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Synergism between Sycp-3 Gene Mutation and DNA Copy Number Variation Increase Genetic Susceptibility in the Cases of Non-Obstructive Azoospermia

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Abstract

Background: In the human, infertility is a serious reproductive health problem in the world and more than 15% couples are infertile. Male infertility is a highly complex phenomenon involving large number of factors including endocrine dysfunction, socioeconomic, life-style, use of alcohol, drugs, exposure with radiations, chemical (pesticides) and automobile fumes. The genetic factors like complex chromosome rearrangements (CCRs), microdeletion of Y-chromosome (AZF regions) and genes assigned on autosomal region of the chromosomes are also associated with male infertility.

Objective: The role of Sycp3 gene mutation in infertile patients is not clear in Indian population. Therefore, the present study has been designed to evaluate the percentage frequency of synaptonemal complex protein-3 (Sycp3) gene mutation and simultaneously also assess the frequency of DNA copy number variations in nonobstructive azoospermic (NOA) cases.

Materials and Methods: Genomic DNA was isolated followed by polymerase chain reaction (PCR) using two different sets of up and down stream amplicons, both from clinically diagnosed cases of NOA and controls of the same age group.

Results: The present study shows mainly categorised, three type of Sycp-3 gene mutations i.e., the complete disappearance bands (absent), over expression and under expression. Interestingly, significant ($p<0.05$) individual variations were observed in the frequency (%) of complete disappearance (null) of 173bp (8.33%) and 568 bp (12.5%) amplicon followed by calculated value of odd ratio 6.45

and 5.00 with confidence interval varies (C.I) at 95% interval 0.76-55.04 and 1.04-24.02, respectively in the cases of NOA when compared with controls. Similarly, the frequency of over expression (up regulation) in 173bp (5.55%) and 568bp (2.77%) amplicons were also showing significant variations ($p<0.05$). Further study was extended to calculate the DNA copy number variations, which again shows statistically significant differences ($p<0.05$) between NOA cases and controls.

Conclusions. The present findings of Sycp-3 gene mutation using two different amplicons in cases of NOA concluded significant variations of Sycp-3 gene mutation of 568 bp amplicon along with DNA CNVs showing negative impact on spermatogenesis leading to male infertility.

Keywords: Sycp-3 gene; Male infertility; Copy Number Variations

Introduction

Reproductive health is a serious and mounting problem in the world, where several factors including climate changes such as global warming resulting in change in sperm quantity according to world health organization (WHO 1999) [1]. The pathogenicity of infertility is highly complex phenomenon affecting approximately 1:20 males during reproductive age due to unknown genetic and epigenetic factors [2-3]. The role of complex chromosomal rearrangement (CCRs) and high incidence of mosaicism are well known in azoospermic patients [4]. More than 20% of *De novo* mutations of USP9Y and PCDH11Y genes were observed in both heterozygous and homozygous conditions that encodes amino acid arginine guanine, isoleucine and leucine modulates early transcription factor such as Sox9 gene in non-obstructive azoospermic (NOA) cases. Similarly, Sox9 gene expression is essential for the normal differentiation of Sertoli cells and DNA copy number variations (DNA CNVs) interfere in germ cell proliferation inside the seminiferous tubule of the testes [5-7].

The study of whole genome sequencing (WES) in male infertility explore new knowledge between the positive interaction to several gene like melatonin receptor1B (MTNR1B), sentrin-specific protease-3 (SENP3), a-kinase anchoring protein3(AKAP3), Procollagen-Lysine,2-Oxoglutrate 5-Dioxygenase 3 (PLOD3) genes assigned on autosomes 11q14.3,17p13.1,12p13.3,11p13.4, respectively, modulates sequential transcriptional activity followed by translational mechanism either due to changes in function of amino acid or loss to binding capacity to the ligand (receptor) during differentiating germ cells in the testis [8-9]. In spite of the fact, still, more than 17% couples fall under the category of unexplained cause of infertility and becomes a major challenge to the scientist as well as for the clinicians to explore the underlying cause. In reproductive medicine, male counterpart is an active and dominant part of the society and testis is highly sensitive towards the exposure of environmental teratogens followed by uninterrupted supply of mature and healthy spermatocytes or sperms in male gonads.

Synaptonemal complex (SC) is a protein structure develop during the pairing between two homologous chromosomes after linkage and crossing over, where the exchange of gametes information take place in zygotene stage of meiosis I of cell-division. Synaptonemal complex-3 (Sycp-3) gene is mapped on chromosome -12 (12q23.2) and consists of 9 exons, spanning 10.5 kb DNA and present outside the AZF region of Y- chromosome [10,11]. However, there is scanty in literature on the functioning of Sycp-3 protein and their association to the meiotic arrest during spermatogenesis. Miyamoto, *et al.* (2003) reported a loss of DNA (>1bp) at 643delA, resulting in origin of truncated sycp3 protein due to the presence of premature stop codon. Arabi, *et al.* (2006) reported debatable findings regarding loss of Sycp-3 gene expression or its restriction to germ cells in azoospermic cases. Therefore, a present study has been designed with the aim to assess the frequency (%) of Sycp-3 gene mutation using two different specific set of primers and the same study was further extended to evaluate the frequency of DNA copy number variations (DNA CNVs) to determine genomic instability in the cases of NOA and compare with controls of the same age group to validate the significance after statistical analysis of the study.

Materials and Methods

Subjects

In the present study, clinically diagnosed cases of NOA were included (n=72) and the finding were compared with control group. The study was approved by Institute Ethical Committee and Institutional Research Committee of All India Institute of Medical Sciences Patna. Written informed consent was taken from the patients.

Isolation of Genomic DNA and RNA

Genomic DNA and RNA was isolated from 1.0 ml whole blood samples using protocol of kit (Promega, USA), quantified by nanodrop spectrophotometer and quality was checked on 1.5 % agarose gel electrophoresis and DNA or RNA bands were visualized after staining with ethidium bromide by Gel Doc system (Bio Rad, USA). The cDNA was prepared by using reverse transcriptase with Oligo (dT) primer and complementary products were stored at -20°C till further analysis.

RT-PCR analysis for the amplification of Sycp-3 gene mutation.

In the present study, Sycp-3 gene mutation and copy number variations (CNVs) were assessed using specific set of forward and reverse primers to amplify the desired regions of exons after confirmation of sequences from NCBI (BLAST/http://blast.ncbi.nlm.nih.gov./Blast.cgi) as documented by Mizutani, *et al.* 2011). For the study of Sycp-3 gene expression RT qPCR-based analysis were carried out using SYBR® Green (10µl) as fluorescence dye, 1µg cDNA with specific primer in 20µl reaction mixture. The amplification was accomplished with a 25 µl reaction mixture containing 50 ng DNA, 1 µl each of 10 pmol of Sycp-3 primer

(forward / reverse), 5x Green GoTaq PCR reaction buffer, dNTPs Mix (10 mM) and 0.2µl of Go Taq DNA polymerase (5U/µl). PCR program for Sycp-3 was as follows: initial denaturation 95°C for 5 minutes, 35 cycles for denaturation at 94°C for 45 seconds, annealing at 48.7°C for 30 seconds, elongation at 72°C for 1 minute, followed by final extension at 72°C for 8 minutes as depicted in Table 1. The amplified products were analysed on 1.5% agarose gel, bands were visualized and characterized on a Gel Doc system (Bio rad, USA), after ethidium bromide staining.

Table 1: Sycp-3 gene mutation were analysed by using two different sets of amplicons (upstream / downstream) in non-obstructive azoospermic cases

S. No.	Types	Primers (forward and reverse)	GC Content	Annealing temp.	Size of the amplicon (bp)
1.	Sycp3F	GATGGCGTGTGCCTATAATCCAAG	50%	48.7°C	173
	Sycp3R	CGTCTTTATTTAATTGACAGTGTTAG	30.8%		
2.	Sycp3F	TCCAATGCTCTGAGAACC	50%	48.7 °C	568
	Sycp3R	TCACCACAGCAAGTTGTG	50%		

DNA Copy Number Analysis of Sycp3 gene.

The DNA copy number variations (CNVs) of Sycp-3 gene analysis were carried out using Image lab software 5.1 in Gel Doc system (Bio Rad, USA) by performing densitometry of individual bands (173 & 568 bp) for two different amplicons. The frequency of CNVs of Sycp-3 gene correlate genomic instability after statistical analysis between the cases of NOA and their respective controls.

Statistical Analysis

To find out the level of significant difference between cases of NOA and their respective controls, p-value was calculated using two tailed chi-square test and compared with controls of the same age groups.

Results

The Sycp-3 gene is mapped on the long arm of chromosome-12 (12q23.2) and encodes the synaptonemal complex protein (Sycp-3). The findings of RT qPCR-based analysis of Sycp-3 gene mutations presented in three different forms-1) the complete disappearance of band 173 and 568 bp, 2) over expression or up regulation and 3) under expression or down regulation using specific forward and reverse set of primers in NOA cases and the same compare with control groups as documented in Figure 1A &B.

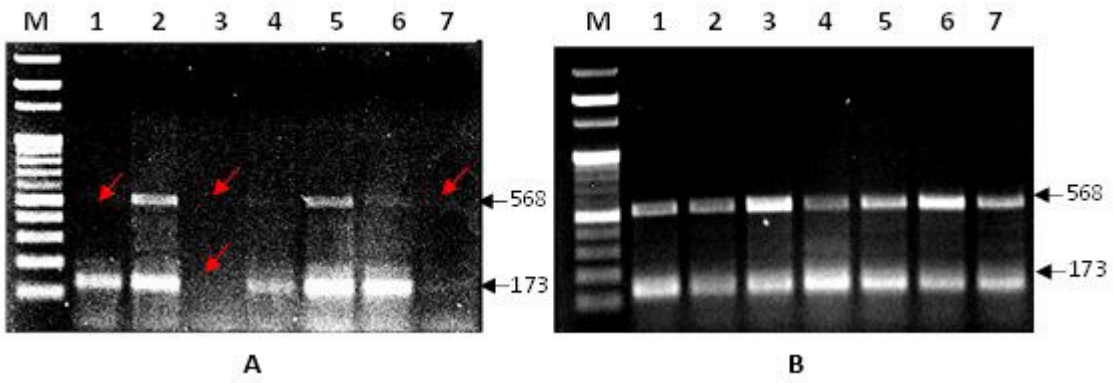

Figure 1: A&B. PCR based analysis of Sycp-3 gene mutation in NOA cases of male infertility using two sets of forward and reverse primers belong to 568 bp and 173 bp respectively, visualize on 1.5 % agarose gel after staining with ethidium bromide (A) and compare with respective controls (B). M= molecular weight marker (100 - bp DNA ladder), lane 1, 3 & 7-cases. (arrow red) showing complete disappearance (null) of 568 bp bands and lane-3 also showing disappearance of band belong to 173 bp while lane-7 showing down regulation

Table 2: PCR based analysis of Sycp-3 gene mutations using two different set of amplicons (173 & 568bp) in the cases of NOA and their respective controls

S.No	Types of Sycp3 Mutation (n=72)	No. & (%) of frequency of Mutation		Odd Ratio (O.R.)	Confidence Interval (C.I.) at 95 %		p -values
		Cases	Control		Min.	Max.	
.	**568 bp**						
1	Down regulation	14 (19.44%)	6 (8.33%)	2.66	0.96	7.36	0.05
	Up regulation	2 (2.77%)	8 (11.11%)	0.23	0.05	1.12	0.04*
	Null/Absent	9 (12.5%)	2 (2.77%)	5.00	1.04	24.02	0.02*
	173bp						
2	Down regulation	10 (13.88%)	3 (4.16 %)	3.71	0.98	14.10	0.04*
	Up regulation	4 (5.55%)	11 (15.27%)	0.33	0.10	1.08	0.05
	Null/Absent	6 (8.33%)	1 (1.38%)	6.45	0.76	55.04	0.05

*Significant differences ($p<0.05$) were observed using x^2-test.

Interestingly, the findings of Sycp-3 gene mutations were assessed between NOA cases and controls as details were documented in Table 2. The significant variations ($p<0.5$) were observed in Sycp-3 gene mutation of 173bp band (complete disappeared) and calculated value of O.R 6.45 with confidence interval (C.I) at 95% varies from 0.76 to 55.04, while for Sycp-3 gene (568bp) showing significant difference ($p <0.05$) with calculated odd ratio (5.00) and C.I. at 95% varying from 1.04-24.02, although, the mutation frequency (12.5 %) was observed higher in Sycp-3 568 bp than band 173 bp in Sycp-3 (8.33%). However, significant variations ($p<0.05$) were also observed in both down/up regulation (over expression) using two different set of Sycp-3 primers 568 and 173bp in the cases of NOA when compared with controls group. Apparently, bar diagram showing lack of definite pattern of mutation frequency after using two different set of amplicons 568bp and 173 bp of Sycp-3 gene - null (absent), over expression or down regulation, but after statistical evaluation significant association ($p<0.05$) were observed when compared with controls (Figure 2).

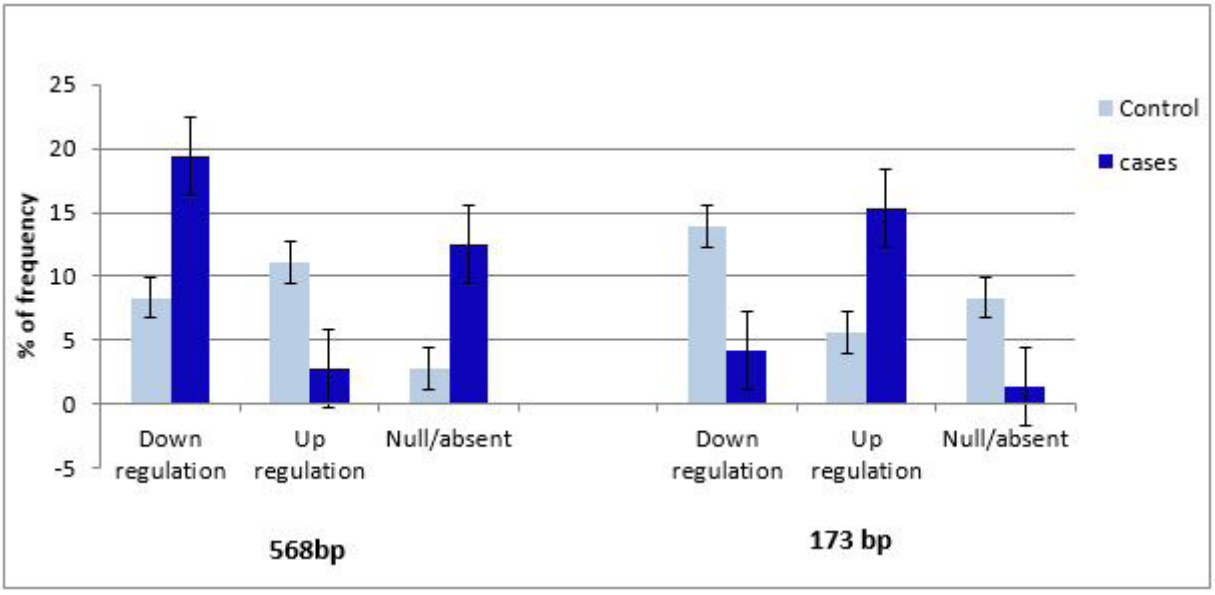

Figure 2: Bar diagram showing Sycp3 gene regulation over expression (up regulation) / under expression (down regulation) / complete disappearance of band (null) in two different set of primers in the cases of non-obstructive azoospermic with respect to controls

The DNA CNVs have shown to be associated with several complex diseases due to genetic susceptibility followed by changes in the behaviour of either exons or introns involving deletions or duplications of DNA segments >1 kb during germ cell differentiation in testes. Figure 3 showing significant association of CNVs of Sycp-3 gene between cases and controls after using two set of forward and reverse primers. The DNA CNVs of Sycp-3 play an important role to determine genomic instability either at leptotene or zygotene stage of meiosis I during crossing over and synapse formation resulting formation of millions of mature and healthy sperm cells through a complex process spermatogenesis. Sycp-3 (568 bp) showing observed mean and s.d values 1595.8 ±1030.37 in NOA cases, while 2067.8 ±188.23 in controls. Similarly, the mean and s.d. values 1730.5 ±920.34 in the cases and in control is 2508.7 ±76.76 for Sycp-3 (173 bp). Apparently, bar diagram (Figure 3) showing high significant ($p<0.01$) differences in 173 bp of Sycp-3 gene with respect to controls between two different amplicons. Although, the detailed p-values after statistical evaluation of DNACNVs between two sets of amplicons (173 bp or 568 bp) are documented in Table 3.

Table 3: Statistical analysis showing DNA Copy Number Variations using two set of amplicons between Cases and Controls

S. No.	Types Sycp3	Mean ± S. D (n=15)		S.E (Standard error)		Significance (p-value)
		Cases	Control	Cases	Control	
1.	568 bp	1595.8 ±1030.37	2067.8 ±188.23	266.04	48.60	0.02*
2.	173 bp	1730.5 ±920.34	2508.7 ±76.76	237.63	20.33	0.01*

*Significant differences ($p<0.05$) were observed after using x^2 -test

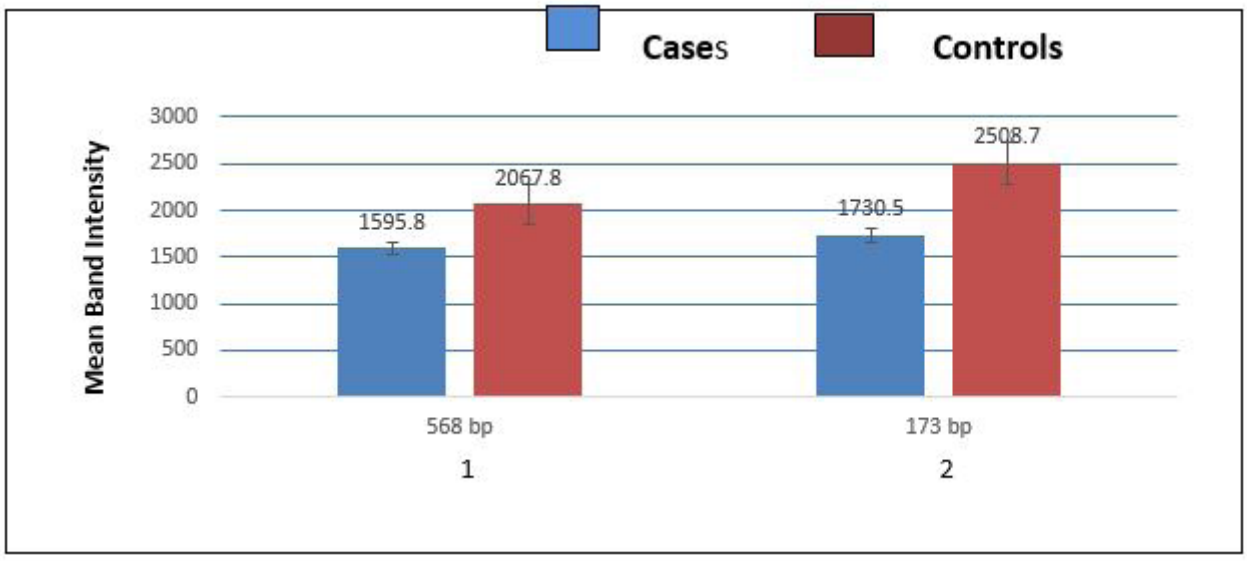

Figure 3: Bar diagram showing mean values of Sycp3 gene between cases and controls in two different set of amplicons belong to 568 bp (Bar1) and 173 bp (Bar 2)

Discussion

During crossing over, synapse is formed where the exchange of genetic information takes place between maternal and paternal homologous chromosomes. Synapse is highly complex and sensitive structure consist of several proteins. However, the synaptonemal complex3 (Sycp-3) play an important role at zygotene stage of cell - division during spermatogenesis. Since Sycp-3 is a prominent DNA-binding protein that encodes an essential component of the axial or lateral filament during formation of synaptonemal complex in gametogenesis. Sycp-3 is mapped on chromosome - 12 (12q23.2) and consists of 9 exons which span 10.5 kb of DNA and exist outside the AZF region of Y- chromosome [10-11]. In the human, the role of Sycp-3 protein is highly debatable in male infertility due to scanty of literature and different views by the different group of the scientists. Hence, curiosity has been developed to assess the role of Sycp-3 gene mutation in terms of either complete disappearance (absent/null) or over expression (up regulation) or under expression (down regulation) along with the DNAcopy number variations using two different set of amplicons (173 bp & 568 bp) in the cases of non-obstructive azoospermia (NOA). Miyamoto, *et al.* (2003) reported in

two cases of azoospermic patients regarding the deletion of 1.0 bp DNA at position 643delA and suggesting the involvement of premature stop codon followed by truncated sycp-3 protein in C-terminal forming region. Although, the present study also showing the deletion of Sycp-3 gene belongs to 568 bp amplicon significantly, but lacking in 173 bp the same cases of NOA with respect to controls, suggesting either sycp3 protein have different isoforms or different physiological identity during meiotic I arrest at zygotene stage of cell division. Further, authors suggested that the discrepancy of Sycp-3 gene mutation frequency is either due to germ cell susceptibility during synapse formation at crossing over in two different set of amplicons or individual's genetic susceptibility in the cases of NOA. However, the controversial findings have been reported earlier by several authors regarding the role of Sycp-3 gene mutation or expression during meiosis in germ cells. However, the authors hypothesized that such mutations (meiotic errors at spermiogenesis) may lead to cause chromosomal translocation resulting spermatogenic arrest, DNA-copy number variation and origin of aneuploid gametes due to non - disjunction event during germ cell differentiation [10-13].

Genes affected by DNAcopy number variations (CNVs) are excellent candidates for reproductive research in diseased conditions associated genetic susceptibility and risk factor. In human, the role of Sycp3 gene is highly ambiguous during spermatogenesis and becomes relevant to extend in the present study to assess the frequency of CNVs shows statistically significant in the cases of NOA when compare the same with controls. Genes affected by CNVs are excellent candidates for genomic research in disease to determine genetic susceptibility. CNVs are structural changes in DNA consisting of deletions or duplications of segments larger than 1bp as compared to reference genome. Because, copy number variations (CNVs) have shown to be associated with several complex diseases and ubiquitous reports have also been documented both in normal and diseased populations, but in some cases show implications of disease like male infertility [3,14,15]. Present study shows significant association in the frequency of Sycp-3 gene mutation and DNA copy number variations (CNVs) to determine "risk factors" of the disease when compare with controls, suggesting penetrance of mutated Sycp3 gene in to probands either from parents or *de novo* mutation. Although there is no direct evidence exist in the literature to correlate CNVs and Sycp-3 gene mutation in male infertility. Earlier study has shown that there is large number gene such as arylsulphatase D (ARSD) polymorphism and cullinring ubiquitin ligase4B (Cul4B) genes has also been associated with male infertility due to non-frame shift mutation (deletion at position 1761-1766 of GGAGGA nucleotide sequences) in homozygous condition, result loss of non-essential amino acid glycine in the cases of male infertility [16, 17].

Beside this, folate metabolism associated MTHFR C677T gene polymorphism is also associated to increase "risk factor" by modulating hormonal dysfunction [2]. Similarly, the role of Nanog and Sox9, the early transcription factor also plays an important role to maintain pluripotency during organogenesis of male gonad (testis)), sex reversal cases i.e. XX male or XY-female and male infertility [18-19].

Conclusion

The present study concludes that Sycp-3 gene mutations play an important role in the cases of NOA either as independent factor or in association with DNA CNVs at early leptotene or zygotene stage of meiosis I during synapse formation. Further, out of different amplicons of Sycp-3, the frequency of amplicon of 568 bp showed higher susceptibility towards mutations leading to male infertility. Such study are warned to determine the role of Sycp-3 in cases of male infertility as an established diagnostic marker and determine the clinical management in Indian population.

Acknowledgement

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Conflict of Interest

There is no conflict of interest between the authors.

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