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RESEARCH ARTICLE

Prevalence of Y chromosome microdeletion in north Indian infertile males with spermatogenesis defect

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Abstract. Deletion of specific genes present in the long arm of Y chromosome has been identified as the most common genetic cause of defective spermatogenesis. Studies have shown that frequency of Y chromosome microdeletion varies in different geographical location and is related to genetic and environmental influence preponderance. Therefore, the present study was carried out to identify the frequency of Y chromosome microdeletion in the northern region of India and to define subgroup of infertile patients who are critically under more risk of having microdeletion. A total of 292 north Indian infertile males with nonobstructive azoospermia and oligozoospermia were selected for screening the Y chromosome microdeletion. Healthy fertile males (n=100) were also enrolled as control subjects. Frequency of Y chromosome microdeletion in north Indian infertile males was found to be about 8.5%, with azoospermia factor (AZFc) region as the most susceptible region for microdeletion. Comparatively microdeletion is more common in patients with nonobstructive azoospermia than oligozoospermia (9.2% versus 7.1%). Statistical analysis also revealed that patients with hormonal FSH level between 20 and 40 mIU/mL have more chances of harbouring microdeletion. Hence, the present study highlights the importance of screening AZFc region among infertile patients with very high serum FSH value.

Keywords. male infertility; spermatogenesis; Y-chromosome; microdeletion; azoospermia factor; spermatogenesis.

Introduction

Infertility is 'a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse' (Zegers-Hochschild *et al.* 2009). Worldwide the incidence of infertility among the general population is estimated to be 10–15% (Bushnik *et al.* 2012) and male factor accounts for almost half of the cases of infertility (Thonneau *et al.* 1991). Several pathological conditions such as immunological factors, infections, exposure to radiation or toxic agents and endocrinological dysfunction may cause loss of the testicular germinal cell which may ultimately leads to male infertility due to spermatogenic failure (Foresta *et al.* 1998). However, the advancement of genetic testing procedures provide an accurate diagnosis of various genetic factors that have ascertained role in the penetrance of male infertility (Vogt *et al.* 1996).

Y chromosome plays a crucial role in the normal process of spermatogenesis and development of germ cells. Microdeletions on the long arm of Y chromosome are the common cause of spermatogenic failure and pathogenesis of idiopathic male infertility (Foresta *et al.* 1997; McElreavey and Krausz 1999). The reported incidence of Y chromosome microdeletion varies from 15–20% in men with idiopathic azoospermia and 7–10% in cases of idiopathic oligozoospermia (Krausz *et al.* 2000; Abur *et al.* 2019). Vogt *et al.* (1996) demonstrated that the defective spermatogenesis is associated with microdeletion in three overlapping regions of Y chromosome and termed them as azoospermia

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factor (AZF)a, AZFb and AZFc. Incidence of microdeletion in AZFc region of Y chromosome are most frequent in infertile male diagnosed with variable phenotype ranging from oligozoospermia to azoospermia, whereas deletion in AZFa and AZFb region are more common in infertile male with spermatogenic arrest and SCO syndrome (Raicu *et al.* 2003; Simoni *et al.* 2004; Sadeghi-Nejad and Farrokhi 2007).

Due to the advancement of modern lab technology there is a sudden increase in the demand of assisted reproduction technology among infertile couples in developing countries. Infertile couple diagnosed with complete absence of sperm in histological examination may undergo sperm retrieval techniques, such as multiple testicular sperm extraction (TESE) for intracytoplasmic sperm injection (ICSI) (Ferlin *et al.* 1999; Xi *et al.* 2019). Spermatozoa may be retrieved up to 50% of these azoospermic patients through TESE and thus increasing the chances of conception (Page *et al.* 1999; van Golde *et al.* 2001; Oates *et al.* 2002). Study of Y chromosome microdeletion has gained importance in infertile male patients with oligozoospermia or nonobstructive azoospermia undergoing ART in the form of *in vitro* fertilization (IVF) or ICSI as conceiving with the help of artificial techniques by pass the critical natural selection barrier and increase the risk of vertical transmission of genetic abnormality to the male offspring (Tournaye *et al.* 1997; Jiang *et al.* 1999).

In the last two decades, various studies have reported high frequency of Y chromosome microdeletion in Indian infertile male population (Suganthi *et al.* 2014; Sen *et al.* 2013; Khan *et al.* 2010; Agarwal *et al.* 2015; Ambulkar *et al.* 2015). However, the observed frequency is variable in different studies which may be due to variable sample size or additional genetic and environmental factors. Thus, there is paucity of data regarding frequency of Y chromosome microdeletion and comparative investigation highlighting correlation between phenotypic and genotypic profile among Indian infertile male patients. Therefore, the present study was aimed to ascertain the frequency of Y chromosome microdeletion in 292 idiopathic cases of infertile male from northern region of India and an attempt was also made to investigate whether the phenotypic profile (size and consistency of testes and level of serum hormone profile) can help to define the subgroup of patients who are at higher risk of harbouring Y chromosome microdeletion.

Materials and methods

Selection of subjects

A total of 292 consecutive Indian infertile men diagnosed with primary infertility due to nonobstructive azoospermia or oligozoospermia were included in this study. Healthy subject (n=100) with sperm count >20 million/mL or fathered a child within one year of sample collection served as controls. Complete clinical data concerning infertility including history and physical examination was also evaluated. Patients were advised for chromosomal analysis before enrollment. Patients with a history of exposure to gonadotoxins, chemotherapy, radiotherapy, or presented with any other chromosomal abnormalities including Klinefelter syndrome were excluded from the study.

Clinical and physical evaluation

Size and consistency of the testis were recorded by a single observer. Semen analysis including volume, pH, sperm count and motility was performed in patients and controls as per World Health Organization guidelines (World Health Organization 2010). Testicular fine needle aspiration cytology (FNAC) was carried out for studying the patterns of spermatogenesis in all patients. Serum hormones FSH, LH and testosterone were estimated by electrochemiluminescence immunoassay using Cobas analysers (Roche Diagnostic, USA). Scrotum ultrasonography was performed to rule out varicocele or other obstruction. After detailed clinical and physical examination by the urologist, all infertile male patients (n=292) were categorized into two groups, namely nonobstructive azoospermia (n=194) having nil sperm count in ejaculate and oligozoospermia (n=98) with sperm count <10 million/mL.

Sample collection and genomic DNA isolation

Around 5 mL of whole blood was drawn and collected in acid citrate dextrose (anticoagulant) vial. Genomic DNA was isolated from whole blood as described by Daly *et al.* (1996). Before proceeding further with molecular analysis, the quality of DNA was verified on 0.8% agarose gel and quantity of DNA isolated was calculated by measuring absorbance at 260 nm using spectrophotometer.

Detection of Y chromosome microdeletion

The screening of Y chromosome microdeletion was carried out using multiplex PCR technology. A series of six STS markers on Yq11 region were used for the detection of submicroscopic deletions according to the European Academy of Andrology (EAA), the European Molecular Genetics Quality Network (EMQN) and previous protocols (Raicu *et al.* 2003; Simoni *et al.* 2004). The STS marker includes AZFa prox-2 and AZFa dist-1 for AZFa region, sY127 and sY134 for AZFb region, sY254 and sY255 for AZFc region. The SRY region of Y chromosome was amplified with each reaction sample to confirm the presence of Y specific DNA. The primer sequence and the size of related PCR products are shown in table 1.

Table 1. STS marker and primer sequence used for screening Y chromosome microdeletions.

STS	Region	Size (bp)	Primer sequence
dist-1	AZFa	390	5'-GGTTCCTGAACAGGGGACT-3' 5'-GGCAGCAGAAGGGCCTCTC-3'
Prox-2	AZFa	220	5'-GGTTCCTGAACAGGGGACT-3' 5'-GGCAGCAGAAGGGCCTCTC-3'
sY134	AZFb	301	F-ACCACTGCCAAAACTTTCAA-3' R-GTCTGCCTCACCATAAAACG-3'
sY127	AZFb	274	5'-GGCTCACAAACGAAAAGAAA-3' R-CTGCAGGCAGTAATAAGGGA-3'
sY254	AZFc	220	5'-GGGTGTTACCAGAAGGCAAA-3' 5'-GAACCGTATCTACCAAAGCAGC-3'
sY255	AZFc	126	5'-GTTACAGGATTCGGCGTGAT-3' 5'-CTCGTCATGTGCAGCCAC-3'
sY1532	SRY	167	5'-TCCTTAGCAACCATTAATCTGG-3' 5'-AAATAGCAAAAAATGACACAAGGC-3'

Table 2. Clinical characteristics of infertile male patients.

Parameters	Nonobstructive azoospermia				Oligozoospermia			
Age (years)	29.2 ± 5.4				33.5 ± 5.5*			
FSH mIU/mL (1.5–12.4 mIU/mL)[a]	29.90 ± 17.57				16.35 ± 9.68			
Testosterone nM/ml (9.9–27.8 nM/L)[a]	15.32 ± 9.66				15.65 ± 5.83			
LH mIU/mL (1.7–8.6 mIU/mL)[a]	10.87 ± 7.79				5.58 ± 2.65			
Small testicular size	83				57			
Undescended testis	3				6			
Y chromosome microdeletion	AZFa	AZFb	AZFc	Total	AZFa	AZFb	AZFc	Total
	0	3	15	18	0	0	7	7

Values are presented as mean ±standard deviation.
[a]Normal hormonal range in male.
*$P < 0.05$.

The amplified PCR products were subjected to electrophoresis on 3% agarose gel, stained with ethidium bromide and visualized under ultraviolet light. A sample was considered negative for STS marker tested when the PCR product of the expected size was not obtained after three PCR attempts. Female genomic DNA was used as negative control in every PCR attempt.

Statistical analysis

Comparisons of various outcomes between two studies group was done by χ^2 analysis to find out their statistical significance. Univariate odds ratio were determined and their 95% CI were calculated to identify predictor for deletion outcome. All statistical tests are two-tailed and $P<0.05$ was considered as significant. The study was approved by the institutional ethics committee at the Post Graduate Institute of Medical Education and Research, Chandigarh (approval no. 877/PG11-1TRG/16814). An information sheet was provided to each patient/family member and written informed consent was obtained.

Results

Demography and clinical variable

In this study, a total of 292 men and 100 healthy fertile controls were analysed for submicroscopic Y chromosome microdeletion. These patients (n=292) were broadly categorized into two groups for instances 194 patients with nonobstructive azoospermia and 98 patients with oligozoospermia having sperm count < 10 million/mL. All the patients with nonobstructive azoospermia showed defective pattern of spermatogenesis in FNAC analysis and no obstruction in reproductive tract during transrectal ultrasonography. The six STS used in this study were all analysed in 100 healthy fertile males and five females before their application to infertile males, to access the specificity and

sensitivity of selected STS. The mean age of the infertile oligospermic male patients at the time of enrollment was 33±5.5 years, which was significantly higher in comparison to the mean age of the patients with nonobstructive azoospermia (table 2). Testicular examination revealed (n=140) 47.9% of infertile males having smaller testicular size and n=9 males were identified with undescended testis (table 2). However, any specific association between observed physical abnormalities with frequency of Y chromosome microdeletion could not be established.

Frequency of Y chromosome microdeletion

Of the 292 infertile male patients screened, Y chromosome microdeletion was observed in 25 patients with an overall frequency of 8.56%. Among the 194 patients diagnosed with nonobstructive azoospermia, Y chromosome microdeletion was observed in 18 cases (9.27%), it is noteworthy here that microdeletion in AZFc region was observed in 7.7% (n=15) patients, whereas deletion in AZFb region was observed in only three patients with a frequency of 1.5%. Strikingly microdeletion was observed only in AZFc region in patients with oligozoospermia (n=98) with a frequency of 7.1% (n=7). Therefore, it can be inferred that microdeletion in AZFc region was most frequent in both the groups and overall, 22 of 292 (7.5%) infertile males were detected with deletion in AZFc region (table 2). However, microdeletion in AZFa region was not detected in any of the infertile males.

Serum hormonal profile and correlation with Y chromosome microdeletion

The mean FSH concentration in azoospermic patients was 26.90±17.57 mIU/mL, while for oligospermic patients it was 16.35±9.68 mIU/mL (table 2). Notably the mean value was much deviated from normal range (1.5–12.4 mIU/mL) in both the groups. When the mean value of FSH was compared with testicular cytopathological parameter, it revealed significantly elevated levels of FSH in SCOS patients compared to patients with hypospermatogenesis or sperm maturation arrest (data not shown here).

The mean testosterone and LH concentration in azoospermic patients were 15.32±9.66 nM/mL and 10.87±7.79 mIU/mL, notwithstanding in oligospermic patients it was 15.65±5.83 nM/mL and 5.58±2.65 mIU/mL, respectively (table 2). The difference in mean FSH, LH and testosterone concentration in patients with or without microdeletion was not found statistically significant (table 3).

Since the patient with microdeletion had elevated FSH level (21 out of 25) an odds ratio was calculated to predict statistically correlation between FSH level and Y chromosome microdeletion. Thus, the patients with high level FSH were categorized into three subgroups (12.4–20 mIU/mL, 21–40 mIU/mL and above 40 mIU/mL). Based on this comparison, an odds ratio was calculated which showed that patients in the 12.4–20 mIU/mL group had two times and patients in the 20–40 mIU/mL had six times increased chances of acquiring Y chromosome microdeletion.

Table 3. Comparison of hormonal profile in patients with or without Y chromosome microdeletion.

Parameters	Patients with microdeletion	Patients without microdeletion	P value
No. of patients	25	267	–
Age (median)	28	30	0.71
FSH mIU/mL	24.20±22.43	21.90±18.05	0.87
LH mIU/mL	9.25±7.01	11.49±9.55	0.48
Testosterone (nM/mL)	17.54±15.66	27.78±8.43	0.91

P<0.05 considered as significant.

Discussion

Microdeletion in the long arm of Y chromosome is among one of the most common genetic cause of male infertility due to defective spermatogenesis. According to various published reports, the frequency of Y chromosome microdeletion among infertile male ranges from 1–55% and the average frequency has been reported to be 15% worldwide (Simoni *et al.* 1999, 2004). In the present study, the estimated frequency of Y chromosome microdeletion was 8.5% which is similar to that reported by other investigators from northern region of India (Thangaraj *et al.* 2003; Dada *et al.* 2003) but lower than that reported from southern India (11.1%–12.9%) (Sakthivel and Swaminathan 2008). This variation in frequency of Y chromosome microdeletion may be due to different selection criteria, sample size, genetic and environmental factors. The number of STS marker selected for screening micro-deletion in different region of Y chromosome may also serve as critical factor in determining frequency of Y chromosome microdeletion. The hypothesis that prevalence of microdeletion varies from different geographical location and is related to genetic and environmental influence was also supported by similar studies done on Caucasian population. For instance, in north European population, the frequency of Y chromosome microdeletion in infertile males was estimated as 1–4% comparatively this frequency is much lower than that found in infertile males from southern Europe 15% (Foresta *et al.* 1997). Hence more comprehensive studies are required to establish exact frequency of Y chromosome microdeletion from different regions of India since India exhibits significant variations in geographic and environmental diversity.

In the present study, the observed frequency of Y chromosome microdeletion in infertile patients with

nonobstructive azoospermia is 9.2% and in patients with oligozoospermia is 7.5%. Although this observed frequency is similar to that reported by other investigators in Indian population, the frequency of deletion in Indian infertile obstructive azoospermic patients is lower than that of estimated range in other population (15-20%) (Krauz *et al.* 2000; Suganthi *et al.* 2009). High frequency of deletion in patients with nonobstructive azoospermia implicate that such patients should be critically analysed for deletion in Y chromosome before adopting any artificial reproduction technology.

In accordance with the previous studies (Ambulkar *et al.* 2015), we found AZFc region as most susceptible for deletion and is observed in 84% of cases. Microdeletion in AZFc region is the most common deletion type both in Indian and European populations and is found in more than 70% of the cases (Foresta *et al.* 2001; Thangaraj *et al.* 2003) The AZFc locus contains 21 candidate genes and 11 families of transcription units specifically expressed in testis (Suganthi *et al.* 2014). The most important and well-studied AZFc gene is *DAZ*. All the members of *DAZ* gene family encode RNA binding proteins, probably involved in the regulation of mRNA translation (Saxena *et al.* 2000). Therefore, deletion in AZFc region may be associated with variable testicular histology ranging from Sertoli cell-only syndrome to spermatogenetic arrest and hypo spermatogenesis.

The reported clinical characteristics of infertile male patients may vary widely depending upon the population under investigation and various other contributing factors for male infertility (Turek *et al.* 1995). Several studies have been implicated to establish any significant association between altered clinical variable and frequency of Y chromosome microdeletion but results have been equivocal utill now. Studies have reported significantly elevated FSH level in azoospermic patients but still any conclusive result to establish correlation between elevated level FSH level and Y-chromosome microdeletion was not shown (Krauz *et al.* 2000; Lammarrone *et al.* 2003). In the present study also, infertile patients with microdeletion present the same clinical characteristics (phenotypic features, hormonal profile and testicular volume) as infertile patients without microdeletions. However, statistically, we have predicted that patients with FSH value between 20 and 40 mIU/mL have six times increased chances of microdeletion in Y chromosome in comparison to infertile male with FSH value less than 20 mIU/mL. Although this is the preliminary investigation and more comprehensive multicentric studies are required to define the subgroup of patients who are at risk of microdeletion, still the present study suggest altered hormonal profile may have some diagnostic role in patients with Y chromosome microdeletion.

In conclusion, incidence of Y chromosome microdeletion are more common in northern region of India with AZFc region as most susceptible for deletion. Therefore, all infertile couples should be advised for Y chromosome microdeletion screening before undergoing any ART procedure. Moreover, further study of large patient's cohort from Indian populations is required to established significant correlation and to distinguish phenotypic profile of patients with higher risk of microdeletion.

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