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MALE INFERTILITY: SCREENING OF AZOOSPERMIA FACTOR (AZF) MICRODELETION IN IDIOPATHIC INFERTILE MEN

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ABSTRACT

Genetic factors cause about 15% of male infertility and microdeletions of Y chromosome is one of the genetic causes in idiopathic infertile men. Azoospermia factors (AZFa, AZFb, and AZFc) on Yq long arm are most important for spermatogenesis. For analysis of microdeletions in the AZF regions by sequence-tagged-site (STS) PCR is important screening method for infertility. An attempt has been made to evaluate the frequencies of microdeletions of AZFa, AZFb, AZFc in idiopathic cases of azoospermic and oligozoospermic subjects. Total 160 subjects (90 oligozoospermia and 70 azoospermia) and 50 control subjects were analyzed in this study. DNA samples were analyzed for microdeletions of Y chromosome by PCR-screening of 18 STS markers from different locus of the AZFa, AZFb, AZFc on Yq and SRY on Yp. The semen analysis was done and infertile men showing normal karyotype only were included in the study. Plasma follicle stimulating hormone (FSH) and leutinizing hormone concentrations were determined to rule out hormonal abnormality. Out of 160 analyzed cases, 17 (10.6%) subjects showed partial deletion of AZF regions, of which deletion in AZFc region was the most common (58.8%) and it was followed by AZFb and AZFa. The four subjects were shown two or more STS primer deleted sites and overall frequency of Y chromosome microdeletion in our subjects is 10.6%. The sites and sizes of deletions varied among patients. No deletions observed in control subjects. The varied frequencies of Y microdeletions are reported in infertile men in Indian population. From the results of this study it can be suggest that the frequency of deletions may be affected by study sample size, selection criteria of subjects and different geographical region. So, the screening of Y microdeletions is necessary along with the chromosomal analysis in case of infertile men.

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1 Introduction

Male infertility affects about 10-15% of couples trying for pregnancy, and approximately 50% man responsible for infertility (Boivin et al., 2007). Infertility is defined as the couple inability to conceive after one year of unprotected intercourse (WHO, 2010). Various causative factors of infertility in men include cystic fibrosis, agglutination of sperm, high semen viscosity, non-motile spermatozoa, low volume of ejaculate, varicocele, obstruction of the spermatic ducts, ejaculatory dysfunction, high sperm density, hormonal imbalance, and diabetes (Ambulkar et al., 2014). In 15% of male infertility cases cause is unknown (idiopathic infertility) (Ambasudhan et al., 2003). Male fertility depends on successful spermatogenesis, in which a number of sex/autosomal genes involved. So, it is evident that deletions or mutations in genes controlling spermatogenesis would result in infertility (Ferlin et al., 2007a).

Molecular studies of Y-chromosome has exposed a region on the short (p) arm specify sex determining region (SRY) that controls sex differentiation and long (q) arm of the Y chromosome having candidate genes that is required for spermatogenesis. Male infertility is associated with long arm of human Y chromosome (Lange et al., 2008). Tiepolo & Zuffardi (1976) reported deletion in non fluorescent euchromatic portion of long arm Y chromosome in azoospermia male and revealed that these regions contains spermatogenesis controlling candidate genes in human male (Tiepolo & Zuffardi, 1976). In fact this microdeletion of long arm of the Y chromosome is involving with spermatogenic failure because of lost spermatogenesis controlling genes (Ferlin et al., 2007a; Ambulkar et al., 2014). Deletion mapping of long arm of Y chromosome directly provide information of genes related to spermatogenesis, and showed three region of Azoospermia Factors (AZFa, AZFb and AZFc) mapped to Yq11.

These region containing candidate genes which involved in azoospermic or severe oligozoospermic male infertility (Ferlin et al., 2007b). In deletion interval 6 AZF region has been mapped in band q11.23 of the Y chromosome that contains 5 million base pairs (Vogt et al., 1996). The physical mapping of these regions has been estimated to be 3 Mb for AZFc and 1-3 Mb for AZFa and AZFb. The important candidate gene in AZFc is the *Deleted in Azoospermia* (DAZ) cluster, four copies of genes transcribed in the adult testis and expressed exclusively in germ cells (Reijo et al., 1995; Skaletsky et al., 2003). DAZ and RBMY genes families express RNA binding proteins with similar structures related to heterogenous nuclear ribonucleoproteins family (hnRNP) involved in RNA metabolism, including packaging of RNA, transporting to cytoplasm and splicing to get matured RNA (Reijo et al., 1995; Page et al., 1999). In AZFa region contains *DFFRY* (*Drosophila Fat Facets Related Y*) and *DBY* (*DEAD box polypeptide Y*) two main candidate genes. *RBMY* (*RNA-binding motif*) gene family whose expression is restricted to the testis is

main candidate gene in AZFb region (Chai et al., 1997). *RBMY* consists of approximately 30 copies of genes and pseudogenes found on both arms of the Y chromosome, but it is suggested that only functional genes are clustered at the long arm of Yq in the AZFb region (Delbridge et al., 1997).

Microdeletions of AZFc regions are causative factor of spermatogenic impairment. Increasing frequencies of *de novo* AZF locus microdeletions indicates that the Y chromosome is highly susceptible to spontaneous loss of spermatogenesis involving genes (Sun et al., 1999). The deletion prone of AZF region has comprised high frequencies of repetitive sequences clustered on Y chromosome, and exist a Y chromosome haplotype that promote deletions of the AZF regions (Thangaraj et al., 2003; Singh & Raman, 2009). Thus, *de novo* deletion may be more susceptible in some infertile individuals than are others. Now a days, intracytoplasmic sperm injection (ICSI) technique is widely accepted procedure to human assisted reproduction in case of male infertility (Silber, 2011). ICSI has changed approach of treatments in males with spermatogenic defects and allowing men to achieve biological paternity of his own children. Transmission of defective AZF sub-region to male child by ICSI has been developed infertility problem in son and lead to familiar infertility (Page et al., 1999).

Due to prognostic value of this type of microdeletion in Y chromosome, present study investigated the frequency of the Y-chromosome microdeletions in idiopathic infertile men in central region of India and to determine the phenotypes associated with specific deletions.

2 Materials and Methods

2.1 Collection of Blood Samples

The present study was carried out on 160 idiopathic infertile patients. All these infertile men were showed different semen profile from oligozoospermia (<20 million/ml sperm) to azoospermia (absent of sperm in semen) according to semen analyses guidelines of WHO 2010 (WHO, 2010). Total 90 oligozoospermia and 70 non-obstructive azoospermia infertile men were studied and all infertile males had recruited from different laboratories, Urology clinics and IVF centre. History of patients such as Diabetes, Cystic fibrosis, testicular-pathies subjects not taken blood sample. After hormonal analysis remaining blood samples were collected from these centres to perform genetic analysis of subjects. Questioner form was filled up from all infertile patients.

2.2 DNA extraction

Human genomic DNA was extracted from peripheral blood lymphocytes using conventional method (Ambulkar et al., 2014) as follow: briefly, 0.5 ml of peripheral blood was collected in vials.

Table 1 STS-Primers and its sequences for screening of AZF region Y chromosome.

STS marker	Forward Sequences	Reverse Sequences	PCR products	Locus
sY746	5'-TTGACTGCTTATTCTACACAA-3'	5'-CAGGGGAAATTGGGTTTT-3'	216 bp	AZF_a
sY86	5'-GTGACACACAGACTATGCTTC-3'	5'-ACACACACACAGACTATGCTTC-3'	320 bp	
sY84	5'-AGAAGGTCTGAAAGCAGGT-3'	5'-GCCTACTACCTGGAGGCTTC-3'	326 bp	
DFFRY	5'-ATGTGGACTATAATTTCTTCCC-3'	5'-CTGTCGTTCCCTCCTACTGG-3'	130 bp	
sY113	5'-GTTCTTTCCACAGCCCATAG-3'	5'-TGGAACACAATCCAAAATTG-3'	304 bp	AZF_b
sY118	5'-GTACCTCTGCAGGCACTGAT-3'	5'-ACACAATCCAACCTGGCTAA-3'	218 bp	
sY127	5'-GGCTCACAAACGAAAAGAAA-3'	5'-CTGCAGGCAGTAATAAGGGA-3'	274 bp	
RBM1Y	5'-ATGCACTTCAGAGATACGGGA-3'	5'-CTCTCTCCACAAAACCAACAGT-3'	800 bp	
XKRY	5'-GACACCATGGCTGGAGTTTT-3'	5'-GGATTGTATGTCCACCTGC-3'	200 bp	
sY134	5'-GTCTGCCTCACCATAAAACA-3'	5'-ACCACTGCCAAAACCTTTCAA-3'	301 bp	
sY143	5'-GCAGGATGAGAAGCAGGAT-3'	5'-CCGTGTGCTGGAGACTAATC-3'	311 bp	
sY153	5'-GCATCCTCATTTTATGTCCA-3'	5'-CAACCCAAAGCACTGAGTA-3'	139 bp	AZF_c
sY148	5'-AAATGAAAAGATACGAATC-3'	5'-GAATCCCACCCAAGAATCTG-3'	202 bp	
sY157	5'-CTTAGGAAAAGTGAAGCCG-3'	5'-CCTGCTGTCAGCAAGATACA-3'	286 bp	
sY255	5'-GTTACAGGATTCGGCGTGAT-3'	5'-CTCGTCATGTGCAGCCAC-3'	126 bp	
sY254	5'-GGGTGTTACCAGGAGGCAAA-3'	5'-GAACCGTATCTACCAAAGCAGC-3'	380 bp	
sY158	5'-CTCAGAAGTCCTCCTAATAG-3'	5'-ACAGTGGTTTGTAGCGGGTA-3'	231 bp	
sY160	5'-TACGGGTCTCGAATGGAATA-3'	5'-TCATTGCATTCTTTCCCAT-3'	236 bp	
sY14 (SRY)	5'-GAATATTTCCCGCTCTCCGGA-3'	5'-GCTGGTGCTCCATTCTTGAG-3'	476 bp	

Table 2 Deleted 17 Azoo/Oligozoospermia cases showed AZF deletion sites and seminal condition.

Deletion in cases	Deleted AZF region	Semen type
P6	AZF _c	Azoospermia
P13	AZF _c	Oligozoospermia
P19	AZF _c	Oligozoospermia
P27	AZF _b +c	Azoospermia
P33	AZF _c	Oligozoospermia
P38	AZF _b	Azoospermia
P41	AZF _c	Oligozoospermia
P53	AZF _c	Oligozoospermia
P69	AZF _b +c	Oligozoospermia
P75	AZF _a	Azoospermia
P89	AZF _a	Azoospermia
P103	AZF _b	Azoospermia
P109	AZF _c	Oligozoospermia
P126	AZF _a +b	Azoospermia
P137	AZF _c	Azoospermia
P143	AZF _b +c	Azoospermia
P156	AZF _c	Oligozoospermia

RBC lysis buffer was added to whole blood. It was following by addition of 20% SDS and proteinase K and incubated for 2 hrs, at 55°C. DNA was extracted by phenol chloroform treatment and 3M sodium acetate and 99.5% ethanol was added for DNA precipitation. DNA sample was washed in

70% ethanol, and dissolved in TE buffer. DNA extracted from each patient was prepared for 100 ng/ml DNA concentration.

2.3 Sequence tagged sites polymerase chain reaction (STS-PCR)

The screening method for microdeletions was based on simplex PCR method using Y-chromosome specific STSs, which corresponded to the AZF_a, b and c regions. Eighteen ICMR recommended Yq STS were used: sY746, sY86, sY84, DFFRY (AZF_a), sY113, sY118, sY127, RBMY, XKRY, sY134, sY143 (AZF_b), sY153, sY148, sY157, sY255, sY254, sY158, sY160 (AZF_c). In addition, the Yp STS sY14 within the SRY gene was tested. Specific primers used for each STS PCR reaction mixture and the expected PCR product lengths are amplified. The analysis of more than three STS loci in each region increases diagnostic accuracy (Table 1), because deletions usually involve more than one STS primer sites loci. Genomic DNA (2µl) was added with 100 mM Tris-Cl (pH 8.3), 5µl 10X PCR assay buffer, 15 mM MgCl₂, 200 mM of dNTP mix, 0.5 mM of each primer pair, 0.2 µl Taq DNA polymerase (NEB, England) in reaction mixture and adjusted to a final volume of 50 µl with sterile triple distilled water. The PCRs were performed according to the following programs for all the STS markers: initial denaturation were 94°C for 4 min subsequently 30 cycles at 94°C for 30 sec, 55-58°C for 30 sec, and 72°C for 1 min and a final extension step at 72°C for 7 min.

Table 3 Regionwise Y microdeletions are detected in 17 in oligo/azoospermia infertile patients.

AZF region deletion	Frequency (n=17)	Percentage
AZF _a	1	5.88%
AZF _b	2	11.76%
AZF _c	10	58.82%
AZF _{a+b}	1	5.88%
AZF _{b+c}	3	17.64%

Every PCR reactions were set with two negative controls as one sample without template DNA and one sample of female genomic DNA. In the event of detecting a microdeletion of a Y-specific sequence tagged sites (STSs) with a primer, the PCR assay were repeated thrice for confirmation.

3 Results

All infertile men were shown normal cytogenetic profile and were subjected for deletion mapping of Y chromosome microdeletions. Y chromosome microdeletions were detected and confirmed with 18 STS primer PCR based analysis in 17 subjects (10.6%) out of the 160 infertile subjects. Out of 17 subjects, 4 (23.5%) were detected with the presence of either two or more deleted STS loci and remaining 13 (76.4%) subjects were shown single STS sties deleted on AZF regions (Figure 1). All deletions were recorded in interstitial region of Yq11 in the AZF locus of 8.8% (8/90) oligozoospermic and 12.8% (9/70) azoospermic infertile men (Table 2). No deletions were identified in any of the 50 fertile men. In normal fertile women, negative control, STS failed to amplify. The relatively high proportion of microdeletions (12.8%) found in 70 azoospermic infertile and (8.8%) found in 90 oligozoospermic infertile men. The Regionwise deletions observed and the percentages of STS markers in AZF locus are shown in Table 3 (Figure 2). All the infertile men with microdeletions had normal serum testosterone concentrations, and serum FSH and LH concentrations had within normal range.

Discussion

Spermatogenesis is controlled by a number of genes on sex chromosome and autosomes (Vogt et al., 1996). Long arm of Y chromosome having three important locus, which contains essential candidates genes for regulation of spermatogenesis. Microdeletions in Y-chromosome are known cause of male infertility (Krausz & Degl'Innocenti, 2006). The incidence of Y chromosome microdeletions varied with the severity of the spermatogenic defect in infertile males (Massart et al., 2012). Over all 10-15% of azoospermic and 5-10% oligozoospermic men show Y microdeletions in idiopathic conditions and till on date STS PCR based technique is used for screening of AZF regions microdeletions on Y chromosome (Sachdeva et al., 2011; Sen et al., 2013).

Now, the diagnosis of genetic aetiology has important clinical significance because assisted reproductive technology helps infertile couple to overcome infertility problem. Intra cytoplasmic sperm injection (ICSI) is prone for transmission of microdeletions to their offspring (Silber, 2011). Hence, STS-PCR based Y chromosome microdeletions screening will provide counselling to infertile couple effectively, regard to the birth of an infertile male offspring (Kuchukaslan et al., 2013; Ambulkar et al., 2015).

In present study, 10.6% microdeletions was reported in the Y chromosome in idiopathic oligozoospermic and azoospermic infertile men. In 90 oligozoospermic and 70 azoospermic infertile males had shown 8 (8.8%) patients and 9 (12.8%) patients Y-chromosome microdeletions respectively. Here, in azoospermia patients the frequency of microdeletion is higher than oligozoospermia (Table 2). The reported frequencies of microdeletions of Yq in different studies range between 2% and 18% of males with non-obstructive azoospermia or severe oligozoospermia in India (Ambasudhan et al., 2003; Dada et al., 2004; Sen et al., 2013). In comparison to the statistical data obtained from all the published report to date, some studies reported 20% of microdeletions in the Y chromosome of infertile males, while others 2%. Further studies had showed an incidence of microdeletions 5.1% to 9.6% in the infertile males (Tuttelmann et al., 2007; Ferlin et al., 2007b). On the other hand, in Italy 55% microdeletions of Y chromosome were found in infertile males (Simoni et al., 2004). Results of present study and prevalence of microdeletions are in accordance with the previously reported results and reported between 10-23% (Ambasudhan et al., 2003; Thangaraj et al., 2003).

Previously published studies have shown a remarkably variation in the microdeletion frequency. This is due to selection criteria of different patients groups and use of different sets of STS marker (Simoni et al., 2004). On Yq region of AZF microdeletions was directly related with the phase in which spermatogenesis was arrested. Microdeletions of each locus cause spermatogenic arrest at a particular stage because each candidate genes of AZF locus specified a different phase of spermatogenesis (Geoffroy-Siraudin et al., 2007). Testicular histology of Y chromosome microdeletions in azoospermia and oligozoospermia infertile male had shown variable phenotypes of spermatogenesis (Singh & Raman, 2005). The microdeletions in AZF_a region was related with SOC syndrome (absence of germ cells and presence of sertoli cells in the seminiferous tubules) and azoospermia (Mitra et al., 2008). The microdeletion of AZF_b was associated with developmental arrest of germ cells and led to meiotic maturation arrest. The microdeletion of AZF_c was associated with arrest of germ cells at spermatid stage and hypospermatogenesis with sperm counts (Chai et al., 1997; Delbridge et al., 1997). Therefore, microdeletions of a AZF regions results in abnormal phenotypes in spermatogenesis and candidate genes at each locus act at a specific stage of germ cell differentiation (Ambasudhan et al., 2003; Massart et al., 2012).

Figure 1 (a & b) Agarose gel analysis shows microdeletions in STS markers of sY127 and sY134 in lane 6.

Y chromosome of AZFc region was most frequently deleted sites in infertile males. DAZ (Deleted in azoospermia) is candidate gene of AZFc region plays important role for spermatogenesis (Zhang et al., 2007). Deletion of AZFc region was detected in ten patients, 58.8% (10/17) of total deleted infertile males. Microdeletions in AZFb region were detected in 2 (11.7%) infertile males and AZFa region was detected in 1 (5.8%) infertile males. Deletion of AZFb+c and AZFb+a regions were detected in 3 (17.6%) and 1 (5.8%) infertile males respectively (Figure 2). Three deletions in AZFc+b and one deletion in AZFb+a are overlapping two regions otherwise all microdeletions are occur in interstitial of AZF regions of Y chromosome. Results of present study showed AZFa and AZFb are less frequently deleted sites than AZFc region (Table 3). DAZ is multiple copy number genes which contains higher number of pallindromic repititive sequences in AZFc region. Skaletsky et al. (2003) was reported that reduction of copy number to have an increased risk in infertility (Skaletsky et al., 2003; Sen et al., 2015). Deletion of AZFa and AZFb of Y

chromosome can cause azoospermia or severe oligozoospermia (Vogt et al., 1996) and these results showed that all AZFa and AZFb microdeletions were present in only azoospermic infertile males (Mitra et al., 2008; Ambulkar et al., 2015).

The present study showed that microdeletions in spermatogenesis involving genes of AZF region cause various abnormal phenotypes in infertile males. These results confirmed that before the infertile couple have adapted Intra cytoplasmic sperm Injection or other assisted reproductive techniques, sets of STS markers of AZF regions should be used routinely as diagnostic genetic test to screen for Y chromosome microdeletions.

Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

Figure 2: Subject wise percentage of AZF region deletion in 17 subjects.

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