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Y chromosome microdeletion analysis reveals predominant AZFc deletions and absence of germline specific deletions among idiopathic infertile males in a south Indian population

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Abstract

Y chromosome microdeletions in any of the three azoospermia factor loci AZFa, AZFb and AZFc affects spermatogenesis causing irreversible male infertility. In the present study Yq deletion frequency, deletion types and associated infertile conditions were analyzed among 240 idiopathic infertile males (102 severe oligospermic and 138 azoospermic) and 100 fertile control individuals of a south Indian population previously not assessed. Polymerase chain reaction analysis for Yq microdeletions was performed using genomic DNA employing eight sequence tagged sites markers. Germ line specific Yq deletions were also assessed among 86 severe oligospermic males using sperm DNA. Apart from these, sperm sex chromosomal aneuploidies were analyzed among 20 severe oligospermic and oligoasthenospermic infertile individuals through fluorescence *in situ* hybridization technique. Among 240 infertile individuals assessed 13 (5.4%) men were identified to harbour interstitial Y chromosome microdeletions. AZFc region deletions involving *DAZ* gene cluster was observed to be predominant (69.2%) followed by AZFb deletions (15.4%) and AZFbc deletions (15.4%). Statistically significant difference was observed in the Yq deletion frequency between the severe oligospermic and azoospermic individuals (χ^2 = 4.13, df =1, p = 0.042). Germ line specific deletions were observed to be completely lacking among severe oligospermic individuals. Insignificant difference was observed in sperm sex chromosomal aneuploidy rates between the oligospermic and control individuals. Influence of genetic and environmental factors, sampling and methodological differences can be attributed to the differences in Yq deletion frequency observed within Indian populations. Germ cell specific Yq deletions and sperm sex chromosome aneuploidy may not be always associated with severe infertility.

Keywords: Azoospermia, Oligospermia, Azoospermia factor regions, Sequence tagged sites, Microdeletions

1. Introduction

During the evolution of human sex chromosomes selective pressures have favoured the gradual concentration and retention of genes regulating spermatogenesis on the Y chromosome (1). Several genes essential for conferring fertility among men are located in the three azoospermia factor (AZF) regions of the Yq arm namely AZFa, AZFb and AZFc region (2). The AZF locus of the euchromatic Yq arm harbours single copy genes namely *USP9Y, DBY* and *UTY* as well as multicopy genes namely *DAZ, RBMY, PRY, CDY* and *XKRY* (3). The AZFa region encompasses ubiquitously expressing genes whereas the AZFb region harbours both testis specific and ubiquitously expressing genes (4, 5). The AZFc region has exclusively testis specific genes indicating functional specialisation (6). A deletion event causing loss of genes from the three AZF regions have been associated with characteristic testicular pathologies rendering the individual infertile. Globally, an estimated 15% couples suffer from infertility and in 50% of all cases a male factor is involved (7). Among male factor infertility, a significant proportion is attributed to genetic disorders of which Yq microdeletions and chromosomal abnormalities are observed to be the major aetiologies (8). Infertile men

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with Yq microdeletions, sperm chromosomal aneuploidies and sperm Yq deletion mosaicism can however circumvent the natural barriers and transmit the abnormality to offspring through Assisted Reproductive Techniques (ARTs) (9).

Previous studies have reported variations in sperm count among different Y haplotypes of the same population indicating a genetic basis for fertility potential (10). Specific haplotypes susceptible to Yq microdeletions have also been identified (11, 12). The AZF spermatogenesis genes lie in the non-recombining region of the Y chromosome. Hence lack of recombination with the X chromosome has led the Y chromosome to evolve in paternal lineages diversifying through deletion, duplication and transposition events (13). These lineages follows a non-random distribution, however genetic drifts, socio-cultural beliefs and practices affects the distribution (14). The rigid social classifications of caste, tribe and religion along with endogamy and long isolation events has led to the tremendous diversification among the Indian ethnic groups (15, 16, 17). A meta-analysis of ethnic based studies on Indian population has unravelled several novel deletion events in the Y chromosome (18). However it is unclear whether a significant number of these studies have been performed on idiopathic or non-idiopathic infertile individuals which can thus introduce a sample bias. Few other studies have not used the recommended STS markers in the EAA/EMQN guidelines. Several studies that listed in the meta-analysis have been performed on a small sample size, a handful of them with less than 100 study individuals, which is insufficient to draw valid statistical conclusions (18).

Hence in the present study Y chromosome microdeletion analysis was performed on confirmed idiopathic infertile males of a Dravidian ethnicity genetically distinct from the north Indian population (16, 18). Most of the studies have focussed on analyzing Yq microdeletions in somatic cell DNA while only a handful of studies have attempted to identify Yq microdeletions confined to germ cell lineage which is of different embryological origin as compared to blood cells (19-21). Hence Yq microdeletion analysis was done in sperm DNA of the oligospermic cohort to identify and characterise possible isolated germ cell Yq microdeletions. Among severe oligo and oligo associated conditions fluorescence *in situ* hybridization (FISH) analysis for sperm sex chromosome aneuploidy was done to identify men at risk of in vitro fertilization (IVF) or intra cytoplasmic sperm injection (ICSI) failure.

2. Materials and methods

2.1. Study subjects

In the present study 273 infertile individuals were initially screened from different hospitals in Mysore, Karnataka, south India. Exclusion criteria involved individuals diagnosed with reproductive tract obstruction, absence of vas deferens, Cryptorchidism, cytogenetic abnormalities, erectile dysfunction, varicocele and hypogonadism. Accordingly 33 individuals were excluded and 240 idiopathic infertile men of age range 22-45 years diagnosed with severe oligospermia (n = 102, mean age = 32.77 ± 4.43) or non-obstructive azoospermia (n =138, mean age = 33.73 ± 4.57) were selected. Clinical examinations, endocrinological evaluation and laboratory assessments were performed to rule out any other cause of infertility. A group of 100 age matched (mean age =30.50 ± 3.61 years) individuals with proven fertility without having undergone ARTs were recruited as control group. Subject history proforma was established after collecting information regarding lifestyle, medical history and details pertaining to the infertile condition. An informed written consent was obtained from the subjects before their inclusion in the study. The present study was initiated after obtaining prior permission from the institutional ethical committee of University of Mysore (IHEC-UOM No.51/Ph.D/2011-12).

2.2. Sample collection and semen analysis

The individuals were instructed to provide semen samples in a sterilized, wide mouthed plastic container by means of masturbation. Sexual abstinence of 3-5 days was recommended to both the control and infertile group. Physical examination (volume, pH, liquefaction time, coagulation) and microscopic examination (count, motility, vitality, morphology) of semen was performed to ascertain the infertile condition (22). 2 ml of venous blood sample was collected in EDTA vacutainer tubes for extraction of DNA.

2.3. Density gradient centrifugation for separation of sperm cells

Sperm pellet free of leukocytes and immature germ cells were obtained by density gradient centrifugation for 86 semen samples of severe oligospermic individuals. Earles balanced salt solution (10x) was prepared by protocol described earlier (22). Isotonic density gradient stock solution was prepared by adding 9 parts percoll reagent (Sigma, USA) and one part Earles salt solution. Further 80% and 40% density gradient solution was

prepared by diluting with Earles salt solution as per the requirement. One ml 80% percoll gradient solution was taken in a 15ml centrifuge tube. 40% percoll gradient solution was layered on top of the 80% gradient solution. Semen samples were mixed well and 1 ml or 0.5 ml of semen was layered over the density gradient media. The tubes were than centrifuged at 1500 rpm for 20 min. The supernatant was then removed and the pellet was suspended in 1X phosphate buffered saline (PBS). The suspension was centrifuged for 10 min at 1500 rpm. The washing step was repeated once again to obtain pure sperm pellet (22).

2.4. DNA extraction and PCR

DNA extraction was performed by using QIAamp DNA blood mini kit (Qiagen, Netherlands) and HiPura Sperm Genomic DNA purification kit (Himedia, Germany). The amount of DNA was quantified spectrophotometrically. DNA extracted from fertile male and female was used as positive and negative control respectively. Double distilled water was used as blank. Y chromosome deletion analysis was performed employing STS markers recommended by the European Academy of Andrology (EAA) and European Molecular Genetics Quality Network (EMQN) guidelines (23). In addition, markers specific to the key candidate genes namely sY627 for *RBMY1* and sY1316 for *USP9Y* gene were also incorporated (24).

PCR was performed in a total volume 12.5µl reaction mixture (Gotaq colorless mastermix and nuclease free water, Promega, USA) containing 100 ng genomic DNA, 2 mM dNTPs, 10 pM forward and reverse primer, 1.5 mM $MgCl_2$ and Taq polymerase enzyme (1X). Thermal cycling involved the following steps beginning with an initial denaturation at 94°C for 4 min; denaturation at 94°C for 30 s, annealing either at 58°C (sY86, sY127, sY254 & sY255) or at 57°C (sY84, sY134, sY1316 & sY627) for 30 s, extension at 72°C for 30 s, for 30 cycles; a final extension at 72°C for 5 min. The PCR products were then electrophoresed at 100V for 1 hour on 2% agarose gel prepared in 1X Tris-Acetate-EDTA (TAE) buffer containing ethidium bromide (0.5µg/ml). The samples were then detected by means of UV trans-illuminator and the absence of visible DNA band of appropriate base pair size was noted as a deletion. Negative results were further confirmed by two repeats of the particular reaction.

2.5. Sperm FISH

Sperm sex chromosomal aneuploidy screening was performed as per protocol previously described (25). Semen sample aliquots of 20 severe oligospermic individuals were liquefied for 30 min and centrifuged at 2000 rpm for ten minutes. The cells were harvested using hypotonic solution and later fixed with fixative (methanol: acetic acid). The pellet was washed and slides were made by dropping the cell suspension. The slides were then stored at -20°C for a minimum of 24 h. Hybridization with probes SE X (DXZ1) and SE Y (DYZ3) and further downstream processes was performed following the manufacturer protocol (Kreatech, Netherlands). The slides were visualized under a fluorescence microscope and images were captured by charge coupled device (CCD) camera. Scoring of sperm nuclei were performed according to previously published criteria (26).

2.6. Statistical analysis

The semen analysis results are depicted as mean and standard error. As the sample sizes were not equal, the data was analyzed by chi-square (χ^2) test through SPSS (version 14), to check for any significant difference in the deletion frequency between the two infertile groups assessed.

3. Results

A total of 240 infertile individuals diagnosed with severe oligospermia or non-obstructive azoospermia were analysed for incidence of Y chromosome microdeletions. A total of 13 (5.4%) infertile individuals were identified to harbour interstitial Y chromosome microdeletions. AZFc region deletions involving *DAZ* gene cluster were observed to be predominant and were detected in 9 out of 13 (69.2%) individuals. The details of the semen parameters observed among the two infertile groups are depicted in Table 1.

Table 1. Physical and microscopic semen parameters of the two class of infertile individuals compared with the control group. (SO - Severe oligospermia; NOA – Non obstructive azoospermia)

Semen Parameter	SO (n=102)	Infertile condition	Control
		NOA(n=138)	(n=100)
Volume (mL)	1.66 ± 0.09	1.73 ± 0.06	2.24 ± 0.13
pH	8.1 ± 0.04	7.94 ± 0.02	7.8 ± 0.03
Count (millions/mL)	3.01 ± 0.15	-	61.68 ± 3.13
Total count (millions/mL)	5.39 ± 0.44	-	137.76 ± 12.22
Motility grade a+b (%)	16.77 ± 1.55	-	61.03 ± 1.84
Motility grade c (%)	83.23 ± 1.55	-	38.97 ± 1.84
Vitality	37.18 ± 1.77	-	67.26 ± 1.79

Values represent mean ± SEM.

The deletion frequency between the severe oligospermic and azoospermic cohort was observed to be statistically significant. The deletion types, deletion frequency and chi-square test results are depicted in Table 2.

Chi square test revealed significant difference in the deletion frequency between the azoospermic and severe oligospermic infertile groups (χ^2 = 4.13, df =1, p = 0.042).

Electrophoresed agarose gel containing the PCR products for an azoospermic individual tested positive for AZFb and AZFc microdeletions are depicted through figure 1a to 1d.

Electrophoresed agarose gel containing the PCR products for a severe oligospermic individual tested positive for AZFc microdeletions are depicted in figure 1c and 1d.

Table 2. Frequency of different types of Yq micro deletions identified among the two infertile groups

Types of deletions	Severe Oligospermia (n = 102) n (%)	Azoospermia (n = 138) n (%)	Total (n = 240) n (%)
AZFa deletions	0	0	0
AZFb deletions	0	2(18.2)	2 (15.4)
AZFc deletions	2(100)	7 (63.6)	9 (69.2)
AZFbc deletions	0	2 (18.2)	2 (15.4)
Total	2(2%)	11 (8%)	13(5.4%)*

*p<0.05, significant. Chi square test revealed significant difference in the deletion frequency between the azoospermic and severe oligospermic infertile groups (χ^2 = 4.13, df =1, p = 0.042).

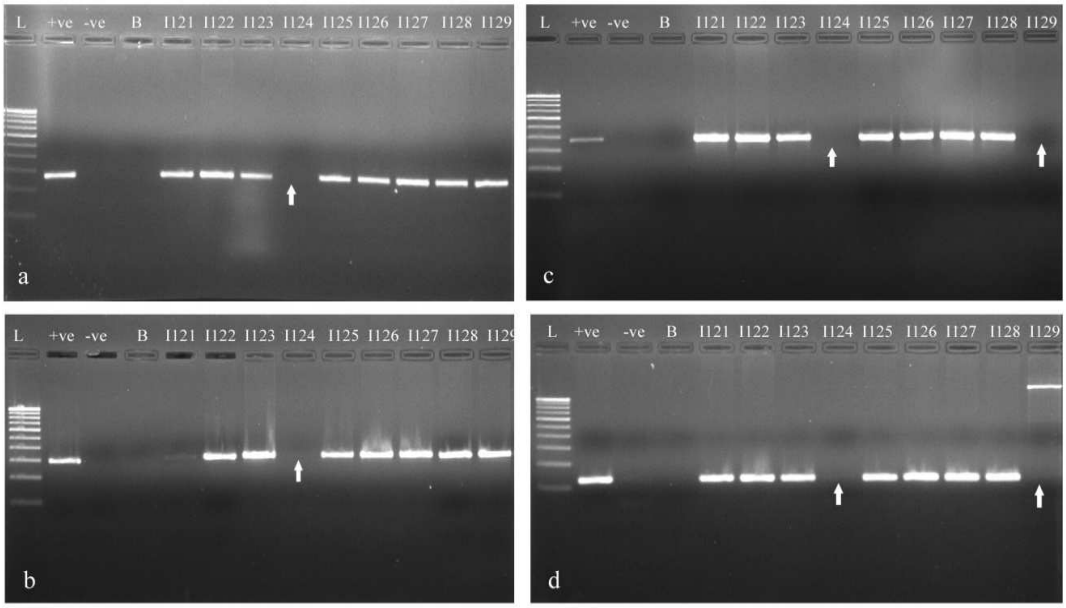

Figure1. Agarose gel electrophoresis image showing PCR products for AZFb and AZFc sequence-tagged sites (STS) markers. Lane L: 100bp DNA ladder, +ve: fertile man, -ve: female genomic DNA, B- Blank, I121 to I129: PCR amplified products of STS markers a) sY127, b) sY134, c) sY254 and d) sY255. I124 and I129 show deletions evident by non-amplification of AZFb and AZFc specific STS markers.

The individual deletion pattern of STS markers among the infertile men found to harbour Y chromosome microdeletions along with the clinical data are represented in Table 3.

Among the oligospermic cohort, blood and semen samples for DNA extraction were available for 86 individuals. Yq deletion analysis in these samples did not reveal the presence of any isolated germ cell Yq deletions.

Figure 2a represents sperm FISH analysis for sex chromosomal aneuploidy in an infertile individual with oligospermia condition and Figure 2b depicts sperm FISH analysis in a control individual with proven fertility. Sperm FISH analysis results showed no significant increase in anomalies in infertile individuals when compared to the control group.

Table 3. Clinical data and details of STS markers deleted among the infertile individuals tested positive for Yq microdeletion (SO- Severe Oligospermia, A- Azoospermia).

Subject Id & Condition	Age	Semen volume (mL)	Semen Concentration (*10^6/mL)	Total Count (*10^6/Ejaculate)	Sperm Motility (a+b %)	STS Deleted
P7 (A)	31	1	0	0	-	sY254, sY255
P18 (A)	34	0.9	0	0	-	sY127, sY134, sY627
P21 (A)	34	1.5	0	0	-	sY254, sY255
P49 (SO)	30	1	1.2	1.2	30	sY254, sy255
P103 (A)	27	1	0	0	-	sY254, sY255
P111 (A)	42	1	0	0	-	sY134, sY627
P120 (A)	33	0.8	0	0	-	sY254, sY255
P124 (A)	38	2	0	0	-	sY127, sY134, sY627, sY254, sY255
P129 (SO)	28	1.2	1	1.2	13	sY254, sY255
P143 (A)	30	1.1	0	0	-	sY127, sY134, sY627, sY254, sY255
P155 (A)	40	1.8	0	0	-	sY254, sY255
P160 (A)	26	1.5	0	0	-	sY254, sY255
P 211 (A)	31	1.4	0	0	-	sY254, sY255

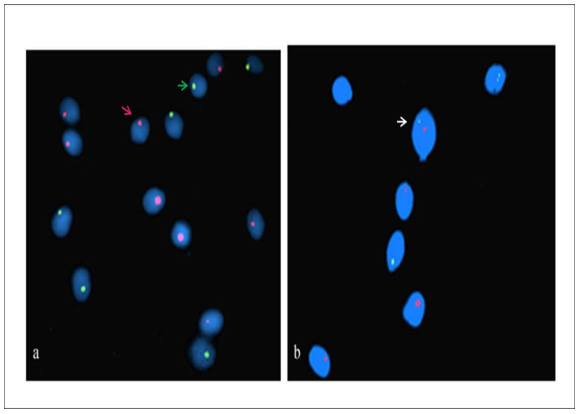

Figure 2 a. FISH on sperm sample of a fertile individual with SE X (DXZ1)/ SE Y (DYZ3) probe. The green signals indicate the X bearing sperms (green arrow) and the red signals indicate the Y bearing sperms (red arrow). b. FISH on sperm sample of an oligospermic infertile individual with SE X (DXZ1)/ SE Y (DYZ3) probe. White arrow indicates a sperm showing sex chromosome disomy.

4. Discussion

Yq microdeletions are inherited from fertile father to male offspring. The progeny has defective spermatogenesis leading to irreversible infertile condition (7). Lack of earlier studies with appropriate study design, stringent inclusion-exclusion criteria and strict classification of infertile groups prompted the present study in a south Indian population. Majority of the deletions arise as *de novo* random meiotic errors in paternal germ cell lineages, however possible pronuclear and embryonic origin of deletions have also been described (27). Existing Yq microdeletion studies deviating from the consensus indicates the influence of genetic (28-30) and environmental factors (31) on male fertility validating the need for population specific Y chromosome deletion analysis.

AZFa deletions are the least frequent single loci deletions reported (<5%) and AZFb deletions have been reported to be more prevalent than AZFa deletions (3 to 10%) in different populations (8, 32). Our study did not identify the presence of any AZFa deletions while single loci AZFb deletions were observed in two non-obstructive azoospermic males. The deletions in these individuals either complete or partial, involves the loss of spermatogenic candidate genes as evident from the total lack of sperms in the ejaculate.

This is in contrast to the AZFc deletions observed in our study where individuals harbouring such deletions were observed to have a phenotype ranging from severe oligospermia to azoospermia indicating polygenic regulation (6).

Yq deletion frequency reported in the present study population is 2 to 3 folds lower then few

existing reports in Indian populations (18). In few instances this can be attributed to the limited choice of STS markers made in non-adherence of the EAA/EMQN guidelines (23). Also few of the studies have employed individuals suffering from mild oligospermia where it has been clearly demonstrated that such individuals do not harbour Y chromosome deletions (8).

A methodological error cannot be ruled out as simultaneous partial deletions involving AZFa, AZFb and AZFc have been reported in Indian populations in huge proportions (18).

Such observations are unique to Indian populations but however these deletions remain non-validated through deletion breakpoint mapping, identifying the recombination substrates and the mechanism of recombination so as to prove such incidences. However in few instances the genetic makeup of the study population might have had a profound influence on the deletion frequency. This can be explained due to the high degree of diversification among the hundreds of Indian ethnic groups that stands second next to African populations (15-17).

All the deletions discussed above are uniform deletions identified in the genomic DNA extracted from the somatic cells (blood) of the infertile individuals. Three independent studies have reported Yq microdeletions confined to germ cell lineage while the somatic cells do not harbour such deletions. A frequency ranging from 5 to 26% has been reported in the sperm DNA of infertile men with intact AZF regions in the somatic DNA (19-21). A thorough review of literature indicates that germ line specific Yq deletions are extremely rare events (27). Hence such incidences must be validated by southern blotting techniques, FISH or single cell PCR, which is not performed by majority of the studies (19). In our study STS markers based PCR assay did not identify any germline specific deletions on the sperm DNA of severe oligospermic infertile individuals.

Other then infertility a much severe phenotype and in certain cases lethal outcomes can occur due to sperm chromosome aneuploidies that can lead to abnormalities in the offspring (33).

Infertile couples opt for ART to have genetically related progeny but the associated genetic risks are often overlooked (25).

Sperm chromosome aneuploidies have been demonstrated to be high in case of infertile men compared to fertile men. A positive correlation has been demonstrated between sperm chromosome aneuploidies and azoospermia (34), low sperm counts (< 5 million per ml) (35), low sperm motility (36) and severe morphologic defects (37). A higher rate of chromosomal anomalies has been observed in embryo and children conceived through ICSI procedure. These aberrations were further related to sperm count, sperm motility and higher rate of chromosome aneuploidies of paternal origin (38). Molecular assays like karyotyping and Yq microdeletions analysis does not completely rule out the possible genetic transmission of the infertile condition through ART. Thus FISH technique is ideal to evaluate autosome and sex chromosome aneuploidy as well as Y chromosome deletion mosaicism in the sperm cells. Our study attempted to identify sex chromosome aneuploidy rates among severe oligo and oligo associated infertile men. However significant difference in sperm sex chromosome aneuploidies between the infertile men compared to control individuals was found lacking. The lack of a positive correlation in our study indicates that the alteration of sperm motility and morphology alone is not necessarily associated with higher sex chromosome anomaly rates in spermatozoa of infertile men.

Conclusions

AZFa microdeletions do not appear to be a risk factor in causing fertility impairment while AZFc microdeletions are the major event of Yq deletions leading to spermatogensis disruption among the infertile group. Y chromosome microdeletion analysis has to be performed with stringent inclusion exclusion criteria to eliminate methodological errors. Until ethnic specific markers are designed for Indian population, studies must follow the EAA/EMQN guidelines as minimum requirement for reporting Yq microdeletions. The lack of germ line specific deletions suggests FISH analysis using probes specific to the AZF regions to identify sperm Y chromosome microdeletions effectively than STS based deletion analysis.

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