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SYCP3 T657C Gene Polymorphism Increases Risk of Developing Infertility in Non-Obstructive Azoospermic Cases in Indian Population

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Abstract

Etiopathology of male infertility is highly complex and chromosome rearrangements play an important role in non-obstructive azoospermia (NOA). Testes is the unique organ, where, spermatogenesis regulates the proliferation of germ cells and non-germinal cells (Sertoli cells & Leydig cells) work together in a synchronized fashion. Infertility is a genetic phenomenon and a variety of structural and numerical chromosome aberrations are well known to interfere with male infertility. The role of Sycp *T657C* gene polymorphism has been poorly documented in the Indian population with special reference to non-obstructive azoospermic (NOA) cases. The present study has been designed with the aim to evaluate the following: 1) Cytogenetics study for evaluating the frequency of both structural and numerical chromosome variations in (NOA) cases by short-term peripheral blood lymphocyte (PBL) cultures; 2) Genetic heterogenicity of the *Sycp3* gene polymorphism by evaluating the frequency of T/C alleles. PCR-based analysis was carried out to characterize bands on agarose gel after ethidium bromide staining and bands were visualized on Gel-Doc system. Karyotype showing two new loci involving chromatid breaks 46,XY2q34, and 46,XY4q32 with the loss of DNA fragment of > 8.0 Mbp. Difference in the frequency of Robertsonian translocation (20%) and mosaicism (46,XY/47,XYY) were observed in the same karyotype. *SYCP3* polymorphism shows significant changes in the frequency of TT genotype (43.33%) in homozygous conditions (wild type) and the calculated value of

odd ratio (0.23) with a confidence interval varying from 0.08 - 0.71, suggesting an increased risk of developing infertility. To enhance the power of significance, two genotypes were combined together CC + TC which again showed significance with respect to controls. Data of the present study concludes that genetic heterogenicity of *Sycp*3 gene polymorphism and chromosomal aberrations with loss of DNA (8 Mbp) together confirm of developing risk of infertility in the NOA population.

Keywords

*SYCP*3 Gene, Male infertility, NOA, Polymorphism, DNA Fragment

1. Introduction

Infertility, emerging as globally reproductive health problem, is suffering from every aspect of society. It is a multifactorial disorder involving 8% - 12% of couples worldwide and approximately 40% - 50% of the cases are contributed by males only. The etiopathology is associated with endocrine dysfunction and genetic abnormalities to confirm failure of spermatogenesis [1] [2]. The prevalence of infertility varies in different geographical regions resulting in poor quality of semen in oligozoospermia, asthenozoospermia, and teratozoospermia patients [3]. However, ~30% of sporadic *de-novo* mutations of complex chromosome rearrangements (CCRs) and single nucleotide gene polymorphism (SNP) with epigenetic factors play a crucial role in reproductive performance in males resulting in infertility [4]. Polymorphic variants of known or unknown are distributed all over the entire genome and show the linkage between autosomes and sex-chromosomes during crossing-over followed by synapse formation have been associated with abnormal spermatogenesis and these variation shows discrepancies in the majority of the idiopathic male infertility [5] [6].

Folate plays an important role in DNA methylation and acts as "epicenter" of folate metabolism during spermatogenesis [7]. Recently, we have demonstrated the involvement of methylenetetrahydrofolatereductase (MTHFR) C677T gene polymorphism as an increasing "risk factor" in other than non-obstructive azoospermic cases in heterozygous conditions [8]. During spermatogenesis, the meiotic events are susceptible to teratogens and determinants for the quality and quantity of spermatozoa output due to abnormal chromosomal behavior at the zygotene or pachytene stage of cell-division [9]-[11]. Synaptonemal complex 3 (*Sycp*3) gene is located on a 12q chromosome with 10.5 kb DNA having nine exons (accession no. NM_153694). The deletion (643delA) of 1bp DNA fragment involved as premature stop codon and truncation of C terminal *Sycp*3 protein resulting in maturation arrest and deteriorated fertility [12]-[14]. *Sycp*3 protein regulates DNA binding to the chromatid-axis, sister-chromatid-cohesion, synapsis-formation, and recombination of gene showing their association in infertility [15] [16]. An earlier study of *Sycp*3 *T*657*C* (rs769825641, NM_001177949.2:c.657T >

C) gene polymorphism associated with recurrent miscarriage (RMC) or pregnancy loss [17] [18]. Earlier study of Zhang *et al*. (2022), recognized the slope of gene mutations involved in synaptonemal complex based on whole genome sequencing in fertility [19]. The DNA copy number variations of *Sycp*3 *T*657*C* 286 bp amplicon increase genomic instability in non-obstructive azoospermic cases [20]. The present study has been designed with the aim to evaluate the frequency of *de-novo* chromosome rearrangements followed by *Sycp*3 *T*657*C* polymorphism-associated "risk factor" in non-obstructive azoospermic cases.

2. Material and Methods

2.1. Subjects

Blood samples (5.0 mL) were collected from the period 2022-2023 after clinical diagnosis total cases were (n = 80) divided in two different studies i.e for cytogenetics (n = 50) and *Sycp*3 polymorphism (n = 30) with equal controls from the OPD in the department of obstetrics and gynecology on the basis of endocrine and semen profile to confirm NOA along with age matched controls (n = 80). The inclusion criteria for the patient study is also record to confirm the nature of disease after construction of pedigree, weather the disease is either familial or spontaneous in nature i.e. exposure with drug or radiation or chemicals. Human testicular biopsy samples were also collected to validate the same study after written consent from the patients. The study is approved by Institute Ethical Committee (IEC) vide no. AIIMS/Pat/IEC/2018/324 dated 27/12/2018, All India Institute of Medical Sciences, Patna.

2.2. Cytogenetics Study

Short-term lymphocytes cultures were set up under sterile condition with the media (RPMI-1640) supplemented with FBS (5%) and phytohemagglutinin-M containing antibiotic (penicillin & streptomycin) for 72hrs at 37˚C. Cultures were harvested after addition of colchicine (1.0 µL) to arrest the dividing cells and KCl (0.056%) solution was used as hypotonic solution. Cells were fixed in acetic acid and methanol (1:3) and well-spread metaphase plates (n = 50) were selected for karyotyping.

2.3. Isolation of Genomic DNA and Quantitative Analysis

The genomic DNA was isolated, after written consent of the patient from blood and tissue biopsy were collected, 1.0 mL & 50 mg, respectively. DNA and RNA were isolated using kit protocol (Promega, USA), after quantitative analysis was carried out by nanodrop spectrophotometer at 260/280 nm and aliquots were stored at −80˚C, till further analysis.

2.4. Polymerase Chain Reaction for Genotyping of *Sycp*3 *T*657*C* Polymorphism

In the present study, the frequency of *Sycp*3 *T*657*C* genotypes was carried out for allele (T or C) using specific forward and reverse primers for separate individual

allele analysis, after the confirmation of sequences from NCBI (BLAST/http://blast.ncbi.nlm.nih.gov./Blast.cgi) as depicted in Table 1 [18]. PCR was carried out in 25 µL of reaction volume containing 50 ng DNA, 1 µL of each 10 pmol allele specific *SYCP*3 primer (forward/reverse), 5x Green GoTaq PCR reaction buffer, 10 mM of each dNTPs and 0.2 µL of Go Taq DNA polymerase (5 U/µL). The amplification product was performed with an initial denaturation step at 95˚C for 5 min; followed by 35 sequential cycles of denaturation at 95˚C for 30 s, primer annealing at 60˚C for 30 s and extension at 72˚C for 1 min with a final elongation step of 5 min at 72˚C. PCR amplified products (286 bp) were visualized on 1.5% agarose gel electrophoresis and further characterized under Gel Doc system (Bio rad, USA), after ethidium bromide staining. The results were interpreted as TT (wild type) and CC (rare) genotype (286 bp) band in homozygous condition, while, CT genotype in heterozygous condition act as risk factor as studied by Sazegari *et al*., 2014 [18].

Table 1. PCR analysis of *SYCP*3 gene polymorphism using specific forward/reverse primers in the cases of NOA.

Subjected exon	Types	Primer Sequence (5'-3') Forward /Reverse	Annealing/ T_m (˚C)	Amplicon Size (bp)
Exon 8	F1	5'-ATGTTGCAAAAAAAAATTATGATGGAAGCT-3'	60˚C	286
	F2	5'-ATGTTGCAAAAAAAAATTATGATGGAAGCC-3'		
	R1, R2	5'-TTGCTGCTGCTGTTTCATG-3'		

T_m = annealing temperature; bp = base pair.

2.5. Statistical Analysis

Significance difference were carried out using SPSS software (SPSS v. 21, SPSS Inc-IBM, USA) to determine the frequency of three genotypes between homozygous (TT, CC) and heterozygous (TC) conditions using χ^2-test for p = values, odd ratio (O.R) and confidence interval (CI at 95% between cases and controls. Allele frequency for T & C allele were also calculated using Hardy Weinberg equilibrium between cases and controls to evaluate genetic heterogeneity.

3. Results

Table 2 shows the details variation of karyotypes and their % frequency in the case of NOA Cytogenetic analysis was carried out to evaluate the individual changes in structural and numerical chromosome variation in NOA. Structural karyotype showing frequency (13.3%) of chromatid breaks at two new different locus 46,XY2q34 and 46,XY, 4q32 with loss of DNA fragments consisting of 8.01 and 8.04 Mbp, respectively, as shown in Figure 1(A). Robertsonian translocation was observed in 20% of metaphase and mosaicism also were observed in karyotypes having 46XY/47,XYY (20%), 46,XY/47,XXY (6.6%) as shown in Figure 1(B). Interestingly, the mosaic pattern 46,XY/47,XYY had typical phenotypic characteristic feature i.e. abnormal ratio of upper and lower arms with aggressive behavior was also observed. The chromosome-1 shows "tetrad" configurations, after the fusion of acrocentric chromo-

somes belonging to D-group as shown in Figure 1(C), and Figure 1(D) showing Robertsonian translocation between D/G group chromosome. The most relevant significant findings were observed in the frequency of numerical chromosomal aberrations in the cases of NOA.

Table 2. Cytogenetic analysis showing the frequency of structural and numerical chromosome variation in the case of NOA.

S.No.	Types	Karyotypic Variations	(%) Frequency in Cases	p-value
1	Normal	46,XY	46.66	0.002
2	D/G association	45,XY, D/G association (15;21)	20.0	0.067
3	Chromatid break	46,XY, 2q34 (8.01 Mbp)	6.60	0.143
		46,XY, 4q32 (8.64 Mbp)	6.60	
4	Tetrad	46,XXY, Tetrad (Chr. 1)	6.60	0.309
5	*Numerical	47,XYY	20.0	*0.014
		47,XXY	6.60	
		46,XY/47,XYY	6.60	

*Statistical analysis (n = 50) showing significant difference ($p < 0.01$) in numerical chromosome variations with respect to controls.

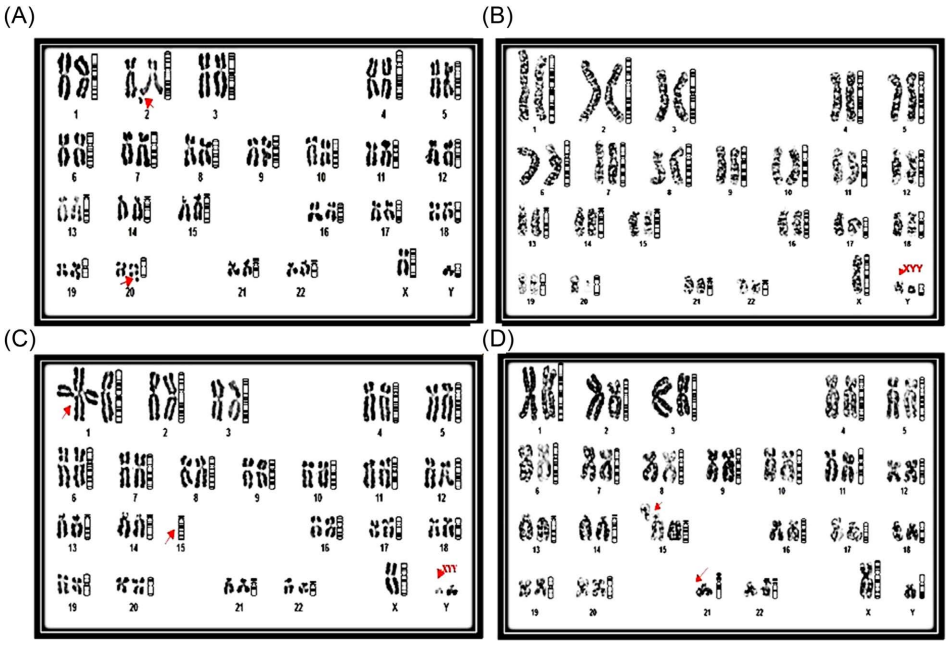

Figure 1. Representative karyotype showing chromatid break points at two different locus in 2q34 and 20q13.3 (arrow) (A), Karyotype showing 47, XYY (B), Karyotype showing 46,XY/47,XYY with rare structural variations showing formation of tetrad, after translocation of acrocentric chromosomes of D-group shift at chromosome-1 (A-group) at centromere region (C), Robertsonian translocation between D/G group chromosome (D).

Sycp3 T657C gene loci mapped on chromosome 12 (12q23.2) with 8 exons showing three different genotypes-TT (rare), CC (wild) in homozygous condition, while, TC (risk) in heterozygous conditions. PCR-based analysis was carried out for *SYCP3*

gene consist of 286 bp amplicons using allelic T/C specific forward and reverse primers, analyzed on agarose gel (Figure 2(A) & Figure 2(B)). PCR amplified products showing disappearance of band 286 bp fragment confirming mutation in the case of NOA. Hardy Weinberg equilibrium was used to determine the frequency of T and C alleles, and showing significant differences ($p < 0.01$) in T alleles between 70% of cases and (86.62%) of controls as detailed in Table 3. The frequency of *Sycp*3 genotypes (TT) also showed significant differences ($p < 0.001$) due to changes in alleles frequency cases (43.33%) with respect to controls (76.66%). Figure 3 shows significant differences ($p < 0.01$) after combining of homozygous and heterozygous genotypes (CC + CT) to increase the power of significance. Further, bar diagram of allele "C" shows a significant decreasing trend (light blue) in NOA cases, when compare with controls (dark blue), suggesting "risk factor" for developing infertility in male. The frequency of mutant allele (T → C) allele increases two folds, in heterozygous condition between cases and control. Furthermore, the same study continues in testicular biopsy (n = 3) to assess the tissue-specific genetic heterogeneity of *Sycp*3 gene polymorphism that again shows the complete disappearance of all three genotypes (TT, CC, TC) as shown in Figure 4(A) & Figure 4(B), lane-1, 2, 3.

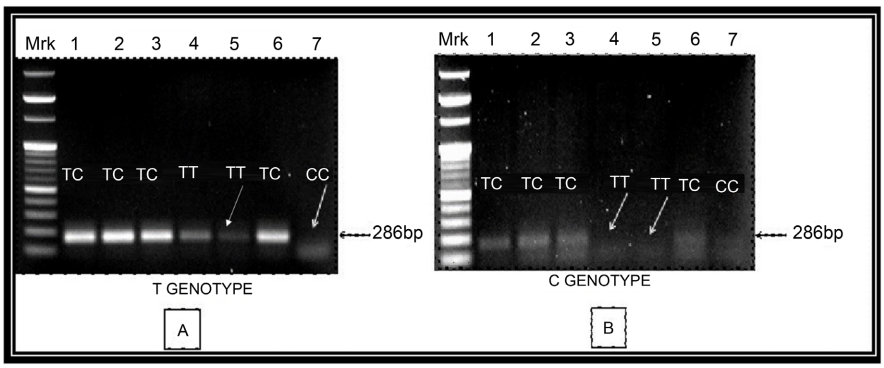

Figure 2. PCR based characterization of *SYCP*3 *T*657*C* gene (286 bp) product of T, C alleles using specific forward/reverse primers. (A) showing combined analysis of T and C allele having three different genotype L1, L2, L3 and L6 = TC in heterozygous condition, L7 = CC (rare homozygous), L4 and L5 TT (wild type) in homozygous condition (B).

Table 3. *SYCP*3 *T*657*C* gene polymorphism showing frequency of genotypes and their alleles in the cases of NOA with respective controls.

Geno types/Alleles	Number of T/C allele and % frequency		Odd ratio	Confidence at 95% Interval		Level of Significance (p-value)
	Controls n = 30	Cases n = 30		Min.	Max.	
TT	23 (76.66%)	13 (43.33 %)	0.23	0.08	0.71	**0.008
CC + CT	7 (CC = 1.0, TC = 6) (23.33%)	17 (CC = 1.0, TC = 16) (56.66%)	4.30	1.41	13.07	**0.008
T	52 (86.62%)	42 (70%)	0.36	0.14	0.91	*0.026
C	8 (13.33%)	18 (30%)	2.79	1.10	7.04	*0.026

Significant differences (*$p < 0.05$ & **$p < 0.01$) were observed between NOA cases and controls (n = 30) using Chi-square test.

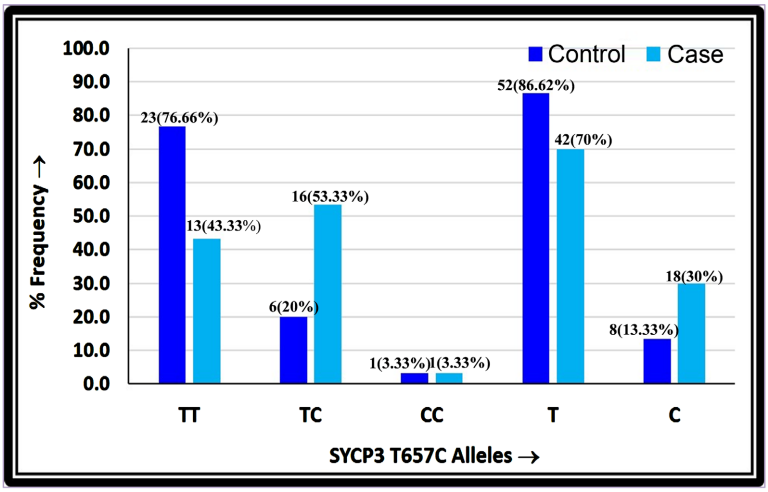

Figure 3. Bar diagram showing the frequency of three genotypes in homozygous (CC, TT) and TC in heterozygous condition and their alleles individual T&C of *SYCP3 T657C* polymorphism NOA cases and compare with their respective controls. Bar diagram of allele frequency of "C" showing significant decreasing trend (light blue) in cases and controls (dark blue).

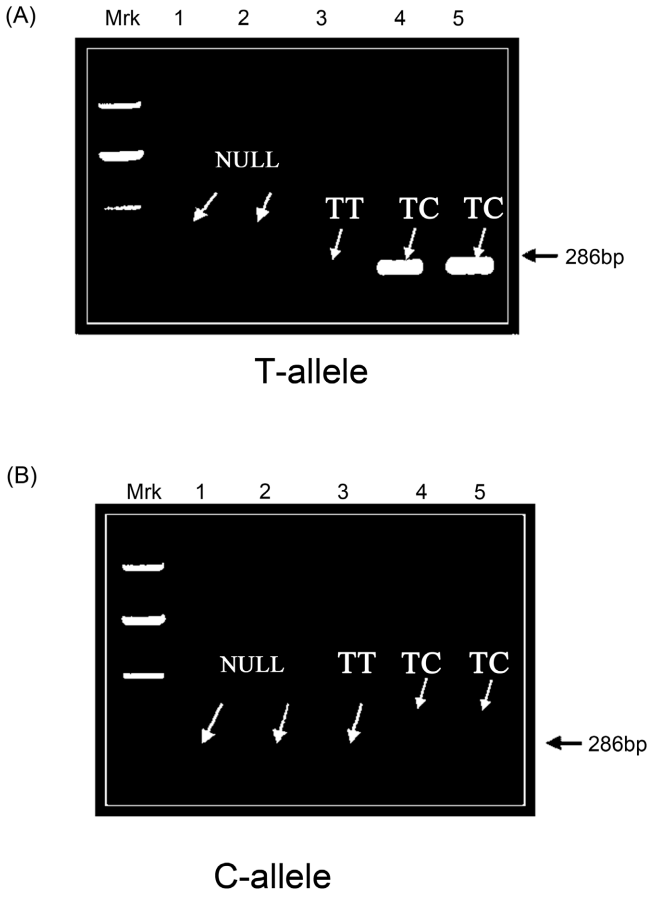

Figure 4. PCR based analysis of *SYCP3 T657C* gene polymorphism of 'T and C' alleles in testicular biopsy samples showing complete disappearance of band (286 bp) consider as mutation (arrow) in L1-L2 (A) and L1, L2, showing mutation, L3 TT (genotype and Lane 4 & 5 showing TC genotype (risk factor) (B).

4. Discussion

Human reproduction is complicated phenomenon and regulated by genetic and environmental factors. Genetic factors are dominant over the complex chromosome rearrangements (CCRs) causing interference to endocrine dysfunction resulting obstruction during proliferation of primary spermatocytes to spermatids. There are numerous reports suggesting that Y-chromosome dependent genes encodes protein essentially to regulate normal spermatogenesis followed by spermiogenesis to form single haploid healthy mature sperm cell [1]. The genes assigned on autosomes are closely linked to the normal development and differentiation of male gonads (testis) in reproductive system. Earlier study shows that chromosome abnormalities (10% - 15%) enhance the incidence of mosaicism (46,XY/47,XYY) due to non-disjunction event during cell-division in male infertility in India [21]. Significance difference in numerical chromosome aberrations might be due stability in nature makes them as genetic biomarker for the disease. Synaptonemal complexes (SCs) protein (*Sycp*3) is a major protein that play a significant role in crossing-over between maternal and paternal homologous chromosomes, where exchange of genetic information takes through 8 exons spanning 10.5Kd DNA exists outside the AZF region of Y-chromosome. The homologues pairing, synapse formation and recombination is a critical process for chromosome segregation during meiotic prophase structure. Synaptonemal complex (SC) consist of series of proteins-1, 2, and 3 (SCP1, SCP2, and SCP3). *Sycp*3 protein is specific for mammals and known to constitute the core of the lateral elements of synaptonemal complex for forming framework and attachment with other proteins. Interestingly, *Sycp*3 *T*657*C* gene mutation does not alters the corresponding amino acid (threonine), and this mutation might have disrupted the splice variant resulting origin of truncated protein that interfere with synapse formation during crossing-over in homologous maternal and paternal chromosomes [18]. The mutation of *Sycp*3 gene either alone or linked with other genes (*AKP*3 or *PLOD*3) may lead to increase the genetic susceptibility followed increase risk of infertility in NOA cases [22] [23]. The frame-shift mutation (c.643delA) in the *Sycp*3 gene has been reported in 2 out of 19 patients (10.5%) of azoospermic cases in heterozygous condition with missense mutation (c.548T>C/p.1183T) [15] [20]. In 2011, similar findings were observed by Mizutani *et al* i.e. the mutation of *T*657*C Sycp*3 gene in heterozygous condition However, the effects of *Sycp*3 mutations on the synaptonemal complex or chromosomes leading to non-disjunction in mammals have not been clarified [24] [25]. Sazegari *et al.* (2014), reported that *Sycp*3 polymorphism has been associated with an increased risk of recurrent pregnancy loss during meiosis-I and affecting pairing in homologous chromosomes. In Spanish population, there is lack of pathogenic mutations of *Sycp*3 gene in infertile men with meiotic arrest, while Turkish azoospermic infertile cases shows chromosome instability [26]-[28], suggesting these controversial findings in the literature makes the study more interesting by accumulating more sample size in different population to reduce ge-

netic heterogenicity. Considering the complexity of meiosis, *Sycp*3 gene likely to become one important candidate gene to explore the mechanism of non-obstructive azoospermic patients. Earlier study of our group shows significant association of *Sycp*3 gene mutations classified in three different forms -1) complete disappearance bands (null), 2) over-expression (up regulation), and 3) under-expression (down regulation) in the cases of NOA. Present study also demonstrates significant variations in the frequency (%) of complete disappearance (null) of two amplicons of 173 bp (8.33%) and 568 bp (12.5%) in NOA, when compared with controls consider as mutation. Further, DNA copy number variations analysis again showing significant up-regulation in both the amplicons, suggesting increase genomic instability [21]. However, the present finding confirms the association of CC/TC genotype of *Sycp*3 *T*657*C* gene even after combination of two (CC + TC) genotypes between homozygous and heterozygous condition1. Thus, present study further strengthens the association of substitution of nucleotide thymidine → cytosine (T → C) of *Sycp*3 *T*657*C* gene in increasing the "risk factor" for fertility. Furthermore, it will be interesting to correlate between cytogenetics findings and *Sycp*3 gene polymorphism. The limitations of the present study is to add more sample size to confirm mutation (disappearance of band (286 bp) and testicular biopsy data unable to analyze statistically because small sample size. Further study is required to confirm mutation data using whole exon sequencing in such patients.

5. Conclusion

Present study includes Karyotypic variation and *Sycp*3 polymorphism in the cases of non-obstructive azoospermic cases that show novel information's of two new loci assigned on chromosome 2 and 20 showing loss of gDNA fragments (>8.0 Mbp) reporting first-time in Indian population. The genetic heterogeneity of *Sycp*3 gene polymorphism supports the data of chromosome aberrations confirming a high risk of interfering with male infertility. Hence, the author concludes that *Sycp*3 may be used as a biomarker for infertility.

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Author's Contribution

AKS; contributed for design the experiments for genetic analysis, CKS, LS help for writing manuscript and S analyzed the data, while MA help for clinical diagnosis and management of the cases, while, LS contributed in execution of experiments and tabulation of data analysis.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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