

## Prevalence of Azoospermia Factor (AZF) Deletions in Idiopathic Infertile Males in North-East India

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**ABSTRACT** The Y-chromosome harbors about 107 genes and pseudo genes. Microdeletions of the Y-chromosome long arm are the most common mutations in infertile males, where they involve one or more “azoospermia factors” (AZF a, b, and c). 100 consecutive infertile men were studied for AZF microdeletions by isolating Genomic DNA from peripheral blood and Polymerase Chain Reaction was carried with Genes: RBMY [RNA-binding motif (RBM), Y chromosome], BPY-2 (Testis-specific basic protein Y 2), STS markers: SY-84, SY-254. Out of 100 infertile males, five males exhibited AZF deletions. RBMY deletion was observed in one male, SY-84 deletion was observed in two males and SY-254 deletion was observed in two males. BPY-2 deletion was not observed in any of the males.

### INTRODUCTION

Infertility is a reproductive health problem that affects many couples in the human population. It is a major health problem today, affecting 10 to 15 percent of couples seeking to have children (Mosher 1985; Tse et al. 2000; Anurag et al. 2008). Male infertility is particularly problematic since it is estimated that the cause of the infertility is unknown in up to 50 percent of the cases (Dublin and Amelar 1971; Hendry et al. 1973; Zhou-Cun et al. 2006). Regardless of whether it is primary or secondary infertility, affected couples suffer from enormous emotional and psychological trauma and it can constitute a major life crisis in the social context. Male infertility problems may be contributory to 30 percent to 40 percent of infertile couples, and in another 20 percent of cases, both men and women are affected. The male factor is, therefore, responsible in about 50 percent of infertile couples. The infertile male partner has qualitative or quantitative abnormalities of sperm production (Dada et al. 2004; Shahira et al. 2009; Viswambharan et al. 2007). In more than 60 percent of cases, the origin of reduced testicular function is unknown (Krausz et al. 2000). Approximately 15 percent of the men seeking help at infertility clinics suffer from idiopathic azoospermia, the absence of mature sperm cells (spermatozoa) in seminal fluid. A significant proportion of infertile male with azoospermia and severe oligozoospermia (sperm counts of less than 5 to 10 million per ejaculate) have a genetic etiology for reproductive failure. In men,

the main causes of infertility are oligozoospermia, asthenozoospermia, teratozoospermia and azoospermia, which account for 20–25 percent of cases (Egozcue et al. 2000; Hargreave 2000). Many cases of idiopathic infertility have either a genetic or molecular basis (Swarna et al. 2003; Omrani et al. 2006). The knowledge of the molecular genetics of male infertility is developing rapidly, new “spermatogenic genes” are being discovered and molecular diagnostic approaches (DNA chips) established (Rabea et al. 2009; Swarna et al. 2003; Omrani et al. 2006; Viswambharan et al. 2007).

The Y-chromosome though representing only 2-3 percent of the haploid genome harbours about 107 genes and pseudogenes. Many of these are responsible for spermatogenesis and other male-related functions and deletion of any of these can result in infertility. Y-chromosome microdeletions are common in about 10-15 percent of men with azoospermia or severe oligozoospermia. These microdeletions are too small to be detected by karyotyping. They can be easily identified using polymerase chain reaction (Verginie et al. 2007; Park et al. 2010).

Over 20 years ago, cytogenetic studies demonstrated that deletion of the distal euchromatin of the Y-chromosome was associated with infertility (Tiepolo and Zuffardi 1976). More recently, the Y-chromosome was mapped with high resolution into a series of seven deletion intervals by analyzing DNA from patients with various deletions of the Y-chromosome (Vollrath et al. 1992). Deletion intervals 1 through 4 comprise the short (p) arm and the centromere, and

deletion intervals 5 through 7 comprise the long (q) arm. The deletion intervals of the Y chromosome have been finely mapped with a series of sequence tagged sites (STSs), aligned along their length (Vollrath et al. 1992). Sequence tagged sites are known sequences of genomic DNA that can be amplified by polymerase chain reaction (PCR). Using this method, much smaller deletions can be detected than is possible with cytogenetic analysis. The cumulative data from these studies demonstrated that there are actually three regions in deletion intervals 5 and 6 where most deletions occur in infertile men. The clustering of deletions in these three regions was first noted in a study by Vogt et al. (1996). These regions were designated AZFa, AZFb, and AZFc. AZFa was found in the proximal portion of deletion interval 5; AZFb was located at the proximal end of deletion interval 6, extending into the distal part of deletion interval 5; and AZFc was found in the distal portion of deletion interval 6. In the study by Vogt et al. (1996), each of the AZF regions seemed to be associated with a distinct testicular histology: deletions in AZFa with Sertoli cells only, deletions in AZFb with spermatogenic arrest at the pachytene spermatocyte stage and deletions in AZFc with spermatogenic arrest at the spermatid stage. The great majority of the reported Y-chromosome deletions found in infertile men have been shown to occur in one of these three regions, with AZFc being the most common site of these microdeletions. However, the correlation between deletions in these regions and particular phenotypes has not been consistently demonstrated in other studies (Pryor et al. 1997).

With the help of Polymerase Chain Reaction (PCR), Y-chromosome microdeletions have been detected in the Azoospermia Factor (AZF) region of DNA from infertile men (Foote et al. 1992; Park et al. 2010). There is lack of adequate data on Azoospermia Factor (AZF) deletions in the North-East Indian population. The present study was undertaken to determine the frequency of AZF deletions in infertile men with azoospermia, severe oligozoospermia and oligozoospermia of North-East India.

## MATERIAL AND METHODS

**Selection of Samples/Patients:** The study population consisted of 100 infertile men compared to 20 normal fertile men seeking androlo-

gic investigation for couples infertility at fertility clinic. The age of infertile men ranged from 25 to 50 years. They were subjected to detailed clinical and biological investigations, including cytogenetic and endocrinology studies, physical examination. On the basis of spermiogram, individuals ( $n = 100$ ) were subdivided into 3 groups: azoospermic (0 spermatozoa spz/mL), severe oligozoospermic (less than  $5 \times 10^6$  spz/mL) and oligozoospermic (less than  $20 \times 10^6$  spz/mL) according to the criteria of the World Health Organization (World Health Organization 1999). 20 males with proven fertility and normal sperm concentration (more than  $20 \times 10^6$  spz/mL) were selected as Control.

**Sample Collection:** Two ml of venous blood was collected in a tube containing ethylenediamine tetraacetate (EDTA) as an anticoagulant for DNA extraction.

**Molecular Investigations:** The molecular investigation was carried out in our Research Laboratory, Cotton College, Guwahati, Assam. Genomic DNA was extracted utilizing the GeneiPure™ Blood Genomic DNA Purification Kit. Polymerase chain reaction (PCR) was performed according to the standard protocol for analysis of the AZF region of the Y-chromosome. Three sub-regions were analyzed: AZFa, AZFb and AZFc, where sequence tagged site (STS) primers were used. As positive control, fertile men with naturally conceived children were used. These STS primers were suggested by the European Academy of Andrology which are able to detect 90% of the deletions in the loci of AZF.

The PCR amplification comprised a total volume of 25  $\mu$ L, which contained 100 to 200 ng of human genomic DNA as template, 2.5mM dNTP's (2.5 mM each of dTTP, dCTP, dGTP, dATP), oligonucleotide primers (0.1 to 2.0  $\mu$ mol/L each of the forward and reverse primers), 10X Taq DNA polymerase assay buffer (Tris with 15mM  $MgCl_2$ ) and 3U Taq DNA polymerase.

The conditions for thermocycling was standardized for the sub-regions, utilizing a TC-512 gradient thermocycler. Samples were subjected to Polymerase Chain Reaction amplification using 35 cycles at 94°C for 30 sec, 53°C for 45 sec and 72°C for 60 sec. Initial denaturation was done at 94°C for 5 min and final extension at 72°C for 10 min. The PCR amplified products were submitted to electrophoresis on 2% agarose gels and stained with 0.5  $\mu$ g/mL ethidium

bromide. Before loading the samples into the wells of the agarose gel, samples were mixed with Gel loading dye in the ratio of 1:1. A visual record of the gels was made with the help of gel documentation system.

### Primers Used for PCR Amplification

SY 84 for amplification of AZFa region.  
Forward: 5'-AGA AGG GTC TGA AAG CAG GT-3'  
Reverse: 5'-GCC TAC CTG GAG GAG GCT TC-3'  
SY 254 for amplification of AZFc region.  
Forward: 5'-GGG TGT TAC CAG AAG GCA AA-3'  
Reverse: 5'-GAA CCG TAT CTA CCA AAG CAGC-3'  
RBMV for amplification of AZFb region.  
Forward: 5'-ATGGTAGAAGCAGATCATC CTGG-3'  
Reverse: 5'-TTAATATCTGCTCGAGTCTC CTTT-3'  
BPY-2 for amplification of AZFc region.  
Forward: 5'-ATGATGACGCTTGTCCCCA GAGCC-3'  
Reverse: 5'-CTTCTGTGATCTGGGCTTCG ACAC-3'

### RESULTS

Among the 100 patients included in this study, 29 (29%) had azoospermia, 17 (17%) had severe oligozoospermia and 54 (54%) had oligozoospermia. Of the 100 infertile men, 5 (5%) were found to have Y-chromosome micro-deletion. The details of the type of infertility and the sperm concentration are presented in Table 1. The type of infertility, nature of deletion and semen analysis are presented in Table 2. Out of 29 azoospermic men, 2 had microdeletions in AZF b (RBMV) and AZF c (sY- 254). Out of 17 severe oligozoospermic males, 1 had microdeletion in AZF a (sY-84). Out of 54 oligozoospermic patients, 2 had microdeletions in AZF a (sY-84) and AZF c (sY- 254).

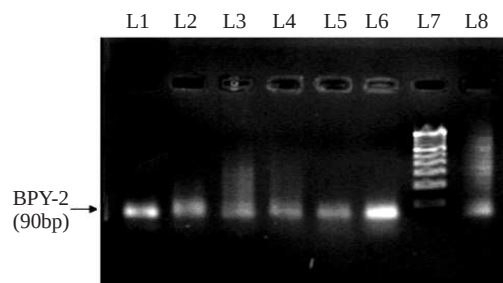
**Table 1: Classification of infertile males with their sperm concentration**

| Number of cases | Sperm concentration 10 <sup>6</sup> /ml | Type of case           |
|-----------------|-----------------------------------------|------------------------|
| 29              | Nil                                     | Azoospermia            |
| 17              | <5                                      | Severe oligozoospermia |
| 54              | >5-20                                   | Oligozoospermia        |

**Table 2: Classification of infertile males with their specific deletion and semen analysis**

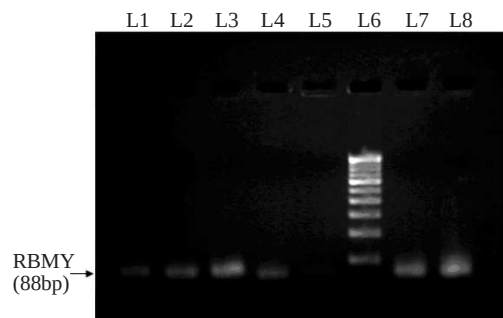
| Type of case           | AZF a deletion | AZF b deletion | AZF c deletion |       | Semen analysis |
|------------------------|----------------|----------------|----------------|-------|----------------|
|                        | sY- 84         | RBMV           | sY-254         | BPY-2 |                |
| Azoospermia            | -              | +              | +              | -     | -              |
| Severe oligozoospermia | +              | -              | -              | -     | 15-30%         |
| Oligozoospermia        | +              | -              | +              | -     | 35-55%         |

No deletion of BPY-2 gene was observed in the infertile males (Fig. 1). Figure 2 shows deletion of RBMY gene in the infertile male, Figure 3 and Figure 4 show deletion of SY-84 STS marker and SY-254 STS marker in the infertile males respectively.



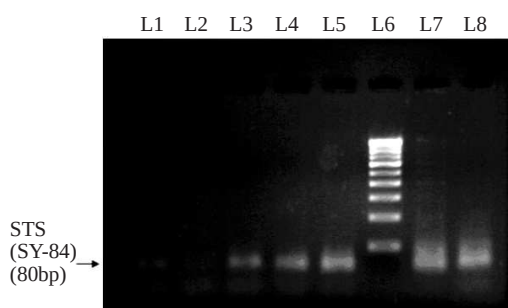
**Fig. 1. PCR analysis on DNA of infertile males using Gene (BPY-2)**

Lane1 : Oligozoospermia male  
Lane2-4 : Severe oligozoospermia males  
Lane5-6 : Azoospermia males  
Lane 7 : Mol.wt marker (100bp DNA ladder)  
Lane 8 : Positive control



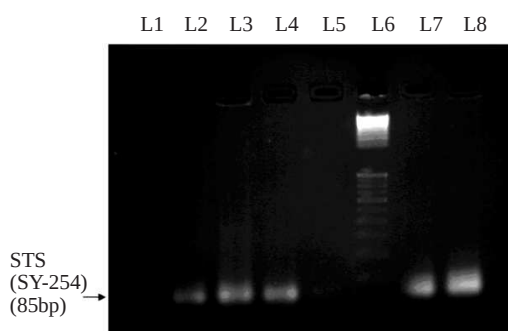
**Fig. 2. PCR analysis on DNA of infertile males using Gene (RBMV)**

Lane1 : Oligozoospermia male  
Lane2-4 : Severe oligozoospermia males  
Lane5 : Azoospermia male (with deletion)  
Lane 6 : Mol.wt marker (100bp DNA ladder)  
Lane 7 : Azoospermia male  
Lane 8 : Positive control



**Fig. 3. PCR analysis on DNA of infertile males using STS (SY-84)**

Lane1 : Severe oligozoospermia male (with deletion)  
 Lane2 : Oligozoospermia male (with deletion)  
 Lane3-5 : Azoospermia males  
 Lane 6 : Mol.wt marker (100bp DNA ladder)  
 Lane 7 : Severe oligozoospermia male  
 Lane 8 : Positive control



**Fig. 4. PCR analysis on DNA of infertile males using STS (SY-254)**

Lane1 : Azoospermia male (with deletion)  
 Lane2-4 : Severe oligozoospermia males  
 Lane5 : Oligozoospermia male (with deletion)  
 Lane 6 : Mol.wt marker (100bp DNA ladder)  
 Lane 7 : Azoospermia male  
 Lane 8 : Positive control

So if we consider the frequency for Y-chromosome microdeletion in azoospermia, severe oligozoospermia and oligozoospermia, the frequency for azoospermia is 2/29 (6.8%), the frequency for severe oligozoospermia is 1/17 (5.8%) and the frequency for oligozoospermia is 2/54 (3.7%).

## DISCUSSION

Spermatogenesis is regulated by a number of genes on the Y-chromosome. Y-chromosome deletions are emerging as a prevalent cause of male factor infertility. Y-microdeletions are specific for spermatogenic failure since no deletions

have been reported in a large number of normospermic men. Although fertility can be compatible with Y deletions, it simply reflects the fact that natural fertilization may occur even with relatively low sperm counts depending on the female partner's fertility status. For this reason it is more appropriate to consider Y deletions as a cause of Oligozoospermia/Azoospermia rather than cause of infertility. Thus, the aim of this study is a PCR-based detection of AZF regions for microdeletions on Y-chromosome in infertile men.

Our study has tried to evaluate the incidence of Y-chromosome microdeletions in North-East Indian infertile male patients. The frequencies of deletions of Y-chromosome reported in different studies, range between 3 percent to 18 percent of males with non-obstructive azoospermia or severe oligozoospermia (Reijo et al. 1995 and Simoni et al. 1998). In our screening of the 100 infertile males, we found 5 patients carrying microdeletions corresponding to a frequency of 5 percent (5 out of 100 patients had microdeletions). Some studies reported 13 percent of infertile microdeletions in the Y-chromosome (Stuppia et al. 1998 and Nakahori et al. 1996), while others reported less than 5 percent (Foote et al. 1992) in comparison to the statistical values obtained from all surveys (Simoni et al. 1998). Another study showed an incidence of microdeletion between 5.1 percent and 9.6 percent in the infertile males (Girardi et al. 1997). Our results are in accordance with the reported results between 3 percent-18 percent (Reijo et al. 1995 and Simoni et al. 1998).

The frequency of deletion of the AZFa and AZFc region was found to be equal as compared to AZFb region in our study. This is in disagreement with earlier studies, which showed that deletion in the AZFc region was high when compared with that in the AZFa and AZFb regions (De Vries et al. 2002). In our present study, 2/100 (2%) infertile males showed deletions in AZFa region, 1/100 (1%) infertile males showed deletions in AZFb region and 2/100 (2%) infertile males showed deletions in AZFc region.

The prevalence of AZF deletion in this study population was 5% which is in contrast to studies of Caucasian populations, in which the deletions were observed in a small proportion (1%) of infertile men with azoospermia (Pryor et al. 1997). In another study, the prevalence of Y-chromosome microdeletions in AZF sub-regions

was reported as 13.3% (12/90) (Zheng et al. 1998). A meta-analysis study has mentioned that the prevalence of Y-chromosome microdeletions was between 0.7-34.5% with an average of 8.2% (Foresta et al. 2001). Many studies concerning AZF microdeletions which have been done in different countries confirmed this meta-analysis (Hellani et al. 2006; Rejeb et al. 2008; Mohammed et al. 2007; Ateyah et al. 2002; El Awady et al. 2004). These studies are in accordance with our result.

### CONCLUSION

Microdeletion analysis using PCR helps to determine the frequency and site of gene deletion. This is the first study of this kind to investigate the prevalence of Y-chromosome deletions in AZF sub regions in North-East Indian men with azoospermia, severe oligozoospermia and oligozoospermia. The frequency of deletion of the AZFa and AZFc region was found to be equal as compared to AZFb region and the prevalence of AZF deletion in this study population was 5%.

### RECOMMENDATIONS

The prevalence of microdeletions in the local population needs to be confirmed by screening more infertile men using more STS markers. Although Y-chromosome microdeletions occur in a subgroup of infertile men, routine screening microdeletions in all male patients is an important prerequisite to their appropriate counseling.

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### REFERENCES

- Ateyah A, Amer M, Helmy N 2002. Incidence of chromosomal anomalies and Y microdeletions in 50 Egyptian males with non-obstructive azoospermia. *Egyptian Journal of Andrology and Reproduction*, 16(1): 13.
- Anurag M, Rima D, Rajeev K, Gupta N, Kiran K, Gupta SK 2008. Screening for Y-chromosome microdeletions in infertile Indian males: Utility of simplified multiplex PCR. *Indian Journal of Medical Research*, 127: 124-132.
- Dada R, Gupta NP, Kucheria K 2004. Yq microdeletions—azoospermia factor candidate genes and spermatogenic arrest. *J Biomol Tech*, 15: 176-183.
- de Vries J W, Hoffer M J, Repping S, Hoovers J, Leschot N J, Fulco V 2002. Reduction copy number of DAZ genes in subfertile and infertile men. *Fertil Steril*, 77: 68-75.
- Dublin L, Amelar RD 1971. Etiologic factors in 1294 consecutive cases of male infertility. *Fertil Steril*, 22: 469-474.
- Egozcue S, Blanco J, Vendrell JM, Garcia F, Veiga A, Aran B, Barri PN, Vidal F, Egozcue J 2000. Human male infertility: Chromosome anomalies meiotic disorders, abnormal spermatozoa and recurrent abortion. *Hum Reprod*, 6: 93-105.
- El Awady MK, El Shater SF, Ragaa E, ATEF K, Shaheen I M, Megiud NM 2004. Molecular study on Y chromosome microdeletions in Egyptian males with idiopathic infertility. *Asian Journal of Andrology*, 6: 53.
- Footo S, Vollrath D, Hilton A, Page DC 1992. The human Y chromosome: Overlapping DNA clones spanning the euchromatic region. *Science*, 258: 60-66.
- Foresta C, Moro E, Ferlin A 2001. Y chromosome microdeletions and alterations of spermatogenesis. *Endocrine Reviews*, 22: 226.
- Girardi SK, Mielnik A, Schlegel PN 1997. Submicroscopic deletions in the Y chromosome of infertile men. *Hum Reprod*, 12: 1635-1641.
- Hellani A, Al-Hassan S, Iqbal MA, Coskun S 2006. Y chromosome microdeletions in infertile men with idiopathic oligozoospermia or azoospermia. *Journal of Experimental and Clinical Assisted Reproduction*, 3: 1.
- Hendry WE, Sommerville IF, Hall RR, Pugh RC 1973. Investigation and treatment of the subfertile male. *Br J Urol*, 45: 684-692.
- Krausz C, Quintana-Murci L, McElreavey K 2000. Prognostic value of Y deletion analysis: What is the clinical prognostic value of Y chromosome microdeletion analysis? *Hum Reprod*, 15: 1431-1434.
- Mohammed F, Al-Yatana F, Al-Bader M, Tayel S M, Gouda S, Naquib K 2007. Primary male infertility in Kuwait: A cytogenetic and molecular study of 289 infertile Kuwaiti Patients. *Andrologia*, 39: 87.
- Mosher WD 1985. Reproductive impairments in the United States, 1965-1982. *Demography*, 22: 415-430.
- Nakahori Y, Kuroki Y, Komaki R, Kondoh M, Namiki M, Iwamoto T, Toda T, Kobayashi K 1996. The Y chromosome region essential for spermatogenesis. *Horm Res*, 46(Suppl 1): 20-23.
- Park MY, Kang HM, Hwang SH, Park HJ, Park NC, Choi KU, Kim HH, Chang CL, Lee EY 2010. Evaluation of a multiplex PCR kit used for detecting Y chromosome microdeletions. *Korean J Lab Med*, 30(4): 432-439.
- Pryor JL, Kent-First M, Muallem A, Van Bergen AH, Noltén WE, Meisner L, Roberts KP 1997. Microdeletions in the Y chromosome of infertile men. *New Engl J Med*, 336: 534-539.
- Reijo R, Lee TY, Salo P, Alagappan R, Laura G, Rosenberg M 1995. Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene. *Nat Gene*, 10(4): 383-393.
- Rejeb R, Maazoul F, Trabelsi M 2008. Y chromosome microdeletions in Tunisian infertile males. *Pathologie Biologie*, 56: 111.



- Simoni M, A Kamischke, E Nieschlag 1998. Current status of the molecular diagnosis of Y-chromosomal microdeletions in the work-up of male infertility-Initiative for international quality control. *Hum Reprod*, 13: 1764-7.
- Stuppia L, Gatta V, Calabrese G 1998. A quarter of men with idioazoospermia display chromosomal abnormalities and microdeletions of different types in interval 6 of Yq 11. *Hum Genet*, 102: 566-570.
- Swarna M, Ramesh Babu S, Reddy PP 2003. AZFc deletions in idiopathic infertile males from South India. *Int J Hum Genet*, 3(1): 1-4.
- Tiepolo L, Zuffardi O 1976. Localization of factors controlling spermatogenesis in the nonfluorescent portion of the human Y chromosome long arm. *Hum Genet*, 34(2): 119-124.
- Tse JYM, Yeung WSB, Lau EYL 2000. Deletions within the azoospermia factor subregions of the Y chromosome in Hong Kong Chinese men with severe male- factor infertility: Controlled clinical study. *HKMJ*, 6: 143-146.
- Virginie R, Jean L B and Fellmann F 2007. Quantitative PCR technique for the identification of microrearrangements of the AZFc region. *Journal of Assisted Reproduction and Genetics*, 24(6): 241-248.
- Viswambharan N, Suganthi R, Simon A M, Manonayaki S 2007. Male infertility: Polymerase chain reaction-based deletion mapping of genes on the human chromosome. *Singapore Med J*, 48 (11): 1140.
- Vogt PH, Edelmann A, Kirsch S, Henegariu O, Hirschmann P, Kiesewetter F et al. 1996. Human Y chromosome azoospermia factors (AZF) mapped to different sub-regions in Yq 11. *Hum Mol Genet*, 5(7): 933-943.
- Vollrath D, Foote S, Hilton A, Brown LG, Beer-Romero P, Bogan JS, Page DC 1992. The human Y chromosome: A 43-interval map based on naturally occurring deletions. *Science*, 258: 52-59.
- Zheng Y, Zhou Z, Wang L 1998. Analysis of the DAZ and DAZh genes in spermatogenesis. *Shengzhi Yixue Zazhi*, 7: 71-74.
- Zhou-Cun A, Yuan Y, Si-Zhong Z, Zhang W, Lin L 2006. Chromosomal abnormality and Y chromosome microdeletion in Chinese patients with azoospermia or severe oligozoospermia. *Acta Genetica Sinica*, 33(2): 111-116.