



ISSN: 0975-833X

Available online at <http://www.journalcra.com>

INTERNATIONAL JOURNAL
OF CURRENT RESEARCH

International Journal of Current Research
Vol. 11, Issue, 02, pp.1373-1379, February, 2019

DOI: <https://doi.org/10.24941/ijcr.34363.02.2019>

RESEARCH ARTICLE

PENETRANCE OF *DE NOVO* MUTATION OF USP9Y AND PCDH11Y GENE IN AZF REGIONS OF NON-OBSTRUCTIVE AZOOSPERMIC POPULATION IN INDIA

*Ajit Kumar Saxena, Meenakshi Tiwari and Aniket Kumar

Human Cytogenetic & Molecular Genetic Laboratory, Department of Pathology/Lab Medicine; All India Institute of Medical Sciences, Patna - 801507, India

ARTICLE INFO

Article History:

Received 14th November, 2018
Received in revised form
27th December, 2018
Accepted 29th January, 2019
Published online 28th February, 2019

Key Words:

Microdeletion of Y-chromosome,
AZF-region, Whole Exome Sequencing,
Non- Obstructive Azoospermia,
PCDH11Y, USP9Y gene.

*Corresponding author: Ajit Kumar Saxena

Copyright © 2019, Ajit Kumar Saxena et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Citation: Ajit Kumar Saxena, Meenakshi Tiwari and Aniket Kumar. 2019. "Penetrance of *de novo* mutation of *usp9y* and *pcdh11y* gene in *azf* regions of non-obstructive azoospermic population in India", *International Journal of Current Research*, 11, (02), 1373-1379.

INTRODUCTION

Reproductive health is a serious problem in the developing countries including India. The male alone contribute more than 30% cases of infertility and approximately half of males underlying cause is unknown (Jungwirth *et al.*, 2012; Saxena & Gupta, 2016). Genetic causes of male infertility are limited because of variation in the frequency of mutation of AZF regions i.e. microdeletion of Y – chromosome and also associated with syndromic case where the frequency varying 10-20% having severe spermatogenic failure. Euchromatic region of Yq11 locus regulate spermatogenesis and azoospermic factor (AZF) has been further divided into four non-overlapping coding regions with varying sizes (1.0 to 3.0Mb) designated as AZFa, AZFb, AZFc and AZFd

associated male infertility (Krausz *et al.*, 2003; Skaletsky *et al.*, 2003). The frequency of microdeletion of Y-chromosome varies from 1% to 50% in idiopathic and non-idiopathic azoospermic patients (Henegariu *et al.*, 1994; Vogt *et al.*, 2004; van der Ven *et al.*, 1997; Foresta *et al.*, 1998) but there is lack of information with a definite genotype-phenotype correlation during AZF mutation. The clustering of gene has been identified to play a significant role during spermatogenesis and three major loci AZFa, AZFb & AZFc which contains 16 coding genes (Vollrath *et al.*, 1992; Vogt *et al.*, 1996). The quantitative role of genetic abnormalities in men is still unexplained due to the presence of several copies of genes assigned on Yq11.23 (Page *et al.*, 1999). The larger deletions or multiple AZFa regions usually been linked to Sertoli cell-only syndrome and AZFb or AZFc regions are restricted to moderate oligozoospermia with abnormal sperm

ABSTRACT

Introduction: Male infertility is a serious problem in developing world where genetic and epigenetic factors play a crucial role in the pathogenesis of the disease. The rationale behind the present study to understand the genetic basis of male infertility, to identify the "novel gene mutation" and also assess the frequency (%) of microdeletion of Y-chromosome i.e. deletion of AZF regions interfere during spermatogenesis. Still, 10-20% cases of infertility fail to identify exact cause of male infertility and are fall in the category of unexplained cause of infertility in non obstructive azoospermia. **Material and Methods:** Blood samples were collected from the cases of clinically diagnosed non obstructive azoospermia (NOA) with respective controls. Study was performed using RT-PCR based analysis using 14 set of STS markers of AZF region allocated on Y- chromosome and NextGen Sequencing. **Results:** Mutational spectra include the individual variations of frequency of AZF gene mutation as a factor responsible for male infertility in eastern part of the country. Genetics analysis of AZF a, b, and c regions showing different frequency of deletion but the deletion of AZFc showing significant difference with respect to controls ($p < 0.001$). NGS play a significant role to explore the involvement of *de novo* mutation of USP9Y and PCDH11Y gene mutation resulting changes in protamines. The deletion frequency AZFa region is 1.0%, while AZFb and AZFc regions showing 6% & 19%, respectively in non obstetric azoospermic cases. Hence, curiosity has been developed further to identify "new mutations" based on Next Gen Sequencing, identifies USP9Y gene of AZFa region showing non-frame shift mutation (insertion of C→G/C→A) at region exon42:c.6996_6997 insCGA in heterozygous condition. Secondly, of AZFb region showing single nucleotide gene polymorphism rs2524543, G→T and rs2563389, T→G of PCDH11Y gene in homozygous condition. **Conclusion:** The identification of causative mutations in the cases of NOA and their penetrance lead to interference in spermiogenesis. Hence, on the basis of mutational spectra, genetic counselling of infertile couples are required before reaching to final decision. No doubt the environmental factors influence the gene-pool lead to altered spermatogenesis in male infertility.

morphological features (Foresta *et al.*, 2001; Sun *et al.*, 2000; Kamp *et al.*, 2000). *De novo* microdeletion of Y- chromosome occurs due to recombination events between repetitive DNA sequences during meiosis in infertility (Ferlin *et al.*, 2006; Liu 2012; Rajender *et al.*, 2006). The study of Yq microdeletion is quite relevant to understand the mechanism of spermatogenesis because every individual belongs to different genetic background. The frequency of deleted regions is quite variable in different populations because of different environmental factors (Krausz *et al.*, 2003; Ma *et al.*, 1993). The rationale behind this study is to identify the frequency of microdeletion of Y- chromosome belongs to euchromatic regions using sequence-tagged sites (STS) specific markers in the cases of non obstructive azoospermia (NOA) in the eastern population where such type of study has not been conducted earlier. However, this study further extended to identify “novel *de novo* gene mutations” and copy number variations based on Next Gen Sequencing (NGS) followed by analysis using of bioinformatics tools.

MATERIALS AND METHODS

In the present study, clinically diagnosed infertile males were include (n=110) referred from OPD of AIIMS, Patna (Bihar, India) for Genetic analysis and compare to aged matched fertile males act as controls (n=42). The study was approved by Institutional Ethical Committee (IEC) and blood samples were collected by written informed consent. The male patients are mainly categorized in three different groups i.e. non-obstructive azoospermia, obstructive azoospermia and oligozoospermia on the basis semen analysis (WHO 1992) (sperm count $>20 \times 10^6$ /ml, progressive motility $>50\%$ and normal morphology $>30\%$) and proven fertility (with one or more children) were included as controls. All patients were initially evaluated by clinician and conventional diagnostic work-up including patient's history, genital examination, ultrasonography and hormone analyses were performed. None of them had any history of childhood disease, environmental exposure, radiation exposure or prescription drug usage that could account for their infertility. The median age of patients included in the study was 35.4 years (range 21-50 years). Sample (2 ml), whole blood was collected in sterile vial from patients male and female partner with their informed written consent. Hormonal screening can be limited to determining follicle stimulating hormone (FSH), luteinising hormone (LH) and testosterone levels in case of abnormal semen parameters. The blood plasma sample from the infertile patients was used for the study hormones (testosterone/luteinizing/follicle stimulating hormone) assay using standard routine laboratory methods (ELISA) with specific antibodies.

PCR based Analysis of STS markers of AZF region of Y-chromosome: Genomic DNA was extracted from peripheral blood samples according to standard procedure of DNA isolation according to the manufacturer's recommendations of the kit (Promega kit USA). Quantitative analysis of the DNA (ng/ul) was measured by Nanodrop spectrophotometer (Thermo.USA). PCR was performed to screen the microdeletions in the AZF region of the Y chromosome. Each patient was analysed by presence of fourteen (14) sequence tagged sites (STS) markers of three different regions (AZFa, AZFb and AZFc) of the Y-chromosome. These primers (forward/reverse) selected according to European Academy of Andrology guidelines having more than 90% detection limit of the mutations of all three major regions of AZF (Lucas *et al*

2000). Table 1 showing details of PCR conditions i.e. amplification conditions and their product size using sense and antisense sequences was performed in 25 μ l reaction volume containing 10x Tris (pH 8.4), 50mM KCl, 25mM MgCl₂, 2.5mM dNTP, 10 pM of oligonucleotide primers, 50-100 ng DNA and 1U Taq DNA polymerase. Further PCR product was analysed on 1.5 % agarose gel electrophoresis using ethidium bromide (1ug/ml) staining, and bands were visualised, characterized on Gel Doc system having software for evaluation of bands intensity to evaluate copy number variation in the same gel (Biorad, USA).

Whole-Exome sequencing for the identification of somatic mutations: Genomic DNA was isolated and purified before initiation of NGS sequencing using Illumina Hiseq 2000 platform with 101 bp paired-end reads. Alignment to the reference genomes (hg19 for human and mm9 for mouse) was performed with Burrows-Wheeler Aligner (BWA). After Next-Generation Sequencing data pre-processing (local realignment, duplicate marking and base quality recalibration) using GATK. The single nucleotide variants (SNVs) and small insertions/deletions were identified for alleles variations with exclusion of non-coding, synonymous and highly repetitive regions as described earlier by Saxena *et al* 2018. DNA sequencing was performed by Illumina HiSeq 2000 platform performed by Xcelris Lab of Ahmadabad, India, where the sequencing data was analysed through (http://www.ensembl.org/Homo_sapiens/Gene/). The mutated region identifies by (http://www.ensembl.org/Homo_sapiens/Tools/Blast/Alignment) during analyses of candidate gene variants when compared to genome-wide data using external available databases such as the 1000 Genomes Project (<http://www.1000genomes.org>). The data was further analysed using large-scale exome sequencing projects including the Exome variant server, NHLBIGO Exome Sequencing Project (ESP), Seattle, WA (<http://evs.gs.washington.edu/EVS/>). To identify exome sequencing candidate variants (http://www.hgvs.org/locus-specific-mutation-databases/?field_hgnc_gene_symbol_title=USP9Y). We also analyzed the sequence data possible functional effects of the mutated region by using two types of prediction programs (<http://www.hgmd.cf.ac.uk/ac/gene.php?gene=USP9Y>) and PolyPhen-2 (Adzhubei *et al.*, 2010). In these tools we are able to predict the possible interaction of protein during spermatogenesis in male spermatogenesis.

Statistical Analysis: The chi square (χ^2) test (two tailed) were apply to find out significance differences (p values) between infertile cases and controls.

RESULTS

The present study of infertile cases of non obstructive azoospermic (NOA) were sporadic in nature with lack of family history associated fertility problem, while remaining patients had a family history of infertility either first or second degree relatives. The median age of patients was 35.4 years (range 23- 27 years). Blood serum was used for hormones assay, showing significant increase (p<0.001) values of FSH (37.27 \pm 32.19), Testosterone (4.2 \pm 2.1 and Luteinizing (15.9 \pm 9.6) were observed when compared with normal /controls. These variations in the hormone profiles may be due to different professions. In the present study PCR based STS markers were used for the screening of recurrent deleted non – overlapping sub regions in proximal, middle, and distal Yq11, designated AZFa, AZFb and AZFc regions.

Table 1. List of the Sense and Antisense Sequences and their PCR condition used in the present study

Gene	STS markers	Sense & Antisense sequences	PCR conditions	Product size(bp)
AZFa	SY84	AGA AGG GTC TGA AAG CAG GT GCC TAC TAC CTG GAG GCT TC	95°C-5'(94°-30sec,56°C-45sec, 72°C-45sec) X35cycles, 72°C-5min	326bp
	SY86	GTG ACA CAC AGA CTA TGC TTC ACA CAC AGA GGG ACA ACC CT	95°C-5'(94°-30sec,56°C-45sec,72°C-45sec) X35cycles, 72°C-5min	320bp
	SY 87	TCT GTT GCT TGA AAA GAG GG GCT GCA GGA AGA ATC AGC TG	95°C-5'(94°-30sec,57°C-1min,72°C-45sec) X35cycles, 72°C-5min	252bp
	SY127	GGC TCA CAA ACG AAA AGA AA CTG CAG GCA GTA ATA AGG GA	95°C-5'(94°-30sec,56°C-45sec,72°C-45sec) X35cycles, 72°C-5min	274bp
	SY134	GTC TGC CTC ACC ATA AAA CG ACC TAC GCC AAA ACT TTC AA	95°C-5'(94°-30sec,56°C-45sec,72°C-45sec) X35cycles, 72°C-5min	301bp
AZFb	SY 141	GCA GTT CCA TTG TTT GCT TC GCA GCA TAA TAG CTA TAC AGT AGT	95°C-5'(94°-30sec,57.8°C-45sec,72°C-45sec) X35cycles, 72°C-5min	290bp
	SY 145	CAA CAC AAA AAC ACT CAT ATA CTC TTG AGA ATA ATT GTA TGT TAC GGG	95°C-5'(94°-30sec,57°C-45sec,72°C-45sec) X35cycles, 72°C-5min	160bp
	SY 152	AAG ACA GTC TGC CAT GTT TCA ACA GGA GGG TAC TTA GCA GT	95°C-5'(94°-30sec,56°C-45sec,72°C-45sec) X35cycles, 72°C-5min	125bp
	SY240	TCA AAT AGC AGC AAT TTA ATA T GCA CCT GAA GAG CTG CTT G	95°C-5'(94°-30sec,56°C-45sec,72°C-45sec) X35cycles, 72°C-5min	247bp
	SY254	GGG TGT TAC CAG AAG GCA AA GAA CCG TAT CTA CCA AAG CAG C	95°C-5'(94°-30sec,56°C-45sec,72°C-45sec) X35cycles, 72°C-5min	350bp
AZFc	SY255	GTT ACA GGA TTC GGC GTG AT CTC GTC ATG TGC AGC CAC	95°C-5'(94°-30sec,56°C-45sec,72°C-45sec) X35cycles, 72°C-5min	126bp
	SY267	GAA TGT GTA TTC AAG GCA TTC TCG TAC TTC CTT CGG GGC CTC T	95°C-5'(94°-30sec,56°C-45sec,72°C-45sec) X35cycles, 72°C-5min	102bp
	SY273	GGT CTT TAA AAG GTG AGT CAA ATT AGA CAG AGG GAA CTT CAA GAC C	95°C-5'(94°-30sec,57°C-1min,72°C-45sec) X35cycles, 72°C-5min	94bp
	SY277	GGG TTT TGC CTG CAT ACG TAA TTA CCT AAA AGC AAT TCT AAA CCT CCA	95°C-5'(94°-30sec,56°C-45sec,72°C-45sec) X35cycles, 72°C-5min	325bp
	Y283	CAG TGA TAC ACT CGG ACT TGT GTA GTT ATT TGA AAA GCT ACA CGG G	95°C-5'(94°-30sec,56°C-45sec,72°C-45sec) X35cycles, 72°C-5min	472bp

Table 2. Individual % frequency of AZF mutations using specific STS markers for microdeletion of Y chromosome in infertile males

S.No.	code ID	Age (years)	AZFa			AZFb					AZFc					
			Sy 84	Sy 86	Sy 87	Sy 127	Sy 134	Sy 141	Sy 145	Sy 152	Sy 240	Sy 254	Sy 255	Sy 273	Sy 277	Sy 283
1.	IF1	25	---	---	---	+	---	+	+	+	+	+	+	+	+	
2.	IF 4	29	---	+	---	---	---	---	---	---	---	---	---	---	---	
3.	IF12	24	---	---	---	---	---	---	---	---	---	---	---	---	---	
4.	IF14	35	---	---	---	---	---	---	---	---	---	---	---	---	---	
5.	IF19	30	---	---	---	---	---	---	---	---	---	---	---	---	---	
6.	IF23	32	---	---	---	---	---	---	---	---	---	---	---	---	---	
7.	IF44	26	---	---	---	---	---	---	---	---	---	---	---	---	---	
8.	IF50	33	---	---	---	---	---	---	---	---	---	---	---	---	---	
9.	IF60	25	---	---	---	---	---	---	---	---	---	---	---	---	---	
10.	IF61	31	---	---	---	---	---	---	---	---	---	---	---	---	---	
11.	IF69	29	---	---	---	---	---	---	---	---	---	---	---	---	---	
12.	IF74	28	---	---	---	---	---	---	---	---	---	---	---	---	---	
13.	IF75	23	---	---	---	+	+	+	+	+	---	---	---	---	---	
14.	IF85	33	---	---	---	---	---	---	---	---	---	---	+	---	---	
15.	IF86	35	---	---	---	---	---	---	---	---	---	---	---	---	---	
16.	IF88	29	---	---	---	---	---	---	---	---	+	---	---	---	---	
17.	IF90	32	---	---	---	---	---	---	---	---	+	+	---	---	---	
18.	IF91	25	---	---	---	---	---	---	---	---	+	+	---	---	---	
19.	IF93	29	---	---	---	---	---	---	---	---	+	+	---	---	---	
20.	IF97	36	---	---	---	---	---	---	---	---	---	---	---	---	---	
21.	IF101	38	---	---	---	---	---	---	---	---	+	+	+	---	---	
22.	IF102	32	---	---	---	---	---	---	---	---	+	+	---	---	---	
23.	IF103	25	---	---	---	---	---	---	---	---	+	+	---	---	---	
24.	IF108	25	---	---	---	---	---	---	---	---	---	---	+	---	---	
25.	IF123	35	---	---	---	---	---	---	---	---	+	---	---	---	---	
26.	IF 140	33	---	---	---	---	---	---	---	---	---	---	+	---	---	
27.	IF202	46	---	---	---	---	---	---	---	---	---	---	+	---	---	
Total			0	1	0	2	1	1	4	3	3	9	7	7	1	8
Mutation frequency (% Total)			0.0	0.9	0.0	1.8	0.9	0.9	3.6	2.7	2.7	8.2	6.4	6.4	0.9	7.3

Table- 3. Table- 3. Statistical analysis showing the microdeletion of Y- chromosome AZF mutation and their% frequency , Odd ratio and confidence interval

Y-chromosome regions	% Mutation frequency	Odd Ratio	C.I. at 95%	p-value
AZFa	1%	1.2000	0.1349 - 3.8880	0.53086
AZFb	6%	0.3143	0.0377 - 2.6231	0.2850
AZFc	19%	0.1222	0.0159 - 0.9412	0.0436

*Significance difference (p>0.05) were observed in AZFc region in case of infertile case and compared with respect to controls.

Table 4. De novo mutation of PCDH11Y & USP9Y genes in homozygous and heterozygous conditions and their % frequency

S.N	Name of the Gene(s)	SNP	Mutation		Gene Detail. Ref gene	Zygoty	Read Depth	Percentage Variation (HQ)
1	PCDH11Y	rs2524543	G	T	Non synonymous	Homozygous	133	100
2	PCDH11Y	rs2563389	T	G	Non synonymous	Homozygous	148	100
3	USP9Y	NA	-	CGA	Non frameshift	Heterozygous	17	20

The PCR products were analysed on 1.5 agarose gel and representative banding pattern showing mutational spectra in figure-1. Table-2 showing the details of individual (%) frequency of microdeletion of Y-chromosome using fourteen set of STS markers in non-obstructive azoospermic cases, which apparently showing the highest frequency of deletion in AZFc regions as represented (+) for mutation. The data was further analysed to identify the total frequency (%) of microdeletion of AZFa, b & c regions which varies from 1%, 6% and 19% in infertile patients respectively as shown in table-3. Statistical analysis showing significant difference ($p < 0.05$) in AZFc region with calculated value of O.R.(0.122) and C.I. at 95% (0.0159-0.9412). Curiosity has been developed further to identify new genetic mutation(s) in a large spanning region of AZF gene of Y- chromosome using NGS for single nucleotide polymorphism, insertion or deletion (frame shift mutation), trinucleotide repeats in heterozygous or homozygous conditions in the same cases of infertility. The whole exome sequencing which is a powerful technique and helps to identify “new *de novo* mutations” was performed in the selected case of NOA. Interestingly, NGS data revealed mutations in USP9Y and PCDH11Y. Figure-2 showing the schematic representation of *de novo* mutation of USP9Y of AZFa and PCDH11Y gene of AZFb regions of Y-chromosomes with fourteen set of sequence tagged site (STS) specific markers associated to spermatogenesis in male infertile patients. After validation and confirmation of USP9Y mutated regions which includes base pairs 12701231 bp to 12860844 bp in azoospermic cases (Homo sapiens Annotation Release 108, GRCh38.p7) <https://www.ncbi.nlm.nih.gov/genome/tools/gdp>. Table-4 showing mutation of two genes such as USP9Y and PCDH11Y candidate consist of mutated variants located on Y-chromosome. The USP9Y gene mapped on Yq11.221 and contains base pairs 12,701,231 to 12,860,844 on the Y chromosome (Homo sapiens Annotation Release 108, GRCh38.p7) (<https://www.ncbi.nlm.nih.gov/genome/tools/gdp>). Present study shows 20% variation of mutation of USP9Y gene, belongs to AZFa region of Y- chromosome, having non frameshift mutation and insertion of CGA trinucleotide in heterozygous condition i.e. allele “C” after mutation it changed into either “C→A” or “C→G” nucleotide which encodes Arginine, required for spermiogenesis as shown in Figure-3.

Further, the sequence of PCDH11Y gene was validated and confirmed their mutation by the single nucleotide polymorphism database (dbSNP) of NCBI (<https://www.ncbi.nlm.nih.gov/snp>). The genetic variation of PCDH11Y gene shows change in the position of nucleotide at position rs2524543 in homozygous condition where the nucleotide Guanine →Thymine and translate into amino acid Cystine → Isoleucine, respectively. Another site rs2563389 in homozygous condition was identified in the same gene, where the nucleotide Thymine → Guanine and translate Serine →Leucine, respectively.

This identification of mutation of SNP in data base are at position rs2524543 and rs2563389 of PHD11Y gene as shown Figure 4.

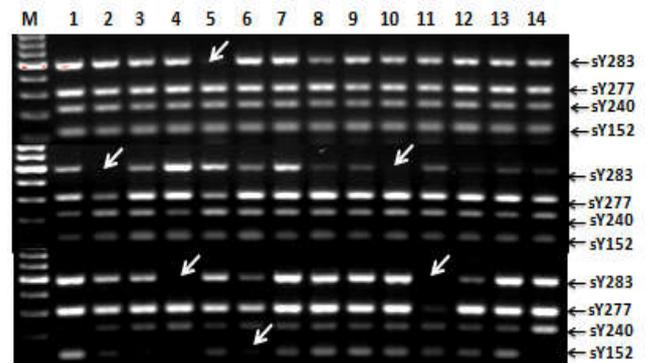


Figure 1. Representative presentation of microdeletion of Y-chromosome regions (AZFa,b,c) using different STS markers for the characterization of mutation in the cases of male infertility

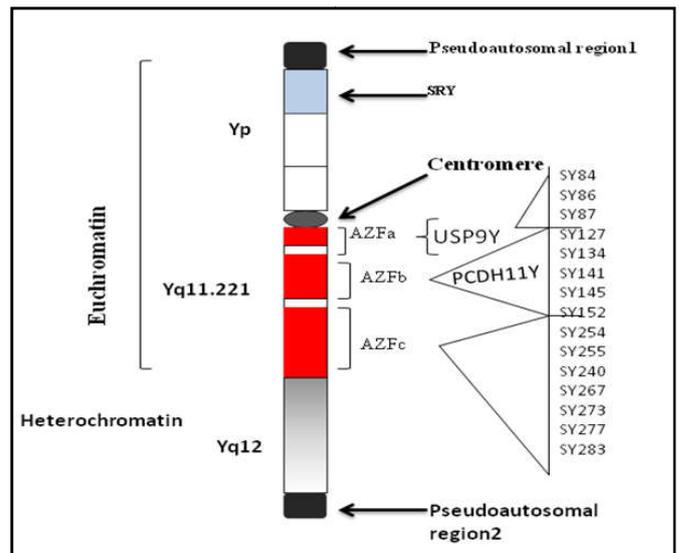


Figure 2. Schematics representation of Y-Chromosome showing AZF regions and their associated genes USP9Y and PCDH11Y regulating spermatogenesis in male fertility

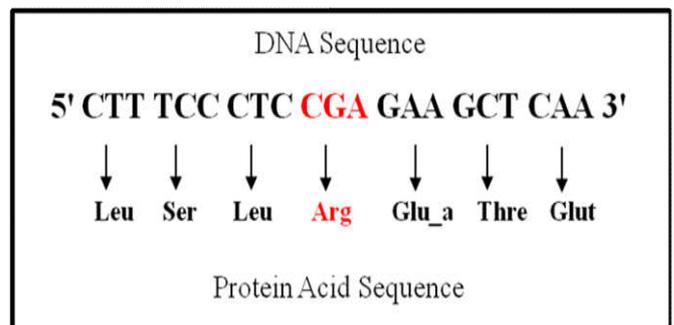


Figure 3. USP9Y gene showing insertion of trinucleotide CGA that encode arginine regulating spermiogenesis in infertile case

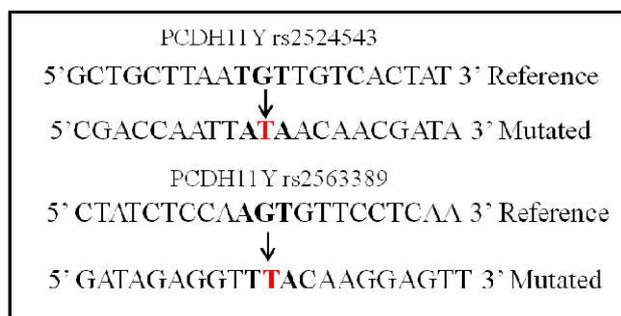


Figure 4. PCDH11Y gene showing variation in SNP at two different locations in the infertile male case as compared with the reference sequence using NCBI

DISCUSSION

In humans, primary genetic factors are responsible for the differentiation of gonads and secondary genetic factors are responsible to regulate the development of gonads (testis) and cellular differentiation regulating spermatogenesis (Ohno *et al.*, 1976). Furthermore, multiple factors including lifestyle and its interactions with genetic factors have significant influence on male fertility (Lin *et al.* 1998). Despite of the major role of genetic factors contributing male infertility new mutations have not yet been well recognized in Indian scenario. Infertility still remains a major challenge for the clinicians as well as the scientist in substantial proportion of cases (32% of infertile men, or 1.5% of the male population), the underlying causes are unknown (idiopathic infertility). The cytogenetic abnormalities including the deletion of short arm of Y chromosome has been well documented in male infertility (Ma *et al.*, 1993). Although, cytogenetic studies reveals that some of the NOA cases are still idiopathic.

They are believed to carry unknown autosomal mutations based on studies of familial male infertility that often involves in two or more siblings. In the present study the variation of hormonal profiles may be due to different lifestyle (diet, smoking, alcohol, drugs, tobacco chewing), different professions including farming (exposure to pesticides) and drivers (exposure to automobile fumes). Further, many unknown factors might also be responsible for testicular functions due to modulation of metabolic endocrine pathways (Sigman and Jarow, 1997). In the present study the elevated levels of FSH seems to be one of the determinant factor for interference in infertility. Similar findings with high level of FSH have also been reported in Danish population (Krasuz *et al.*, 2001, Frydelund-Larsen *et al.*, 2002). In such cases, the hypothalamus-pituitary-testis axis might be affected and responsible to down-regulate FSH level (Trumble *et al.*, 2010). Male infertility is a multifactorial disorder including genetic and environment might have influence to change the phenotypic variability in individual and regulating meiotic events during spermatogenesis. The microdeletion of the Y chromosome has been associated to the spermatogenic failure where the mutation frequency varies from 1-55% in infertile man depending on inclusion criteria (Tiepolo and Zuffardi 1976, Voget *et al.* 1996, Krausz *et al.*, 2001). Although, there is no direct evidence of existing relationship between microdeletion of Y- chromosome and hormone profile in the cases of male infertility. However, in some of the cases of infertility can't be explained by genetic alterations and fall under the category of "unexplained cause of infertility". Interestingly the present study also showed a significant loss of

AZFc region which may be due to either natural transmission from father to child or may be sporadic in nature (Pryor *et al.*, 1997; Chang *et al.*, 1999). Tiepoli and Zuffardi (1976), shows that the mutation of 5.3% frequency of AZFc region where as in the present study reveals statistically significant mutation frequency (19%) of the same locus which was more than three times higher in Eastern part of India. Although, there is a variation in the frequency of microdeletion of AZFc region showing variation of 3% - 3.5% between South Indian and Eastern Indian populations, respectively (Thangaraj *et al.*, 2003; Pandey *et al.*, 2010). Similar findings has also been reported by Kerr *et al.*, 2000, in the frequency of microdeletion upto 20% in Swedish population and New Zeland population. Such high frequency of AZFc mutation in this region may be due to endocrine disorders or disrupter, lifestyle (poor socio-economic status) or polluted environment, exposure of chemicals in the form of pesticides in the present population. Interestingly, in the present study the loss of AZFc regions may be either due to common locus or natural transmission from paternal side to the proband (Vogt *et al.*, 1996, Pryor *et al.*, 1997). It has been identified that the regulation of the complex process during spermatogenesis depends on interaction of many genes including ARSD (Arylsulfatase D) present on X chromosome (Saxena *et al.*, 2018) or other autosomes (unpublished data from the laboratory). In addition to these, several genetic polymorphisms have been demonstrated to be significantly associated with male infertility (Ceylan *et al.*, 2009). Recent advancements in molecular biology techniques, whole exome sequencing (WES) has emerged as a reliable toll to identify "new gene mutations" based on SNP, insertions/deletions (indel mutations) and a cost-effective tool for detecting rare mutations within a patient population. More than 30 Mendelian disease genes have been identified based on NGS, including recessive and dominant in nature. NGS data can also be used to discover linkage and homozygosity intervals, as well as determine copy number of specific intervals (Krawitz *et al.* 2010; Becker *et al.* 2011).

Interestingly, our data reveals a variety of "new mutations" that were identified on Y- chromosome that included single nucleotide polymorphism (SNP) and insertion non-frame shift mutation belonging to AZF regions in their heterozygous or homozygous conditions. Although, WES covers only the coding regions of the genome and the data are easy to interpret with a high probability to identifying significant gene variants, i.e. 85% of disease-causing mutations are thought to occur in gene coding regions (Bamshad *et al.*, 2011). In the present study, the alleles variation and their heterogeneity may be due to regional variation in the population. Multiple members of the same family may be affected by infertility with similar or different presentations of testicular failure with different pathologies. Genetic causes of male factor infertility can be broadly assumed to be a result of either *de novo* mutations either inherited by the patient from one of his parents or sporadic circulating in the population. In both cases, a search for potential genetic causes for infertility must rely on comparison of the DNA sequence of the patient with a controlled fertile member of his family. At the same time non-coding regions of the genome should not be forgotten in the biomedical research and consequently may be helpful to identify the unknown genetic factors in the case same cases of Infertility. But some of the non-coding regions were found to be useful for diagnosis of unexplained cause of male infertility, prognosis and therapeutics (Taft *et al.*, 2010).

The penetrance of USP9Y and PCDH11Y gene and their *de novo* mutations in AZF regions provide genomic instability and gonadal dysfunction leading to male infertility. The gene mutation of USP9Y in the form of insertion where the CGA encodes for arginine residue is required for chromatin modeling and required for formation of sperm head (de Kretser, *et al.* 1998; Balhorn R, 2007). The *de novo* mutation of PCDH11Y gene where Serine →Leucine and Cystine→Isoleucine are also associated during chromatin remodeling, histone methylation (H2A and H2B) during meiosis-responsible for elongation of spermatids (spermiogenesis) (Rathke *et al.*, 2014). Although, it has been established that *de novo* mutation of USP9Y belonging to AZFa region is responsible for complete absence of germ cells in syndromic cases, while PCDH11Y of AZFb is responsible for chromatin folding during maturation of sperm in spermiogenesis which might be a causative factor in NOA cases.

Conclusion

The findings of new genetic recombination of *de novo* mutation of AZF regions and changes in protamines may hampered infertility either due to insufficient sample size or ethnic variations between heterogeneity in two groups of population. However, the linkage between the genetic and non-genetic factors associated in azoospermic cases remain obscure due to disequilibrium between the primary and secondary event of gonadal dysfunction. Hence, the present study are beginning to shed additional light on the genetic architecture of male infertility, based on NGS to proven effective efficiency to identifying novel genetic causes of in male infertility in eastern region of India.

Acknowledgement

AKS thankfully acknowledges Director, AIIMS Patna for valuable suggestions and the DBT project (No.BT/PR14671/MED/12/487/2010) for providing financial assistance to carry out the work.

REFERENCES

Adzhubei IA., Schmidt S., Peshkin L., Ramensky VE., Gerasimova A., Bork P., Kondrashov AS., Sunyaev SR. 2010. A method and server for predicting damaging missense mutations. *Nat Methods* 7(4):248-249.

Balhorn R. 2007. The protamine family of sperm nuclear proteins. *Genome Biol.*, 8(9):227.

Bamshad MJ., Ng SB., Bigham AW., Tabor HK., Emond MJ., Nickerson DA., Shendure J. 2011. Exome sequencing as a tool for Mendelian disease gene discovery. *Nat Rev Genet.* 12:745-55.

Becker J., Semler O., Gilissen C., Li Y., Bolz HJ., Giunta C., Bergmann C., Rohrbach M., Koerber F., Zimmermann K. *et al.* 2011. Exome sequencing identifies truncating mutations in human SERPINF1 in autosomal-recessive osteogenesis imperfecta. *Am J Hum Genet.* 88:362-71.

Ceylan GG., Ceylan C. 2015. Genetics and male infertility. *World J Clin Urol* 4:38-47.

Chang PL., Sauer MV., Brown S. 1999. Y chromosome microdeletion in a father and his four infertile sons. *Hum. Reprod.* 14: 2689-2694.

de Kretser DM., Huidobro C., Southwick GJ., Temple-Smith PD. 1998. The role of the epididymis in human infertility. *J Reprod Fertil Suppl.* 53:271-5.

Ferlin A., Arredi B., Foresta C. 2006. Genetic causes of male infertility. *Reprod Toxicol.*, 22(2):133-41.

Foresta C., Ferlin A., Garolla A., Moro E., Pistorello M., Barbaux S., Rossato M. 1998. High frequency of well-defined Y-chromosome deletions in idiopathic Sertoli cell-only syndrome. *Hum. Reprod.* 13: 302-307.

Foresta C., Moro E., Ferlin A. 2001. Y chromosome microdeletions and alterations of spermatogenesis. *Endocr. Rev* 22, 226-39.

Frydelund-Larsen L., Krausz C., Leffers H., Andersson AM., Carlsen E., Bangsboell S., McElreavey K., Skakkebaek NE., Rajpert-De Meyts E. 2002. Inhibin B: a marker for the functional state of the seminiferous epithelium in patients with azoospermia factor C microdeletions. *J. Clin. Endocrinol. Metab.* 87: 5618-5624.

Henegariu O., Hirschmann P., Kilian K., Kirsch S., Lengauer C., Maiwald R., Mielke K., Vogt P. 1994. Rapid screening of the Y chromosome in idiopathic sterile men, diagnostic for deletions in AZF, a genetic Y factor expressed during spermatogenesis. *Andrologia* 26: 97-106.

Jungwirth A., Giwercman A., Tournaye H., Diemer T., Kopa Z., Dohle G. 2012. European association of urology guidelines on male infertility: The 2012 Update. *Eur Urol.* 62(2):324-32.

Kamp C, P. Hirschmann, H. Voss, K. Huellen, P.H. Vogt. 2000. Two long homologous retroviral sequence blocks in proximal Yq11 cause AZFa microdeletions as a result of intrachromosomal recombination events. *Hum Mol Genet.*, 9: 2563-2572.

Kerr NJ., Zhang J., Sin FY., Benny P., Sin IL. 2000 Frequency of microdeletions in the azoospermia factor region of the Y-chromosome of New Zealand men. *N. Z. Med. J.* 113: 468-70.

16. Krausz C, Forti G, Meclraeavey. 2003; The Y – chromosome and male infertility. *International J of Andrology* 26:70-75.

Krausz C., Rajpert-De Meyts E., Frydelund-Larsen L., Quintana-Murci L., McElreavey K., Skakkebaek NE. 2001; Double-blind Y chromosome microdeletion analysis in men with known sperm parameters and reproductive hormone profiles: microdeletions are specific for spermatogenic failure. *J. Clin. Endocrinol. Metab.* 86: 2638-2642.

Krawitz PM., Schweiger MR., Rödelsperger C., Marcelis C., Kölsch U., Meisel C., Stephani F., Kinoshita T., Murakami Y., Bauer S. 2010. Identity-by-descent filtering of exome sequence data identifies PIGV mutations in hyperphosphatasia mental retardation syndrome. *Nat Genet.*42:827-29.

Lin WW., Lamb DJ., Lipshultz LI., Kim ED. 1998. Absence of cyclic adenosine 3':5' monophosphate responsive element modulator expression at the spermatocyte arrest stage. *Fertil. Steril.* 69: 533-538.

Liu RZ. 2012. AZF deletions and male infertility. *Zhonghua Nan Ke Xue* 18: 963-8.

Lucas H., Patrat C., Jouannet P., Beldjord C., Bienvenu T. 2000. A novel, rapid, and accurate method for detecting microdeletion involving the DAZ gene in infertile men. *Fertil. Steril.* 73: 242-7.

Ma K., Inglis JD., Sharkey A., Bickmore WA., Hill RE., Prosser EJ., Speed RM., Thomson EJ., Jobling M., Taylor K. *et al.*, 1993; A Y chromosome gene family with RNA-binding protein homology: candidates for the azoospermia factor AZF controlling human spermatogenesis. *Cell* 75:1287-1295.

- Ohno S., Christian LC., Wachtel SS., Koo GC. 1976. Hormone-like role of H-Y antigen in bovine freemartin gonad. *Nature.*, 261:597-9.
- Page DC., Silber S. and Brown LG. 1999. Men with infertility caused by AZFc deletion can produce sons by intracytoplasmic sperm injection, but are likely to transmit the deletion and infertility. *Hum. Reprod* 14: 1722-6.
- Pandey LK., Pandey S., Gupta J., Saxena AK. 2010. Loss of the AZFc region due to a human Y-chromosome microdeletion in infertile male patients. *Genet Mol Res.*, 2010 Jun 29;9:1267-73.
- Pryor JL., Kent-First M., Muallem A., Van Bergen AH., Nolten WE., Meisner L., Roberts KP. 1997; Microdeletions in the Y chromosome of infertile men. *N. Engl. J. Med.*, 336:534-539.
- Rajender S., Rajani V., Gupta N., Chakravarty B., Singh L., Thangaraj K. 2006. SRY-negative 46,XX male with normal genitals, complete masculinization and infertility. *Molecular Human Reproduction* 12:341-346.
- Saxena AK., Gupta RK. 2016; Microdeletion of Y-chromosome associated male infertility-impediment and consequence. *J of Basic Clin Rep Sciences*: 5 (2): 57-60.
- Saxena AK., Tiwari M., Agarwal M. 2018. Single Nucleotide Polymorphism of Arylsulfatase D Gene (ARSD) and their association with male infertility. *J Clin Gen Genomics.*, 1:11-13.
- Sigman M., Jarow JP. 1997. Endocrine evaluation of infertile men. *Urology* 50: 659-664.
- Sun C., H. Skaletsky, S., Rozen, J. Gromoll, E. Nieschlag, R. Oates, Page D.C. 2000. Deletion of azoospermia factor a (AZFa) region of human Y chromosome caused by recombination between HERV15 proviruses. *Hum Mol Genet* 9: 2291-2296.
- Taft RJ., Pang KC., Mercer TR., Dinger M., Mattick JS. 2010. Non-coding RNAs: regulators of disease. *J Pathol.* 220(2):126-39.
- Thangaraj K., Gupta NJ., Pavani K., Reddy AG., Subramanian S., Rani DS., Ghosh B., Chakravarty B., Singh L. 2003. Y chromosome deletions in azoospermic men in India. *J. Androl.* 24: 588-597.
- Tiepolo L., Zuffardi O. 1976. Localization of factors controlling spermatogenesis in the nonfluorescent portion of the human Y chromosome long arm. *Hum. Genet.* 34: 119-124.
- Trumble BC., Brindle E., Kupsik M., O'Connor KA. 2010. Responsiveness of the reproductive axis to a single missed evening meal in young adult males *Am J Hum Biol.*, 22:775-781.
- van der Ven K., Montag M., Peschka B., Leygraaf J., Schwanitz G., Haidl G., Krebs D. van der Ven H. 1997. Combined cytogenetic and Y chromosome microdeletion screening in males undergoing intracytoplasmic sperm injection. *Mol. Hum. Reprod.* 3: 699-704.
- Vogt PH. 2004. Molecular genetic of human male infertility: from genes to new therapeutic perspectives. *Current Pharmaceutical Design* 10:471-500.
- Vogt PH., Edelmann A., Kirsch S., Henegariu O., Hirschmann P., Kiesewetter F., Köhn FM., Schill WB., Farah S., Ramos C. *et al.*, 1996; Human Y chromosome azoospermia factors (AZF) mapped to different subregions in Yq11. *Hum Mol Genet* 5:933-43.
- 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* 2;258:52-9.
