IJBPAS, November, 2016, 5(11): 2828-2839

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

SCREENING OF Y-CHROMOSOME MICRODELETION IN THE AZFC SUB-REGION OF INFERTILE MEN, GUJARAT, INDIA

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Received 10th July 2016; Revised 22nd Aug. 2016; Accepted 20th Sept. 2016; Available online 1st Nov. 2016

ABSTRACT

Objectives: The aim of this study is to identify the AZFc microdeletion which are risk factors for infertility in men. Deletion of AZFc sub-region in the long arm of Y-chromosome is most frequent genetic cause in male infertility which leads to spermatogenic arrest. *Methods:* Total 50 infertile men with abnormal seminogram and 50 normozoospermic men were analysed for the detection of microdeletion using different sequence tagged site markers specific to AZFc sub-region of Yq chromosome. *Results:* Five patients (10%) showed microdeletions in AZFc loci out of 50 infertile men. Our results suggested that AZFc deletions are significantly associated with male infertility risk. *Conclusion:* In this study, we have observed the occurrence and frequency of partial AZFc microdeletion in infertile as well as fertile patients. The finding may be useful for the couple attending fertility clinics for ART treatment, to know and diagnose AZFc microdeletion. Strong correlation of microdeletion with male infertility risk, might contribute to spermatogenic arrest in Gujarati population.

Keyword: Male Infertility, AZFc, Azoospermia, Oligospermia, Y-chromosome microdeletions

1. INTRODUCTION

Infertility is a major social problem affecting about 10-15% of all couples who wish to have a child [1] and male is traced to be responsible for infertility in approximately 50% of the cases [2-4]. There are number of causes- genetic and non-genetic responsible for male infertility. A survey of literature suggests that after Klinefelter syndrome, there is a major involvement of the Y chromosome microdeletion in the male infertility [5, 6]. Y-chromosome microdeletions in men have been identified in 7–13% with azoospermia and oligozoospermia [7, 8].

Y-chromosome is the sex determining chromosome and smallest chromosome of approximately 60 Mb [9]. Azoospermia factor (AZF) region in the long arm of euchromatic region of Y chromosome (Yq) is important in male fertility as it contains genes necessary for spermatogenesis and is divided into sub regions AZFa, AZFb and AZFc [10]. The cytogenetic location of AZF region is q11.23 of Y-chromosome [11]. Azoospermia means the absence of spermatozoa and oligozoospermiameans reduced sperm production below the lower reference limit i.e. $15x10^6$ per ml according to WHO manual 2010 [12].

AZFa sub-region contains two important genes- *USP9Y* (Ubiquitin specific protease 9) and *DBY* (DEAD/H box polypeptide), deletion in this sub-region is rare but have severe consequences [13]. AZFb sub-region have one main candidate gene, the RNA binding motif gene- *RBMY*, Y-chromosome [14]. AZFc sub-region have genes which shows testis specific expression [15]. The most important candidate gene of AZFc sub-region is *DAZ* (deletion-in-azoospermia), most frequently deleted sub-region in men with infertility. Spermatogenesis will be disturbed if any one of these loci had spontaneous mutation in the paternal germ line [16]. Recently, many types of AZFc sub-deletions has been observed which includes gr/gr, b2/b3 and b1/b3 partial deletion [17].

In this study, we sought to find out the Y-chromosome microdeletion in azoospermic and oligozoospermic men by several sequence tagged site (STS) markers on AZF region of long arm of Y-chromosome as Y-chromosome microdeletion frequency vary with different ethnic populations. Interestingly, to the best of our knowledge, this study of AZFc screening is the first original report from Gujarat, India and also it will provide an interesting vision about the Gujarat population.

2. MATERIALS AND METHODS

2.1 Study population

In this study, semen and blood samples of infertile patients were collected from

different areas of Gujarat, which was approved by Institutional Ethics Committee, S. G. Patel Ayurveda Hospital & Maternity home, New V.V. Nagar, Gujarat. This prospective study was carried out in Ashok & Rita Patel Institute of Integrated Study & Research in Biotechnology and Allied Sciences (ARIBAS), New V.V. Nagar. A total of fifty infertile men were recruited from *in vitro* fertility Clinics and Pathology laboratories. Semen Analysis was done according to criteria of World Health Organization (WHO), 2010 [12]. Patients with sperm count less than 15 million per mL were classified as having oligozoospermia, Asthenozoospermia patients with lower reference limit for total motility (PR + NP) is 40% and those with no sperm in semen as azoospermia according to the clinical diagnosis. Fifty normozoospermic men were clinically healthy and exhibited normal semen sample were recruited as positive control and two healthy female blood samples as negative control. Age of men in both the groups ranged from 22 to 45 years. Each patient gave patient information sheets and informed consent forms to participate in genetic analysis of their donated semen and blood samples. The basic infertility evaluation included semen analysis. Person with proven immunological, hormonal or other causes of male infertility, age above 45 years and secondary infertility were excluded from this study.

2.2 Study procedures

2.2.1 DNA analysis by PCR

Blood samples of azoospermic men and semen samples of oligozoospermic men were taken from all the subjects for DNA analysis. The DNA was extracted by organic solvent extraction method. The quantity and quality of DNA was checked by NANO drop ND-1000 spectrophotometer and agarose gel electrophoresis respectively followed by PCR amplification which was carried out using specific primer sets. All the samples tested for the presence of SRY (sY14) as an internal control followed by AZFc DAZ gene deletions using STS markers sY254, and sY255 according to the EAA/ EMQN guidelines [18]. Also, we screened specific STS markers sY153, sY1291 for gr/gr deletion, sY1191 for b2/b3 deletion and sY1197 along with sY1291 for b1/b3 deletion. The details of primer used for detecting Y-chromosome microdeletions in AZFc sub-region are shown in **Table 1**. PCR amplification was carried out in a final volume of 25 µL. A mega mix for all samples was prepared using 12.5 µL of 2X master mix (EmeraldAmp® GT PCR Master Mix), 7.4 µL of deionized water, 0.1 µL of 5% DMSO and 1 µL of forward

and reverse primer each, per reaction. Three µL of genomic DNA (20 ng/µL) was added in each PCR tube to make the final volume of 25 µL. PCR was carried out by adding different components as mentioned above for SRY (sY14), sY153, sY254, sY255, sY1191, sY1197 and sY1291 in a final volume of 25 µL in a 200 µL capacity PCR vials. The thermal cycle parameters consist of initial denaturation of 3 mins at 94^{o}C, followed by 34 cycles of denaturation for 1 min at 94°C, annealing for 1 min at varying temperature according to the STS primer (**Table 1**). Extension was preferred for 1 min at 72°C, with a final extension at 72°C for 5 min. Samples in which deletions were detected was reconfirmed by repeating PCR analysis for three times.

2.2.2 Analysis and visualization of PCR product

To confirm the targeted PCR amplification, 8 µL of PCR product from each tube was electrophoresed along with 100 bp DNA ladder (Low range DNA Ladder, Fermentas) and negative control on 2% agarose gel, stained with ethidium bromide (0.5 µg/mL) in 1X TBE buffer. The amplified product from SRY, sY153, sY254, sY255, sY1191, sY1197 and sY1291 was visualized as a compact single band of expected size under U.V. transilluminator.

3. RESULTS

3.1 Sample collection: Among the 100 patients included in study, 15 (15%) were Azoospermic, 20 (20%) were Oligozoospermic, 15 (15%) Asthenozoospermic and 50 (50%) were Normozoospermic. Also we have included two female sample as a negative control. The patient's age ranged from 22 to 45 years.

3.2 DNA isolation: DNA was isolated from all the 85 semen and 17 blood samples by phenol:choloroform method and subjected to electrophoretic analysis on 0.8% agarose gel. The electrophoretic pattern of representative isolated DNA is presented in **Fig. 1** indicates good quality of DNA.

DNA quantification of 85 semen and 17 blood samples by using nanodrop spectrophotometer revealed absorption ratio at 260/280 between 1.8 - 2.00 in majority of samples, indicate good quality of DNA. Variation in the DNA concentration (14.1 ng/µL - 2135.5 ng/µL) might be due to the variable quantity of spermatozoa and variation in count of spermatozoa of the individuals.

3.3 STS-PCR: PCR is a very sensitive technique to detect constitutional Yq microdeletions. Guidelines for diagnostic testing region, according to European Academy of Andrology [19] and most

recent works [20, 21], included sY254 and sY255 of AZFc sub-region STS primers as the first choice. After these primer, we evaluated for sY153 and the distributions of partial AZFc deletions in 50 infertile patients and 50 normozoospermic men for sY1191, sY1197 and sY1291.

Data analysis revealed no microdeletion out of 50 infertile men (0%) with spermatogenesis impairment were found to have classical AZF deletions using STS analysis of genomic DNA. Two type of partial AZFc deletion in 2 patients (4%) with gr/gr and 2 patients (4%) with b2/b3 partial deletion was found. In addition to this, 1 patient (2%) with sY153 and 3 patients (6%) with sY1197 microdeletions were also observed. No microdeletions were found in the normozoospermic men. The observed microdeletions in AZFc and the STS markers involved are schematically shown in **Fig. 1**. The distribution and the percentage of all microdeletions detected is illustrated in **Table 3**.

Table 1: Primer Details of STS Primers for AZFc sub-region

STS	Primer sequence (5'-3')	PCR product size	STS position*	Annealing temperature (^{0}C)	GeneBank Accession no.
sY14	F- GAATATTCCCGCTCTCCGGA R- GCTGGTGCTCCATTCTTGAG	472 bp	2787066- 2787535	56	G38356
sY153	F- GCATCCTCATTTTATGTCCA R- CAACCCAAAAGCACTGAGTA	139 bp	24500247- 24500385 22866498-22866636	60	G12004
sY254	F- GGGTGTTACCAGAAGGCAAA R- GAACCGTATCTACCAAAGCAGC	380 bp	24851664+2485204324840816+248411952322642923226804824806117-2480649623191734-2319211323180886-2318126523170046-23170425	56	G38349
sY255	F- GTTACAGGATTCGGCGTGAT R- CTCGTCATGTGCAGCCAC	123 bp	24804741+24804864 23190358+23190481 23179510+23179633 23168670+23168793 24853296-24853419 24842448-24842571 23228061-23228184	56	G65827.1
sY1191	F- CCAGACGTTCTACCCTTTCG R- GAGCCGAGATCCAGTTACCA	385 bp	22729473-22729857	58	G73809
sY1197	F- TCATTTGTGTCCTTCTCTTGGA R- CTAAGCCAGGAACTTGCCAC	453 bp	22377471+22377923	58	G67168
sY1291	F- TAAAAGGCAGAACTGCCAGG R- GGGAGAAAAGTTCTGCAACG	527 bp	23358923- 23359449	61	G72340

***from UCSC Genome Browser**

Table 2: Microdeletions in AZFc sub region of Y chromosome

STS markers	Patient AS19	Patient A28	Patient O10	Patient O52	Patient A53
sY153	-	Del	-	-	-
sY254	-	-	-	-	-
sY255	-	-	-	-	-
sY1191	-	-	Del	Del	-
sY1197	-	-	Del	Del	Del
sY1291	Del	Del	-	-	-

Del: microdeletion

Table 3: Frequency of AZFc Y chromosome microdeletions

Class	Total Samples	Frequencies of AZFc microdeletion						Total
		sY153	sY254	sY255	sY1191	sY1197	sY1291	
Azoospermia	15 (15%)	2% (1/50)	0% (0/50)	0% (0/50)	4% (2/50)	6% (3/50)	4%% (2/50)	16% (8/50)
Oligozoospermia	20 (20%)							
Asthenozoospermia	15 (15%)							
Normozoospermia	50 (50%)	0% (0/50)	0% (0/50)	0% (0/50)	0% (0/50)	0% (0/50)	0% (0/50)	0% (0/50)

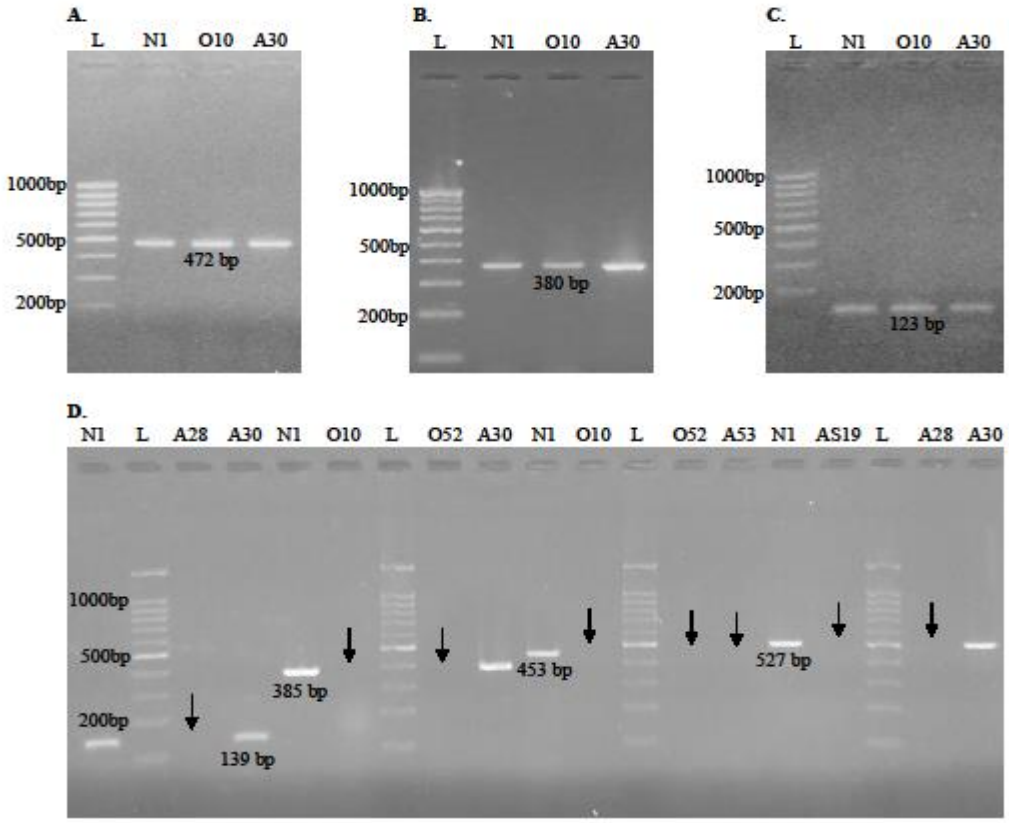

Figure 1: Electrophoretic pattern of PCR products generated by amplification of DNA using
A.SRY (sY14);
B. sY254;
C. sY255;
D. sY153 (Lane 1 to 4), sY1191 (Lane 5 to 9), sY1197 (Lane 10 to 14) and sY1291 (Lane 15 to 19) STS specific primer.
N: Normospermic semen samples, O: Oligospermic semen samples, A: Azoospermic blood samples, AS: Asthenozoospermic semen samples & L: DNA ladder (100 bp)

4. DISCUSSION

Many factors, incorporating genetics causes play a vital role in diagnosis of infertility in men. If we compare Y chromosome to other chromosomes of human, it contain less number of genes. For normal spermatogenesis, the integrity of AZF region in the Y-chromosome is necessary as numerous genes in this region are required for the process [22, 23]. On the basis of conventional cytogenetic techniques, Tiepolo and Zuffardi in 1976 discovered the deletions in the long arm of Y chromosome in azoospermic men. According to their study, the presence of an Azoospermia factor (AZF) region on

proximal Yq arm is involved in spermatogenesis. This was further studied at sub-microscopic level for the characterization of Yq11 region, which was further divided into AZFa, AZFb and AZFc sub-region [10]. If we consider all the three sub-regions, AZFc is the most frequently deleted region of Yq11 in men with infertility as compared to AZFa and AZFb [24-27]. AZFc sub-region on Yq11 is about 500 kb [25]. Deletion in the AZFc sub-region, reduces sperm density quantitatively. In 1995, Reijo et al. first studied the AZFc microdeletions in azoospermic men with 13% frequency [25]. AZFc complete deletions are associated with hypospermatogenesis condition causing abnormal and decrease sperm production, whereas the AZFc sub-deletions in few copies of eight gene families results in spermatogenic impairment [28].

Not only complete deletion but also partial deletions are also associated with spermatogenetic arrest. Male with partial AZFc deletion may transmit the mutation to the offspring. This may increase the population of infertile men which is the main concern. There are multiple gene family located in AZFc- two copies of *CDY1*, three copies of *BPY2* and four copies of *DAZ* [29]. The gr/gr sub deletion is the group of deletions which is due to recombination flanking g1/g2, r1/r3 and r2/r4 amplicons. These deletions results in loss of half of the AZFc sub-region in which combinations of *DAZ, CDY1* and *BPY2* gene copies are deleted. Thus, decrease the sperm count [30-31].

A total of 85 semen samples and 15 blood of fertile and infertile men along with 2 female control were screened in the present study for sub-microscopic Y-chromosome deletions. Microdeletions in the AZFc sub-region were detected and confirmed with PCR-based analysis for 5 out of the 50 infertile patients, corresponding to a proportion of 10%. No microdeletions were identified in any of the normozoospermic males. The frequency of microdeletions was 13.33% (2/15) in the azoospermic group, 10% (2/20) in the oligozoospermic group and 6.66% (1/15) in the asthenozoospermic group. Further, SRY (sY14) gene screening was normal in all infertile and the fertile group.

In our study, we found an increased partial AZFc deletions prevalence in infertile men, which gives an idea that AZFc mutations may be considered as a risk element for male with infertility. Screening for AZFc microdeletions was performed on azoospermic, oligozoospermia (Severe and mild) and asthenozoospermic men. Association of partial AZFc microdeletions was found in azoospermia, severe to mild

oligozoospermia. Two patient with oligozoospermia showed deletion of sY1191 and sY1197 (patient O10 and patient O52), another patient with azoospermia had a deletion involving sY153 and sY1291 (patient A28). The gr/gr and b2/b3 group of deletions was present in patient cohorts at similar frequencies of 4% and 4% respectively.

The relatively large proportion of microdeletions found in study population of 50 infertile men suggest the need for strict patient selection to avoid unnecessary screening for long arm Y-chromosome microdeletions. Among the 5 patients, 2 (O10 and O52) oligozoospermic men, 1 (AS19) asthenozoospermic male and 2 (A28 and A53) azoospermic men may have interstitial deletions that could not be identified by classical cytogenetic analysis. This variations is probably due to the selection criteria of the patients and STS markers.

Therefore, Y-chromosome microdeletion screening of partial AZFc deletion provide a useful molecular diagnostic tool in the work-up of patients with infertility. If we consider the evidence of partial AZFc microdeletion, it may be considered that molecular analysis should be carried out for the infertility candidates who attend the fertility clinic for assisted reproduction techniques and further genetic counselling of the couple should be done. Our findings are in agreement with other preliminary reports on the partial AZFc microdeletions [32-34].

5. CONCLUSION

Azoospermia factor (AZF) microdeletions are responsible for spermatogenic arrest and lead to Oligozoospermia, Asthenozoospermia or Azoospermia condition. Our current study, for the first time in Gujarat population suggest evidence for the association of partial AZFc deletions with spermatogenic failure in men with infertility. Because of high incidence of Y chromosome microdeletions among infertile patients, routine molecular screening may be advised to infertile patients especially before they undergo assisted reproductive techniques (ART) treatment, as Y chromosome microdeletions are vertically transmitted from father to son.

CONFLICT OF INTEREST

No conflict of interest.

ACKNOWLEDGEMENTS

The authors are grateful to Charutar Vidya Mandal and SICART for providing research platform. Our special thanks to Dr. Nilanjan Roy, Head of Institute, ARIBAS his generous support and inputs during our work. The study was is a result of INSPIRE fellowship to Miss Mili Nailwal by Department of Science and Technology,

New Delhi, India. Authors are thankful to all the patients and gynaecologist for providing samples.

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