, the most prevalent is gr/gr deletion [17], results in the removal of two of the four copies of DAZ (deleted in azoospermia) gene, one of the two CDY1 (chromodomain protein on Y, 1) and BPY2 (basic protein Y, 2) genes. Thus, gr/gr deletions lower the number of copies of gene families on the Y chromosome but do not fully eliminate any genes exclusive to the testis. In comparison to gr/gr deletion, the b2/b3 is a slightly larger deletion (1.6 Mb versus 1.8 Mb) and removes almost the same genetic segment as the gr/gr deletion, while the b1/b3 deletion, which differs from the gr/gr and b2/b3 deletions by its overlap with the part of the AZFb segment, results in the loss of the six copies of RBMY1 (ribonucleic acid binding motif, Y, 1) and both functional copies of PRY (PTPN13 Like Y-Linked) gene [18, 19].

Beside genetic risk, epidemiological risk factors such as an incorrect lifestyle and addiction have been reported as risk factors for infertility among men [20, 21]. Different chemical and physical agents [22], including the use of tobacco (both in smoke and chewing forms), have been identified as potential epidemiological risk factors that impair spermatogenesis. Tobacco use is known to affect sperm concentration, motility, and morphology, as well as cause DNA damage [23–28]. Surprisingly, studies on the combined effects of genetic risk factors like partial Y microdeletion and tobacco exposure on infertility among males are almost lacking. But it is of the utmost interest to know how genetic and habitual risk factors act synergistically to increase the risk of spermatogenic impairment.

To explore this possibility, we designed the current study considering Y-choromosomal AZFc partial deletion as a genetic risk and habits of smokeless chewing tobacco (SCT) as an epidemiological/habitual risk. We have taken opportunity of a large Indian Bengali male population sample that is unique owing to their SCT use habits. We tried to address two basic research questions. First, is SCT use associated with the incidence of AZFc partial deletion? Second, if these two factors are associated, how do they interact with each other to increase the risk of infertility among men?

We, for the first time ever, have taken the initiative to get a holistic overview and insight into the multifactorial risk of spermatogenic failure, which will reveal the Gene × environment model for male infertility.

Results

Prevalence of AZFc partial deletion:

We scored an incidence of 7.06% AZFc partial deletions in our sample cohort, including cases and controls (70 out of 992). Frequency was recorded much higher in the infertile group than in the control fertile group; we found 8.52% (62 out of 728) and 3.03% (8 out of 264) of participants carried partial deletions from the case and control groups, respectively. The frequency distribution of each type of partial deletion and its combination among the case sub-groups and fertile controls is presented in Table 3.

Table 1. The STS primer sets used in the study show annealing temperature and product size. bp base pair

STS primers	Primer sequence Forward (F) and Reverse (R)	Annealing temp. (°C)	Product size (bp)
sY1191	F : 5'-CCAGACGTTCTACCCTTTCG- 3' R : 5'-GAGCCGAGATCCAGTTACCA- 3'	59	385
sY1291	F : 5'-TAAAAGGCAGAACTGCCAGG - 3' R : 5'- GGGAGAAAAGTTCTGCAACG - 3'	59	527
sY1161	F : 5'-CGACACTTTTGGGAAGTTTCA - 3' R : 5'-TTGTGTCCAGTGGTGGCTTA- 3'	56	377
sY1206	F : 5'-ATTGATCTCCTTGGTTCCCC - 3' R : 5'-GACATGTGTGGCCAATTTGA - 3	55	394
sY1201	F : 5'-CCGACTTCCACAATGGCT- 3' R : 5'-GGGAGAAAAGTTCTGCAACG- 3'	65	677
sY14 (SRY)	F: 5'-GAATATTCCCGCTCTCCGGA- 3' R: 5'-GCTGGTGCTCCATTCTTGAG- 3'	57	470

Table 2. Deletion specific coverage of STS makers in AZFc regions. For each STS marker, plus (+) and minus (-) signs, respectively, denote the presence or absence of the amplicon.

Type of AZFc partial deletion	Y chromosome AZFc partial deletion specific STS primers				
	sY1161	sY1191	sY1291	sY1206	sY1201
gr/gr deletion	+	+	-	+	+
b1/b3 deletion	-	-	-	+	+
b2/b3 deletion	+	-	+	+	+

Table 3
Incidence of AZFc partial deletions in different case sub-groups and fertile controls.

Subjects	Sample size (N)	AZFc partial deletion			
		gr/gr (%)	b1/b3 (%)	b2/b3 (%)	Total (%)
Azoospermic	332	32 (9.64; 32/332)	4 (1.20; 4/332)	0	36 (10.84; 36/332)
Oligozoospermic	93	8 (8.60%; 8/93)	2 (2.15; 2/93)	0	10 (10.75; 10/93)
Severe-oligozoospermic	67	3 (4.48; 3/67)	2 (2.99; 2/67)	0	5 (7.46; 5/67)
Asthenozoospermic	62	0	0	0	0
Normozoospermic	174	11 (6.32; 11/174)	0	0	11 (6.32; 11/174)
All Cases	**728**	**54** **(7.42; 54/728)**	**8** **(1.10; 8/728)**	0	**62** **(8.52; 62/728)**
Controls (Proven fertile)	264	8 (3.03; 8/264)	0	0	8 (3.03; 8/264)
Total (Cases + Controls)	992	62 (6.25; 62/992)	8 (0.81; 8/992)	0	70 (7.06; 70/992)

gr/gr deletion:

We found 54 (7.42%) gr/gr deletions among 728 case samples. Of them, 32 (9.64%) were found in azoospermic, 8 (8.60%) in oligozoospermic, 3 (4.48%) in severe-oligozoospermic and 11 (6.32%) in infertile-normozoospermic sub-groups, respectively. Out of 264 fertile control males, 8 (3.03%) exhibited the presence of this deletion. No such deletion was found in the asthenozoospermic sub-group. The frequency of gr/gr deletion was found to be significantly different in pair-wise comparison among the azoospermic, oligozoospermic, and fertile control groups, with P = 0.011, P = 0.0015, and P = 0.0382, respectively. We did not find a significant association for severe oligozoospermic (P = 0.4703) and normozoospermic (P = 0.1483) groups when compared to that of the control group.

b1/b3 deletion:

We observed a b1/b3 deletion in 8 (1.10%) subjects from infertile groups, while no such deletion was detected among the fertile controls. In the infertile group, 4 (1.20%) patients were from the azoospermic, 2 (2.15%) were from the oligozoospermic, and 2 (2.99%) were from the severe-oligozoospermic subgroup. However, this type of sub-deletion was unidentified, neither in normozoospermic nor in asthenozoospermic individuals. However, when we compared to control, we did not obtain any significant difference in all pair-wise comparisons (P = 0.0707), azoospermic (P = 0.1335) and oligozoospermic (P = 0.0673) except between control and the severe oligozoospermic (P = 0.0405) sub-group.

Combined incidence of AZFc sub-deletions:

We combined all sub-deletions (gr/gr and b1/b3, no b2/b3 sub-deletions were observed) and found 62, i.e., 8.51% of the infertile subjects (N = 728), carried deletions in contrast to 8, i.e., 3.03% of the fertile controls (N = 264). Of the 62 partial deletion-bearing case subjects, 36 (10.84%) were from the azoospermic (N = 332) group, 10 (10.75%) from the oligozoospermic (N = 93) group, 5 (7.46%) from the severe oligozoospermic group (N = 67), and 11 (6.32%) from the infertile normozoospermic (N = 174) group. Thus, the frequency of sub-deletions (combining all partial deletions) was much higher among the subjects than in fertile controls, and the difference was found substantially significant (P < 0.05).

The summary of the results of the association study for all the partial deletions with their respective odd ratio (OR), 95% confidence interval (CI), and P values at < 0.05 level of significance is presented in Table 4.

Table 4
Association of AZFc partial deletions with the cases and controls. Fisher's exact test was done to compare test groups and P value was fixed at < 0.05. N/n n odd ratio, CI confidence interval.

Subjects	AZFc partial deletion										Combination of AZ	
	gr/gr				b1/b3				b2/b3			
	Frequency, n (%)	OR	95% CI	P value	Frequency, n (%)	OR	95% CI	P value	Frequency, n (%)	P value	Frequency, n (%)	OR
Controls (N = 264) (Proven fertile)	8 (3.03%)	-	-	-	0	-	-	-	0	-	8 (3.03%)	-
Cases (N = 728)	54 (7.42%)	2.564	1.203–5.463	**0.0111***	8 (1.10%)	7.731	0.451–132.48	0.0707	0	-	62 (8.51%)	2.97
Azoospermic (N = 332)	32 (9.64%)	3.413	1.545–7.541	**0.0015***	4 (1.20%)	7.247	0.388–135.30	0.1335	0	-	36 (10.84%)	3.89
Oligozoospermic (N = 93)	8 (8.60%)	3.012	1.096–8.273	**0.0382***	2 (2.15%)	14.454	0.687–304.10	0.0673	0	-	10 (10.75%)	3.85
Severe-oligozoospermic (N = 67)	3 (4.48%)	1.5	0.387–5.816	0.4703	2 (2.99%)	20.191	0.957–425.97	**0.0405***	0	-	5 (7.46%)	2.58
Asthenozoospermic (N = 62)	0	-	-	-	0	-	-	-	0	-	0	-
Normozoospermic (N = 174)	11 (6.32%)	2.16	0.850–5.484	0.1483	0	-	-	-	0	-	11 (6.32%)	2.16

***** indicates value significantly different from fertile counterpart

Genetic × Environmental risks: Synergistic effect of AZFc partial deletion and SCT use:

We observed frequent occurrences of SCT use (gutkha, chopped tobacco leaf and jarda) among the participating subjects through the scouting of information collected in family records. We decided to take this opportunity of this unique population sample to look into whether SCT use has some synergistic effect along with partial microdeletion on male infertility. In other words, we were interested in designing gene × environment effects on male infertility, considering partial microdeletions are genetic risk factors and SCT use an environmental/epidemiological risk factors. As far as published literature is concerned, no such study has been undertaken anywhere in the world.

We observed 102 (14.01%; 102/728) SCT users from the case subjects (N = 728) in comparison to 15 (5.68%; 15/264) from the control groups (N = 264). Among the case subjects who bear AZFc sub-deletions (N = 62), 48 (~ 77.42%) were recorded as SCT users, whereas 14 (22.58%) were non-users. In contrast, subjects without microdeletions exhibited 54 (8.10%) SCT users and 612 (91.89%) SCT non-users. Among the fertile controls, 8 individuals were identified with partial deletions, of whom 3 (37.5%) were SCT users and 5 (62.5%) were SCT non-users (Supplementary Table S1).

We designed a logistic regression model, considering microdeletions and SCT use as 'predictors' and fertility status as 'outcome'. In binary logistic regression, we tested three interactions among the predictors, where SCT non-user × mDel (partial microdeletion) absent group was considered as the reference group. We found a statistically significant interaction between SCT use and microdeletion (SCT user × mDel) in the infertile group (P = 0.002), with significantly elevated odds (OR = 6.38) compared to the other two interactions, i.e., SCT non-user × mDel (OR = 1.11; P value = 0.834) and SCT user × mDel absent (OR = 1.79; P value = 0.075) (given in Table 5). This finding suggests SCT has a strong interaction with partial micro-deletion among infertile Bengali-speaking men, and it may be a significant epidemiological risk factor for developing infertility among men.

Table 5
Logistic regression analysis revealed significant interaction between the predictors SCT use and partial microdeletion. The p value < 0.05 is considered statistically significant.

Total infertile individuals (N = 728)	SCT use status	Partial mDel status	Regression Analysis			
			Interactions	OR	95% CI	P Value
	Non-user (N = 626)	Absent (N = 612)	SCT non-user × mDel absent	Reference		
		Present (N = 14)	SCT non-user × mDel present	1.11	0.397–3.132	0.834
	User (N = 102)	Absent (N = 54)	SCT user × mDel absent	1.79	0.943–3.412	0.075
		Present (N = 48)	SCT user × mDel present	6.38	1.968–20.673	**0.002***

***** indicates value significantly different in the analysis

Discussion

Y chromosome microdeletion (YCMDs) is one of the major genetic causes of male infertility and it leads to the quantitative reduction of spermatozoa and causes spermatogenic failure [29–31]. We have analysed YCMDs of AZF sub-regions in this population in our previous study [32]. AZFc is a hotspot of structural mutations that cause significant spermatogenic impairment in nearly all males. Besides complete AZFc deletion (b2/b4), smaller sub-deletions (gr/gr, b2/b3 and b1/b3) in the AZFc region are also common due to frequent intrachromosomal recombinations between repeat elements [14, 18]. These partial deletions result in a diverse range of phenotypes, from normozoospermic to azoospermic, depending upon the genetic backdrop and exposure to confounding environmental/epidemiological agents [4, 33, 34].

In present study, we took an approach to characterising the AZFc partial deletions in an Indian Bengali-speaking population, one of the large ethnic groups from South Asia that had not yet been tested in this regard. Additionally, we were interested to see if the habit of SCT use could increase the risk of infertility in the genetic backdrop of Y-chromosomal partial deletion. We demonstrated that two types of AZFc sub-deletions, gr/gr and b1/b3, are prevalent among infertile Bengali men. The gr/gr deletion was found to be more frequent among the case subjects (7.42%) than the b1/b3 deletion (1.10%). Together, 8.52% of the infertile males exhibited the presence of any of the partial deletion types, in contrast to 3.03% in the control group. In addition, we did not find any incidence of the b2/b3 sub-deletion in our study cohort. The reason behind this is not clear. This may be a chance that our sample did not include b2/b3 deletion; a larger sample could have been presented with all the sub-deletion categories. Alternatively, it may be a true reflection of the fact that Bengali men do not carry b2/b3 deletion.

Our findings receive its supports from previous studies that report frequent occurrence of gr/gr deletion than other types. Since the initial finding of gr/gr sub-deletion [18] among male subjects, several independent studies have reported incidence of gr/gr deletion, though results were inconsistent. Three meta-analyses [17, 35, 36] suggested a significant association between the gr/gr deletion and infertility, and one meta-analysis [37] inferred that such deletion significantly reduced the sperm count. A population-based survey on 20,000 Y chromosomes revealed gr/gr deletion causes severe spermatogenic failure (SSF) [38]. Several independent case-control analyses conducted in different ethnic populations identified gr/gr deletion as a risk factor for male infertility [39–45]. In contrast, a large number of independent investigations from other populations failed to demonstrate such phenotypic impact in the context of gr/gr deletion, and they proposed that this deletion might not be connected to the failure of spermatogenesis that results in male infertility [46–56].

In India, previous studies have reported gr/gr deletions among infertile men from North India (P = 0.02; P = 0.0004) [36, 57], Western India (P < 0.05) [58], and Central India (P = 0.0002) [59]. All these data are concordant with what we observed in our present study population from the eastern part of India. Our findings suggest men diagnosed as azoo/oligozoospermic experienced frequent occurrences of gr/gr deletions, and the association was significant (P = 0.0015, OR = 3.413 for azoospermic; P = 0.0382, OR = 3.012 for oligozoospermic) when compared to fertile controls (Table 4). Surprisingly, we observed the presence of gr/gr deletions among normozoospermic infertile men (11/174) and proven fertile controls (8/264), though the association was found to be

insignificant. Presence of gr/gr deletions in normozoospermic/fertile men was also reported in other studies [36, 37, 57, 60, 61]. Thus, from the above discussion, it can be inferred that the implication of gr/gr deletion in clinical manifestation of infertility is still a matter of dispute. This disparity in the findings across studies developed as a result of study designs, association analysis techniques, geographical differences, and ethnic heterogeneity [62]. In addition, the Y chromosome background of the study group (allelic variations of other spermatogenic regulator genes) might influence the risk of developing spermatogenic impairment due to such sub-deletions in the AZFc region [63]. When we compared the outcome of our study with that obtained from other Indian populations, we found the frequency of gr/gr deletion among Indian Bengali-speaking men is higher (7.42%; 54/728) than the findings from two North Indian (7.17%; 5.84%) sample populations and South Indian population (6.25%) [36, 56, 57], but lower than the Western Indian population (9.90%) [58]. Our population showed a significant association of this sub-deletion with male infertility (P = 0.0111), which is consistent with the findings from Asian and Caucasian men [36, 38].

Besides gr/gr deletion, we recorded b1/b3 in our infertile study cohort (1.09%, 8/728), and no such deletion was detected among control groups. This deletion was reported to cause spermatogenic failure [38] and maturing arrest [64]. In our study, b1/b3 deletion was recorded in severe oligozoospermic subjects (P = 0.0405) (Table 4), which is consistent with the previous findings. Contrary to this, a recent independent study on Northwest Chinese men showed that the b1/b3 deletion was more prevalent among control men, and the authors suggested that the deletion may not be a risk factor for male infertility (P = 0.089) [45]. Studies conducted previously on Indian men revealed an association between b1/b3 sub-deletion and male infertility in the North [57] and Western Indian [58] cohorts. Support for this association comes from a recent study that reported that 2.7% (26/973) of the study cohort had b1/b3 deletion [59]. Thus, considerable population-specific variations in phenotypic manifestations of these deletions made the genotype-phenotype relationship enigmatic, and this needs more investigation in other populations as well.

Genetic risk factors for male infertility depend not only on ethnicity or demographic attributes for variable phenotypic manifestations but also on exposure to different epidemiological, lifestyle, and environmental factors. The genetic make-up act as loaded gun, while environmental factors act as trigger [65]. Several studies have confirmed association of tobacco and alcohol use with infertility or altered semen parameters [20, 21, 27, 66–70]. In India, a number of studies have revealed the adverse impact of smokeless chewing tobacco (SCT) on semen quality [23, 25, 26, 28]. At the beginning of the current study, when we analysed the collected epidemiological data, we found the habit of SCT use was more frequent among infertile men. We stratified the study subjects as SCT users and SCT non-users and blindly screened participants for AZFc partial deletion without looking into their SCT use status. Then the two sets of data, i.e., SCT use status and occurrence of partial deletions, were compiled (as shown in Supplementary Table S1) to check whether SCT use potentially interacts with partial deletion and increases the risk of male infertility. The synergistic or interactive effect of SCT use and partial deletions was checked using a logistic regression model, and the result showed the SCT users harbouring AZFc partial deletions were at significantly higher risk (OR = 6.38; P = 0.002) of being infertile than the other two groups, SCT non-users carrying those deletions (OR = 1.11; P = 0.834) and SCT users not bearing those sub-deletions (OR = 1.79; P = 0.075), while SCT non-users carrying no such deletions are taken as the reference group in this study (Table 5). This is a novel observation, and it helps us realise that the underpinning cause of variable phenotypic or clinical manifestations of Y chromosome partial deletions in relation to male infertility is exposure to life-style/habitual or epidemiological factors. In other words, lifestyle and epidemiological factors impose confounding effects on the genetic make-up that result in variable clinical sub-types of compromised semen parameters among men. Previously, SCT use has been identified as a risk factor for chromosome 21 nondisjunction in the oocyte that leads to Down syndrome birth [65, 71] and for shorter telomere and accelerated molecular ageing among women bearing Down syndrome children [72]. At this point, it is difficult to say whether SCT use instigates aberrant intrachromosomal recombination and YCMD or whether their association is just a stochastic event. Nevertheless, our study is the first ever report regarding the interaction and synergistic effects of YCMD and SCT use. More insightful studies in this regard need to be conducted in India and in other ethnic populations for a better understanding of the aetiology of infertility in men in the light of gene-environment interactions.

Conclusion

In summary, the present study highlights the facts that AZFc partial deletion and smokeless chewing tobacco (SCT), when present together, interact with each other and increase the risk of infertility among men. We succeeded in figuring out for the first time ever that genetic and environmental risk factors act synergistically to manifest phenotypic variations. Additionally, allelic variations of other genetic loci with which genes from AZFc interact to regulate spermatogenesis contribute to the clinical variability of semen quality that surrogates infertility phenotype. Our study provides the foundation for the Gene × Environment model that can be successfully applied to understate the overall risk of female infertility or other lifestyle disorders in which both genetic make-up and environmental factors play pivotal roles.

Material and methods

Ethics declarations:

We followed the guidelines as specified in the “Declaration of Helsinki” and the Indian Council of Medical Research (ICMR) in working with human subjects and for the collection of biological samples. The study design was approved by the Institutional Ethics Committee constituted by the University of Calcutta, West Bengal, Kolkata, India. (Approval No. CU/BIOETHICS/HUMAN/2306/3044; Dated 04/12/2017).

Consent statement

All participating individuals involved in this study provided their informed consent in the pre-printed questionnaire.

Inclusion and exclusion criteria of the study subjects:

We recruited study subjects who were diagnosed clinically as the cases of compromised fertility following semen sample study. Individuals with chromosomal aberrations, abnormal karyotypes, endocrinal abnormalities, immunological defects, and infections were excluded. Furthermore, infertile cases with obstructive azoospermia, congenital/acquired anatomical defects, or any history of surgical intervention of the urogenital system were all excluded. Besides, cases with classical AZF microdeletions who participated in our previous study [32] were not considered in the current study.

Proven age-matched fertile individuals from the same ethnic group and having homogeneity in demographic attributes with case subjects were recruited as 'control' subjects. They reported to the clinic for health issues other than infertility and were proven fertile as they all had their own biological offspring.

Study subjects:

Based on the above-mentioned criteria, we recruited a cohort of 728 infertile male patients (randomly and blindly), with an age ranging from 20 to 50 years (with an average age of 36.2 ± 4.53 years), from the Institute of Reproductive Medicine (IRM), Kolkata, from December 2018 to January 2023. Simultaneously, a group of 264 proven fertile men who fathered at least one child and had the same age range (average age of 32.7 ± 5.17 years) were recruited as controls. All participants in our study were Bengali-speaking, demographically homogeneous, and residents of the state of West Bengal, India, over generations. These criteria negate the possibility of population substructure within the sample cohort. The participants were interviewed in person to collect epidemiological information regarding their personal, conjugal, and lifestyle attributes in a pre-printed questionnaire. To ensure the utmost level of confidentiality for all participants, all records were kept in the laboratory anonymously with a specific code. We obtained informed consent from the participants for the use of their donated tissue (blood and semen) samples in the study while maintaining confidentiality.

Stratification of study groups:

The clinician collaborators from IRM categorised them as azoospermic (N = 332; non-obstructive azoospermia, NOA; absence of sperm in the ejaculate), oligozoospermic (N = 93; sperm concentration: < 15 million/ml of ejaculate), severe-oligozoospermic (N = 67; sperm concentration: ≤ 5 million/ml of ejaculate), normozoospermic (N = 174; sperm concentration: > 15 million/ml of ejaculate) with idiopathic infertility, and asthenozoospermic (N = 62; sperm motility: < 32% and sperm count: ≥ 15 million/ml of ejaculate) individuals following guidelines laid by the World Health Organization, 2010 [73]. It is important to mention here that we did not define 'case' and 'control' based on their semen criteria. This is because 'normozoospermia' and 'fertility' are not synonymous; we classified normozoospermic men who were reported for infertility as 'idiopathic cases'. On the other hand, all the 'control' subjects were reported to have fathered at least one child and found to be normozoospermic on testing their semen samples.

Tissue sample collection:

The blood samples from each of the participants were taken with the help of laboratory technicians through the venepuncture method and collected in an EDTA vacutainer tube (BD, USA).

DNA extraction:

Genomic DNA (gDNA) was extracted from whole blood samples with the help of DNA extraction and purification kit (QIAamp® Blood Mini Kit, Qiagen, Germany), as directed by the manufacturer's protocol. Following that, the quality (purity) and quantity of the isolated DNA were analysed through 0.8% agarose (Sisco Research Laboratories Pvt. Ltd.) gel electrophoresis and NanoPhotometer® (Implen, Germany), respectively. Then the gDNA was stored at -20° C for subsequent analysis.

Screening for AZFc partial deletions:

A total of six sequence-tagged site (STS) markers were used, out of which five are of Y chromosome AZFc partial deletion-specific (sY1191, sY1291, sY1161, sY1206 and sY1201) [18]. The marker sY14 was taken as internal control and is specific to the sex-determining region on Y-chromosome (SRY). Detailed information of each of the STS markers, reaction conditions, and amplicon size is presented in Table 1. Failure of amplification of a certain STS marker or group of markers defines the span of partial Y-chromosome sub-deletions of AZFc region. The gr/gr partial deletion is characterised by the absence of sY1291, while the lack of sY1191 detects the b2/b3 sub-deletion. On the other hand, the b1/b3 sub-deletion was validated by the absences of sY1161, sY1191, and sY1291. Thus, we used a conventional plus (+) or minus (-) sign for denoting the presence or absence of the sub-deletions, and the genotyping result is presented in Table 2.

All PCR were conducted in a simplex reactions. Each PCR contained a total of 10 µl of reaction mixture consisting of PCR master mix (GoTaq Green Master Mix, Promega), nuclease-free water (Promega), STS marker-specific forward and reverse primers (Integrated DNA Technologies, IDT), and gDNA template (concentration of 25 ng). PCR amplification was carried out in a thermal cycler (Applied Biosystems, Thermo-Fischer Scientific) with the reaction conditions of initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds with respective annealing temperatures (Table 1), and extension at 72°C for 30 seconds. The final extension was performed at 72°C for 5 minutes before being kept at 4°C. The amplified PCR products were separated on a 2% agarose gel for 1 hour at 90 V and 400 mA current. The Gel Doc EZ imager (Bio-Rad) was utilised to visualise the amplicons and document the results. All samples were tested for the presence of SRY gene (sY14). We repeated all the deletion-positive reactions at least three times to ensure the presence of such deletions in a given subject.

Statistical analysis:

The differences among frequencies of AZFc partial sub-deletions were calculated and compared between several case groups and control groups using Fisher's exact test using GraphPad InStat® software (GraphPad Software, Inc., San Diego, USA, version 3.06). Logistic regression was performed to analyse the effect of smokeless chewing tobacco (SCT) use as a potential epidemiological risk factor on infertility among the partial sub-deletion carrying subjects. We consider SCT as 'epidemiological/environmental predictor' and partial deletion as a 'genetic predictor'. On the other hand infertility was the 'outcome'. This analysis was carried out using the software package STATA 13 (StataCorp LP, College Station, Texas). P values < 0.05 were considered statistically significant.

Odds ratios (ORs) with their respective 95% confidence intervals (CI) were computed to determine the risk of developing the condition. To verify the findings, statistical tests were conducted at least twice.

Declarations

Data availability:

All data generated and analysed in this study are included in this article.

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Author contributions:

S.G. conceptualised and conceived the study; S.G., P.G., and S.D. designed the study; S.D., P.P., and S.P. helped in collection of samples, interviewed the couples, and recorded the epidemiological details. R.C. and G.B. cared for the participants and helped in sample collection and subsequent categorization. S.D., P.P., S.P., and S.S. performed experimentation and analysed the results; S.G., P.G., and S.D. wrote the manuscript. All authors read and approved the final version of the manuscript.

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Competing interests:

The authors declare no competing interests.

Additional information:

Supplementary information attached.

References

1. Ferlin, A. *et al.* Male infertility: role of genetic background. *Reprod. Biomed. Online* **14,** 734–745 (2007).
2. Poongothai, J., Gopenath, T. S. & Manonayaki, S. Genetics of human male infertility. *Singapore Med J* **50,** 336–347 (2009).
3. Rao, L. *et al.* Chromosomal abnormalities and y chromosome microdeletions in infertile men with varicocele and idiopathic infertility of South Indian origin. *J Androl* **25,** 147–153 (2004).
4. O'Flynn O'Brien, K. L., Varghese, A. C. & Agarwal, A. The genetic causes of male factor infertility: a review. *Fertil. Steril.* **93,** 1–12 (2010).
5. Tiepolo, L. & Zuffardi, O. Localization of factors controlling spermatogenesis in the nonfluorescent portion of the human Y chromosome long arm. *Hum. Genet.* **34,** 119–124 (1976).
6. Vogt, P. *et al.* Microdeletions in interval 6 of the Y chromosome of males with idiopathic sterility point to disruption of AZF, a human spermatogenesis gene. *Hum. Genet.* **89,** 491–496 (1992).
7. Pieri, P. D. C. *et al.* A cost-effective screening test for detecting AZF microdeletions on the human Y chromosome. *Genet Test* **6,** 185–194 (2002).
8. Shamsi, M. B., Kumar, K. & Dada, R. Genetic and epigenetic factors: Role in male infertility. *Indian J Urol* **27,** 110–120 (2011).
9. Krausz, C. *et al.* EAA/EMQN best practice guidelines for molecular diagnosis of Y-chromosomal microdeletions: state-of-the-art 2013. *Andrology* **2,** 5–19 (2014).
10. McElreavey, K. & Krausz, C. Sex Chromosome Genetics' ' '99. Male infertility and the Y chromosome. *Am. J. Hum. Genet.* **64,** 928–933 (1999).
11. Krausz, C., Quintana-Murci, L. & McElreavey, K. Prognostic value of Y deletion analysis: what is the clinical prognostic value of Y chromosome microdeletion analysis? *Hum. Reprod.* **15,** 1431–1434 (2000).
12. Cram, D. S. *et al.* Y chromosome analysis of infertile men and their sons conceived through intracytoplasmic sperm injection: vertical transmission of deletions and rarity of de novo deletions. *Fertil. Steril.* **74,** 909–915 (2000).
13. Foresta, C., Moro, E. & Ferlin, A. Y chromosome microdeletions and alterations of spermatogenesis. *Endocr. Rev.* **22,** 226–239 (2001).
14. Kuroda-Kawaguchi, T. *et al.* The AZFc region of the Y chromosome features massive palindromes and uniform recurrent deletions in infertile men. *Nat. Genet.* **29,** 279–286 (2001).
15. Repping, S. *et al.* Recombination between palindromes P5 and P1 on the human Y chromosome causes massive deletions and spermatogenic failure. *Am. J. Hum. Genet.* **71,** 906–922 (2002).
16. Skaletsky, H. *et al.* The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. *Nature* **423,** 825–837 (2003).
17. Stouffs, K., Lissens, W., Tournaye, H. & Haentjens, P. What about gr/gr deletions and male infertility? Systematic review and meta-analysis. *Hum. Reprod. Update* **17,** 197–209 (2011).

18. Repping, S. *et al.* Polymorphism for a 1.6-Mb deletion of the human Y chromosome persists through balance between recurrent mutation and haploid selection. *Nat. Genet.* **35,** 247–251 (2003).
19. Colaco, S. & Modi, D. Genetics of the human Y chromosome and its association with male infertility. *Reprod Biol Endocrinol* **16,** 14 (2018).
20. Rai, T. & Rai, G. Cigarette smoking and alcohol consumption are enemy of male fertility? A patho-radiological correlation study. *Int. J. Res. Med. Sci.* 847–854 (2016). doi:10.18203/2320-6012.ijrms20160530
21. Barratt, C. L. R. *et al.* The diagnosis of male infertility: an analysis of the evidence to support the development of global WHO guidance-challenges and future research opportunities. *Hum. Reprod. Update* **23,** 660–680 (2017).
22. Leisegang, K. & Dutta, S. Do lifestyle practices impede male fertility? *Andrologia* **53,** e13595 (2021).
23. Dikshit, R. K., Buch, J. G. & Mansuri, S. M. Effect of tobacco consumption on semen quality of a population of hypofertile males. *Fertil. Steril.* **48,** 334–336 (1987).
24. Sofikitis, N. *et al.* Effects of smoking on testicular function, semen quality and sperm fertilizing capacity. *J. Urol.* **154,** 1030–1034 (1995).
25. Said, T. M., Ranga, G. & Agarwal, A. Relationship between semen quality and tobacco chewing in men undergoing infertility evaluation. *Fertil. Steril.* **84,** 649–653 (2005).
26. Sunanda, P. *et al.* Prevalence of abnormal spermatozoa in tobacco chewing sub-fertile males. *J Hum Reprod Sci* **7,** 136–142 (2014).
27. Sharma, R., Harlev, A., Agarwal, A. & Esteves, S. C. Cigarette Smoking and Semen Quality: A New Meta-analysis Examining the Effect of the 2010 World Health Organization Laboratory Methods for the Examination of Human Semen. *Eur. Urol.* **70,** 635–645 (2016).
28. Borkhataria, B., Dhameliya, J., Mavani, D. & Dhameliya, J. Effects of tobacco chewing habits on male infertility. *Int. J. Res. Med. Sci.* **8,** 2589 (2020).
29. Vogt, P. H. *et al.* Human Y chromosome azoospermia factors (AZF) mapped to different subregions in Yq11. *Hum. Mol. Genet.* **5,** 933–943 (1996).
30. Krausz, C. & Degl'Innocenti, S. Y chromosome and male infertility: update, 2006. *Front. Biosci.* **11,** 3049–3061 (2006).
31. Krausz, C. & Riera-Escamilla, A. Genetics of male infertility. *Nat. Rev. Urol.* **15,** 369–384 (2018).
32. Dutta, S. *et al.* Prevalence of Y chromosome microdeletion in azoospermia factor subregions among infertile men from West Bengal, India. *Mol. Genet. Genomic Med.* **9,** e1769 (2021).
33. Repping, S. *et al.* A family of human Y chromosomes has dispersed throughout northern Eurasia despite a 1.8-Mb deletion in the azoospermia factor c region. *Genomics* **83,** 1046–1052 (2004).
34. Navarro-Costa, P., Gonçalves, J. & Plancha, C. E. The AZFc region of the Y chromosome: at the crossroads between genetic diversity and male infertility. *Hum. Reprod. Update* **16,** 525–542 (2010).
35. Tüttelmann, F., Rajpert-De Meyts, E., Nieschlag, E. & Simoni, M. Gene polymorphisms and male infertility--a meta-analysis and literature review. *Reprod. Biomed. Online* **15,** 643–658 (2007).
36. Bansal, S. K. *et al.* Gr/gr deletions on Y-chromosome correlate with male infertility: an original study, meta-analyses, and trial sequential analyses. *Sci. Rep.* **6,** 19798 (2016).
37. Visser, L. *et al.* Y chromosome gr/gr deletions are a risk factor for low semen quality. *Hum. Reprod.* **24,** 2667–2673 (2009).
38. Rozen, S. G. *et al.* AZFc deletions and spermatogenic failure: a population-based survey of 20,000 Y chromosomes. *Am. J. Hum. Genet.* **91,** 890–896 (2012).
39. Giachini, C. *et al.* The gr/gr deletion(s): a new genetic test in male infertility? *J. Med. Genet.* **42,** 497–502 (2005).
40. Lynch, M. *et al.* The Y chromosome gr/gr subdeletion is associated with male infertility. *Mol. Hum. Reprod.* **11,** 507–512 (2005).
41. Ferlin, A. *et al.* Association of partial AZFc region deletions with spermatogenic impairment and male infertility. *J. Med. Genet.* **42,** 209–213 (2005).
42. de Llanos, M., Ballescà, J. L., Gázquez, C., Margarit, E. & Oliva, R. High frequency of gr/gr chromosome Y deletions in consecutive oligospermic ICSI candidates. *Hum. Reprod.* **20,** 216–220 (2005).
43. Navarro-Costa, P. *et al.* Characterizing partial AZFc deletions of the Y chromosome with amplicon-specific sequence markers. *BMC Genomics* **8,** 342 (2007).
44. Choi, J. *et al.* Impaired spermatogenesis and gr/gr deletions related to Y chromosome haplogroups in Korean men. *PLoS One* **7,** e43550 (2012).
45. Liu, C. *et al.* The association of partial azoospermia factor C deletions and male infertility in northwestern china. *Hum. Hered.* **84,** 144–150 (2019).
46. Hucklenbroich, K. *et al.* Partial deletions in the AZFc region of the Y chromosome occur in men with impaired as well as normal spermatogenesis. *Hum. Reprod.* **20,** 191–197 (2005).
47. Carvalho, C. M. B., Zuccherato, L. W., Bastos-Rodrigues, L., Santos, F. R. & Pena, S. D. J. No association found between gr/gr deletions and infertility in Brazilian males. *Mol. Hum. Reprod.* **12,** 269–273 (2006).
48. de Carvalho, C. M. B. *et al.* Study of AZFc partial deletion gr/gr in fertile and infertile Japanese males. *J. Hum. Genet.* **51,** 794–799 (2006).
49. Ravel, C. *et al.* GR/GR deletions within the azoospermia factor c region on the Y chromosome might not be associated with spermatogenic failure. *Fertil. Steril.* **85,** 229–231 (2006).
50. Fernando, L., Gromoll, J., Weerasooriya, T. R., Nieschlag, E. & Simoni, M. Y-chromosomal microdeletions and partial deletions of the Azoospermia Factor c (AZFc) region in normozoospermic, severe oligozoospermic and azoospermic men in Sri Lanka. *Asian J Androl* **8,** 39–44 (2006).
51. Lardone, M. C. *et al.* AZFc partial deletions in Chilean men with severe spermatogenic failure. *Fertil. Steril.* **88,** 1318–1326 (2007).
52. Imken, L. *et al.* AZF microdeletions and partial deletions of AZFc region on the Y chromosome in Moroccan men. *Asian J Androl* **9,** 674–678 (2007).
53. Stouffs, K. *et al.* Do we need to search for gr/gr deletions in infertile men in a clinical setting? *Hum. Reprod.* **23,** 1193–1199 (2008).

54. Stahl, P. J. *et al.* Diagnosis of the gr/gr Y chromosome microdeletion does not help in the treatment of infertile American men. *J. Urol.* **185,** 233–237 (2011).
55. Alimardanian, L., Saliminejad, K., Razi, S. & Ahani, A. Analysis of partial azoospermia factor c deletion and DAZ copy number in azoospermia and severe oligozoospermia. *Andrologia* **48,** 890–894 (2016).
56. Vijesh, V. V., Nambiar, V., Mohammed, S. I. K., Sukumaran, S. & Suganthi, R. Screening for AZFc partial deletions in Dravidian men with nonobstructive azoospermia and oligozoospermia. *Genet. Test. Mol. Biomarkers* **19,** 150–155 (2015).
57. Shahid, M., Dhillon, V. S., Khalil, H. S., Sexana, A. & Husain, S. A. Associations of Y-chromosome subdeletion gr/gr with the prevalence of Y-chromosome haplogroups in infertile patients. *Eur. J. Hum. Genet.* **19,** 23–29 (2011).
58. Sen, S. *et al.* Susceptibility of gr/gr rearrangements to azoospermia or oligozoospermia is dependent on DAZ and CDY1 gene copy deletions. *J. Assist. Reprod. Genet.* **32,** 1333–1341 (2015).
59. Rani, D. S. *et al.* High frequencies of Non Allelic Homologous Recombination (NAHR) events at the AZF loci and male infertility risk in Indian men. *Sci. Rep.* **9,** 6276 (2019).
60. Machev, N. *et al.* Sequence family variant loss from the AZFc interval of the human Y chromosome, but not gene copy loss, is strongly associated with male infertility. *J. Med. Genet.* **41,** 814–825 (2004).
61. Sato, Y. *et al.* Replication study and meta-analysis of human nonobstructive azoospermia in Japanese populations. *Biol. Reprod.* **88,** 87 (2013).
62. Krausz, C., Escamilla, A. R. & Chianese, C. Genetics of male infertility: from research to clinic. *Reproduction* **150,** R159–74 (2015).
63. Yang, Y. *et al.* Y chromosome haplogroups may confer susceptibility to partial AZFc deletions and deletion effect on spermatogenesis impairment. *Hum. Reprod.* **23,** 2167–2172 (2008).
64. Li, Q. *et al.* Relationship between AZFc deletions and testicular histology in infertile South Chinese men with azoospermia and severe oligospermia. *Springerplus* **5,** 1805 (2016).
65. Halder, P. *et al.* Understanding etiology of chromosome 21 nondisjunction from gene × environment models. *Sci. Rep.* **11,** 22390 (2021).
66. Marinelli, D., Gaspari, L., Pedotti, P. & Taioli, E. Mini-review of studies on the effect of smoking and drinking habits on semen parameters. *Int J Hyg Environ Health* **207,** 185–192 (2004).
67. Yao, D. F. & Mills, J. N. Male infertility: lifestyle factors and holistic, complementary, and alternative therapies. *Asian J Androl* **18,** 410–418 (2016).
68. Sharma, A. Male infertility; evidences, risk factors, causes, diagnosis and management in human. *Ann. Clin. Lab. Res.* **05,** 3:188 (2017).
69. Sansone, A. *et al.* Smoke, alcohol and drug addiction and male fertility. *Reprod Biol Endocrinol* **16,** 3 (2018).
70. Gunes, S., Metin Mahmutoglu, A., Arslan, M. A. & Henkel, R. Smoking-induced genetic and epigenetic alterations in infertile men. *Andrologia* **50,** e13124 (2018).
71. Ghosh, S. *et al.* Epidemiology of Down syndrome: new insight into the multidimensional interactions among genetic and environmental risk factors in the oocyte. *Am. J. Epidemiol.* **174,** 1009–1016 (2011).
72. Ray, A. *et al.* Maternal telomere length and risk of down syndrome: epidemiological impact of smokeless chewing tobacco and oral contraceptive on segregation of chromosome 21. *Public Health Genomics* **19,** 11–18 (2016).
73. World Health Organization. *WHO Laboratory Manual For The Examination And Processing Of Human Semen*, fifth edition, 1–287 (WHO press, 2010).

Figures

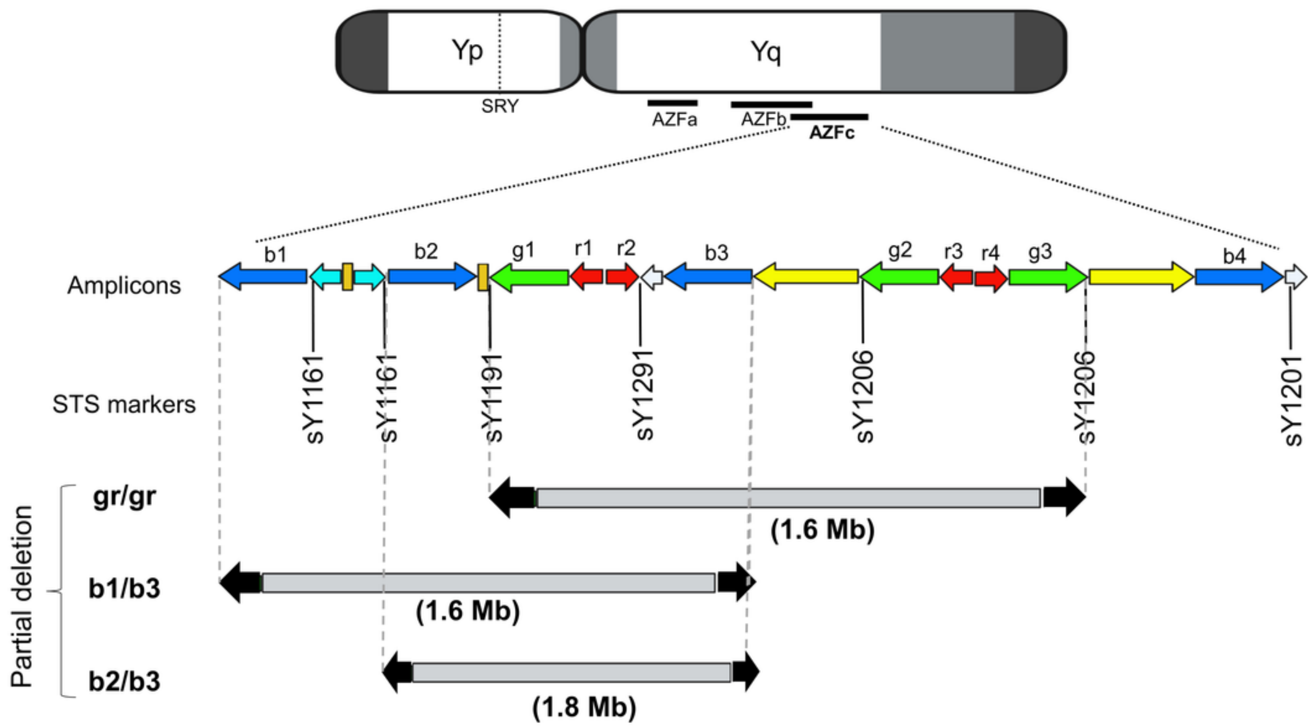

Figure 1

Y chromosome with AZFc sub-deletions defined by STS markers. A schematic presentation shows the AZFc region with three partial deletions, gr/gr (1.6 Mb), b1/b3 (1.6 Mb), and b2/b3 (1.8 Mb) on the long arm (Yq) and SRY on the short arm (Yp) of the Y chromosome. The partial deletions are identified by specific STS markers used in screening.

Supplementary Files

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- SupplementaryTableS1.pdf