

## Microdeletions of *AZFc* Region in Infertile Men with Azoospermia and Oligoasthenoteratozoospermia

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**KEYWORDS** Male Infertility. Azoospermia. Microdeletion. Azoospermia Factor

**ABSTRACT** Considerable progress has been made to study the pathophysiology of male infertility in the last few years. About 10-15% of genetic causes such as chromosomal abnormality, single gene and multifactorial mutations may be a causative factor. Submicroscopic microdeletions on chromosome Y is considered to be the most frequent genetic defects associated with impaired spermatogenesis. Therefore, the aim of this present study was to assess pregnancy outcome of intracytoplasmic sperm injection (ICSI) in infertile men from 285 study subjects; fertile controls ( $n=110$ ) and cases ( $n=175$ ) with sperm count less than 5 millions/ml [azoospermia ( $n=150$ ) and oligoasthenoteratozoospermia ( $n=25$ )]. Microdeletions located on chromosome Y-sY84, sY86 (*AZFa*), sY127, sY134 (*AZFb*), and sY254, sY255 (*AZFc*) were confirmed with Polymerase Chain Reaction (PCR). A 12.56% was observed in infertile men in one or more STS with a percentage of 1.14, 2.28, 9.14 for *AZFa*, *b* and *c* respectively. The azoospermia factor region *c* (*AZFc*) loci showed an increase in frequency when compared to *AZFb* and *a* regions. Intracytoplasmic Sperm Injection (ICSI) could be offered or an alternative method to patients with *AZFc* deletion.

### INTRODUCTION

Infertility has been defined by World Health Organization as inability of couples to conceive naturally after at least one year of unprotected intercourse (Dohle et al. 2002). About 10-15% of married couples worldwide suffer from infertility (Dada et al. 2003), of which approximately 50% is attributed to male factor; clinically presenting as either absent or low sperm counts (less than 5 millions/ml) resulting from altered spermatogenesis (Hellani et al. 2006). The etiology of male infertility is complex which includes life style, environmental and genetic factors. The genetic basis may be due to chromosomal, single gene or mitochondrial DNA (mt DNA) mutations, Y chromosome microdeletions and multifactorial disorders (Gianotten et al. 2004; Shamsi et al. 2011).

A significant pathogenetic defects associated with male infertility is microdeletions located on long arm of chromosome Y (Yq) a most frequent

target for genetic damage (Poongothai et al. 2009). The role of Y chromosome was first elucidated in 1976 when Tiepolo and Zuffardi have proposed the factor which controls spermatogenesis are encoded by a gene that is localized within the euchromatic region of (Yq11) Y chromosome, called as azoospermia factor (*AZF*) (Tiepolo and Zuffardi 1976). It is divided into seven deletion intervals that are divided into sub-intervals (A, B, C D, E, F and G). Vollrath et al. in 1992, constructed a 43-interval deletion map that contains an array of sequence tagged sites (STS) which spans entire length of the Y chromosome (Vollrath et al. 1992). The short arm (Yp) and centromere contain deletion intervals 1-4 from distal to proximal; euchromatic part of the Yq is represented by intervals 5 and 6 that is, proximal to distal; the heterochromatic region is defined as interval 7. Deletion interval 5 and 6 corresponds to Yq11.21 through the middle part of Yq11.22, and Yq11.22-Yq11.23. Originally, three regions were defined: *AZFa*, *AZFb* and *AZFc* (azoospermia factor), which map on the long arm (Yq) from the centromere to telomere; a fourth region, named *AZFd*, located between *AZFb* and *AZFc* is associated with precise testicular histology (Esteves and Agawal 2011)

The incidence of microdeletions varies from 1-55% in *AZF* regions of infertile men. This phenotype had shown a marked variation in dele-

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tion frequency among different population and also has featured to selection of distinct patient groups and use of marker sets (Simoni et al. 1999). In north European populations such as Scandinavian countries, France, Germany, Netherlands for instance, the frequency of Y-chromosome deletion in infertility cases is low (1–4%), while it is greater than 15% in Italy (Foresta et al. 1997). The frequency revolves around 10% with available data from Australia, New Zealand and Southeast Asian countries (China, Japan, Korea, Philippines etc.). In earlier reported cases from Asian populations, vast majority of deletions is confined to *AZFc* region, only rare cases of showed *AZFa/b* deletions. Vogt et al. in 1998 correlated the position of the *AZF* deletion with a phase in which particular stage of spermatogenesis arrest can be represented at each specific *AZF* locus (Vogt et al. 1998). Therefore, this present study was aimed to correlate the prevalence and frequency of microdeletions in *AZFa*, *b* and *c* subregions of azoospermic and oligoasthenoteratozoospermic infertile men.

## MATERIAL AND METHODS

The study was approved by the Institutional Ethics Committee (IEC: SRMC/RP/4505). EDTA blood samples were collected after obtaining informed consent from the study subjects with detailed medical history. To investigate microdeletions on chromosome Y, genomic DNA was isolated from peripheral blood with the manufacturer's protocol (Bioserve-DNA Isolation Kit). Microdeletions were detected by performing STS PCR based techniques with controls ( $n=110$ ) and case's ( $n=175$ ). All primer sequences were obtained from published literature (Simoni et al. 1999). The azoospermia factor regions (*AZF*) were studied using *AZFa*-sY 84 (326 bp), sY 86 (320 bp), *AZFb*-sY 127 (274 bp), sY 134 (301 bp) and *AZFc*- sY 254 (400 bp), sY 255 (126 bp). Materials required for PCR experiments were commercially procured (Fermentas 2X-DreamTaq PCR Master Mix). The PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide (0.5 $\mu$ g/ml) and observed under UV-transilluminator. The amplified PCR products showed presence or absence of a band (expected size) for all the six loci and documented. Fertile male and female samples were used as positive and negative controls. The deletion frequency between the cases

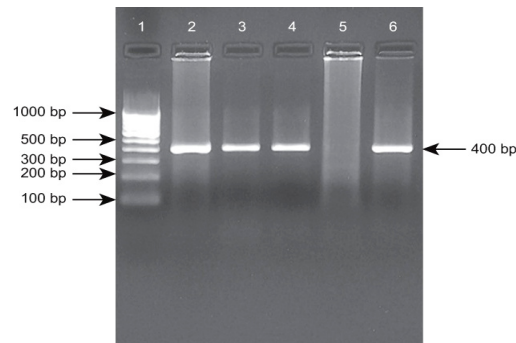
and controls were compared and level of significance was calculated using student's "t" test.

## RESULTS

The frequency of microdeletions was found to be 9.14% and 3.42% in the azoospermic and oligoasthenoteratozoospermic infertile men. The results obtained from fertile control subjects did not display any deletion in either *AZF a*, *b* or *c* region ( $n=110$ ), whereas a 12.56% of infertile subjects showed microdeletions in one or more sequence tagged sites (STS) with a percentage of 1.14, 2.28, 9.14 for *AZFa* (sY84, sY86), *AZFb* (sY127, sY134) and *AZFc* (sY254, sY255) respectively (Table 1). A representative picture shows PCR amplicons of sY 254 region (Fig. 1).

**Table 1: Yq microdeletions associated with azoospermia and Oligoasthenoteratozoospermia (OAT)**

Abnormal spermogram	Microdeletions observed in AZF region			
	<i>AZF a</i>	<i>AZF b</i>	<i>AZF c</i>	Total
Azoospermia	2.0 (1.14)	4.0 (2.28)	10.0 (5.72)	16.0 (9.14)
OAT	0.0	2.0 (1.14)	4.0 (2.28)	6.0 (3.42)



**Fig. 1. Displays the PCR amplicons of *AZF c* (sY 254) region**

Lane 1: Molecular weight ladder (100bp); Lane 2,3,4 and 6: shows the presence of PCR amplicons and Lane 5: absence of PCR amplicons

## DISCUSSION

Genetic factors contribute significantly to infertility of which approximately 50% are due to male factor (Vollroth et al. 1992). Spermatogenesis is highly disorganized and still healthy fertile men may have certain percentage of sperm with abnormal morphology. It was reported that microdeletions in *AZF* region can lead to a vari-

able phenotype with a significant reduction in sperm count and secondarily to an increased loss of germ cells and progressive decline in semen quality. The incidence of Yq microdeletions ranges from 7–21% in azoospermic men and 0–14% in oligozoospermic patients (Foresta et al. 2001; Poongothai et al. 2009). Babu et al. (2002) found 15% of *AZF* deletions in azoospermic and severely oligozoospermic men in a population of 20 patients (Babu et al. 2002). Thus, the incidence of microdeletion of chromosome Y is higher when patients are selected by their testicular histology (Ferlin et al. 2005; Esteves et al. 2011).

The deletion frequency in the present study was found to be 9.14% in azoospermia and 3.42% in oligoasthenoteratozoospermia cases. However, Foresta et al. (1997) found a very high percentage (55.5%) of Italian infertile men to carry these Y-chromosomal microdeletions in azoospermia cases (Foresta et al. 1997). Kuroda-Kawaguchi et al. (2001) have also reported a higher deletion frequency in azoospermia of 12% and 6% in severe oligoasthenoteratozoospermia cases (Kuroda-Kawaguchi et al. 2001). Mohammed et al. (2007) reported 2.6% ( $n=7/266$ ) of microdeletions in the *AZFc* and *c* regions (Mohammed et al. 2007). Dada et al. in 2003 reported the frequency of Y microdeletions as 9.63% among 83 infertile Indian men studied using six STS primers (Dada et al. 2003).

On the basis of testicular histology, the deletion of *AZFa* was associated with complete absence of germ cells, presence of sertoli cells in seminiferous tubules, characteristic of sertoli cell-only syndrome (SCO) associated with azoospermia. The main candidate gene in the *AZFb* region has a restricted expression in the testis (Ferlin et al. 2003) which is associated with developmental arrest of germ cells at pachytene stage and leads to meiotic maturation arrest (Frydelund et al. 2002). Deletions in *AZFc* regions are (Raicu et al. 2003) associated with developmental arrest of germ cells at spermatid stage, hypospermatogenesis, maturation arrest and low sperm counts. Thus, deletion of a particular *AZF* locus results in a characteristic phenotype, and genes at each locus act at a particular stage of germ cell differentiation.

Krausz et al. (2006) found that these microdeletions may cause deregulation of gene expression by position effect and interfere with post-transcriptional modification of gene expression,

or result in the absence of genes critical for spermatogenesis (Krausz et al. 2006). The Y chromosome has a highest spontaneous loss of genetic material in the human genome. Most of the ancestral genes are functionally intact on X chromosome, which undergoes crossing over; but because of the lack of XY recombination, there is monotonic decline in gene function on chromosome Y and thus the accumulation of deleterious mutations. More than one SNPs and point mutations in any specific genes of Y-chromosome, deletions of large regions within *AZF* region have been found to be more frequent in cases of idiopathic infertility.

In the present study, 12.56% of infertile men have shown Yq microdeletion in 175 cases, while no microdeletion was observed in control samples. A 2% of the fertile men might harbor microdeletions of chromosome Y that involving non-coding region (Choi et al. 2004). The relative frequency of individual microdeletions is reported to be 5%, 16% and 60% for *AZFa*, *AZFb* and *AZFc* regions respectively. The first major gene identified in *AZFa* is sY84, but its role in spermatogenesis remained to be confirmed. To date only three infertile patients have been reported carrying a deletion of this gene, loss of these genes through *AZFa* have been seen to give rise to more severe phenotypes such as SCO syndrome (Sargent et al. 1999). One of the hallmarks of the Y-chromosome is the high frequency of amplified repeat sequences dispersed throughout the euchromatic and heterochromatic regions. This genetic instability arises from the presence of highly repetitive segments in the long and short interspersed repeats and from a large portion of Y chromosome (95%) that does not undergo recombination during meiosis (Sun et al. 2000). Microdeletions restricted to *AZFb* or *c*, can result in a range of phenotypes from sertoli cell-only syndrome to moderate oligozoospermia. The *DAZ* gene cluster localized on the distal euchromatic region of the Y chromosome *AZFc* region is one of the most important candidate genes involved in infertility. Another study from South India (Swarna et al. 2003) reported 4/50 infertile men with *AZFc* deletions. The deletions in *AZFc* often include all the copies of *DAZ* gene, and they are frequently associated with azoospermia, rarely with oligozoospermia (Reijo et al. 1995). The presence of multiple copies is to create redundancy in this important gene if mutation damages one of those genes.

Estimations of the molecular extensions of *AZF* regions in numerous patients using PCR multiplex interval mapping suggest similar breakpoints in Yq11 for *AZFa* (Qureshi et al. 1996; Vogt et al. 1996), *AZFb* (Girardi et al. 1997; Vogt 1998) and *AZFc* patients (Girardi et al. 1997; Qureshi et al. 1996; Reijo et al. 1995; van der Ven et al. 1997; Vogt et al. 1996). This indicates breakage hotspots in Yq11 to be at their borderlines. Such hotspots are frequently represented by homologous loci chromosome-specific repetitive DNA blocks, (Yen 1998) these deletions probably occur due to unequal intrachromosomal crossing-over events at meiosis during spermatogenesis in the father. This would explain for higher frequency of *AZFc* deletions than *AZFa* and *b* deletions, as local repetitive DNA blocks are enriched in distal Yq11 in the neighborhood of highly repetitive heterochromatic Yq12 region. However, to enable a more detailed examination of *AZF* deletion's origin, single cell deletion analyses in Yq11 of the spermatozoa of sterile patients and fertile control men might be an essential prerequisite. Therefore, it should be of critical interest to take geographical, environmental and ethnic axis into consideration on the genetic basis of infertility. In a multifactorial disorder, such as idiopathic infertility, where environment and genetic components interact variously, data from more regions need to be generated to develop further realistic picture.

### CONCLUSION

The present study has shown the prevalence of Y microdeletions as 12.6% in infertile men with an increase in the frequency of microdeletion in *AZFc* loci when compared to *AZFb* and *a* regions. The occurrence of Y microdeletions is reported to be *de novo*, but there is a risk of transmission of the Yq microdeletions from father to son. Therefore, Y microdeletion analysis by STS-PCR is a simple, highly sensitive method and molecular analysis can be a useful tool in identifying affected infertile men so that appropriate counseling can be given before treatment by assisted reproductive technology. Despite the improvement of assisted reproductive technology, ICSI could be offered only to patients with an *AZFc* deletion. Insemination with donor sperm is a potential alternative for other patients with *AZFb* and  $\alpha$ .

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