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GENETIC HETEROGENECITY OF MTHFR C677T ALLELE MODULATE HORMONAL DYSFUNCTION ASSOCIATED RISK FACTORS IN THE CASES OF MALE INFERTILITY

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ABSTRACT

Male infertility is a serious problem in the developing world. Spermatogenesis is highly sensitive phenomenon due to involvement of environmental and genetic factors. The present study has been designed with the aim to evaluate the frequency of MTHFR C677T gene polymorphism, DNA copy number variations, hormone profile changes, and karyotypic alterations to evaluate the 'risk factor' in male infertility. Blood samples (n=72) were collected from clinically diagnosed cases of non obstructive azoospermia (NOA) and sever oligozoospermic patients with respective control. MTHFR C677T allele frequency and hormone profile (FSH, LH, Testosterone) was evaluated by ARMS-PCR and ELISA, respectively. Karyotypes were developed for confirmation of chromosomal aberrations as end point for genetic marker in infertility. Statistical analysis shows significant ($p<0.05$) variation in FSH, LH and testosterone in serum level in the cases of azoospermia and oligozoospermia. The frequency of CT allele shows (15.15%) genetic heterozygocity in the cases of azoospermia. Karyotype shows highest frequency of mosaicism (46, XY/46, XYY), suggesting chromosomal imbalance occurs due to non - disjunction events during meiosis I , which not only increase the frequency of heterozygocity of MTHFR gene but may also modulate endocrine dysfunction during spermatogenesis resulting in an increase of "risk factor" for developing male infertility.

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INTRODUCTION

The pathogenecity of male infertility is highly complex due to involvement of genetic and epigenetic factors. Large number of genetic factors (chromosomal variations) including microdeletion of Y- chromosome regions has been known to play significant role during spermatogenesis (1-6). There are diversified views between various group of scientist due to lack of knowledge of endocrine disruptor including hormone regulation of follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone (T) with folate metabolism in male infertility. Only 65 % cases shows confirm diagnosis based on genetic abnormalities and remaining 35% cases fall under the category of "unknown" cause of infertility (7). The physiological equilibrium of these hormones FSH, LH and testosterone are essential for the proliferation of germ cells and regulate the event of spermatogenesis in mammals. Earlier study has been shown that microdeletion of Y- chromosome regions modulates hormone profile changes in azoospermic and oligozoospermic cases (8-9). Methylenetetrahydrofolate reductase (MTHFR C677T) gene polymorphism plays a central role in folate metabolism to maintain intracellular folate "pool" required for DNA synthesis during spermatogenesis (10). MTHFR (C677T) gene has been assigned on chromosome 1p36.6 locus with gene consists of 2.2 kb having 11 exons and substitution (point mutation) of single nucleotide C → T transition (point mutation) converts a cytosine (C) to a thymine (T), resulting alanine is replaced by valine reducing stability of thermolabile enzyme. The enzymatic activity of MTHFR C677T enzyme is reduced up to 60% affecting DNA methylation followed by increase of serum homocysteine levels (11). Experimental studies further suggests that MTHFR gene mutation affecting processes of spermatogenesis due to depletion of serum folate in blood (12-13). The findings of meta-analysis of MTHFR gene C677T polymorphism are highly controversial in two group of population between Asians and Caucasians (14-15). Therefore, the present study becomes more imperative with the aims - first to evaluate the frequency of MTHFR C677T allele

to determine genetic heterogenecity, correlate with DNA copy number variations (CNVs), and secondly to correlate the karyotyping findings with hormonal profiles alterations in three different hormone profile FSH, LH and Testosterone either together or individually in two different clinically diagnosed group of infertility i.e. azoospermic and oligozoospermic with respective aged matched controls.

MATERIALS AND METHODS

Present study were carried out in preclinically diagnosed cases (n=71) of infertility along with respective age matched controls and referred from OPD of AIIMS, Patna for Genetic analysis. Blood samples were collected after written informed consent and the study was approved from Institutional Ethical Committee (IEC). The median age group was 35.4 years, non-obstructive azoospermia (n=21) and oligozoospermic (n=16) were pathologically differentiated on the basis of semen profile according to protocol of WHO guide lines (16), sperm count $>20\times10^6$/ml, progressive motility >50% and normal morphology >30% and proven fertility (with one or more children) were included as controls. Brief history of all patients were recorded on prescribed performa such as genital examination, ultrasonography, hormone profile, any history of childhood disease, environmental exposure towards drugs , chemicals such as pesticides , antenatal exposure with drug and radiation exposure might interfare to the reproductive life of an individual affecting risk factor for proband. Blood samples (5.0 ml) were collected in sterile EDTA vial after their informed written consent and used for hormone assay and DNA was isolated for MTHFR C677T polymorphism. Karyotypes were developed after using short term lymphocyte cultures in RPMI 1640 medium with Phytohematogglutinine for 72 hrs at 37^0C under sterile conditions as described previously (17). Karyotype were prepared after GTG banded technique according to the recommendations of the International System for Chromosome Nomenclature (ISCN) using applied spectral imaging software system (Genesis USA) in the cases of male infertility. Hormonal assay was carried out using standard routine laboratory procedure for ELISA with specific antibodies for determining FSH, LH and testosterone levels from serum andcompare with aged match controls.

Selection of primers for ARMS PCR for MTHFR C677T allele analysis

The primers for tetra plex real-time PCR assay were designed for genotyping of MTHFR C677T (http://cedar.genetics.soton.ac.uk/public_html/primer1.html) and further confirmed by BLAST program at http:// www.ncbi.nlm.nih.gov/blast to determine the specificity of the primers. To increase the specificity of the reaction a mismatch at the 2 position of the 3' end both the allele-specific primers were selected and further confirm their specificity by software as documented earlier in the literature (18). To obtain amplicons with distinct melting points, the '*Tm'* values were calculated using analyzer (http://eu.idtdna.com/analyzer/Applications/OligoA). The selection of the primers were based on the amplicons '*Tm'* values and following primers were used to determine the genetic heterogeneity - MTHFR-T, 5' – GCACTTGAAG GAGAAGGTGTCTGCGGGCGT-3'; MT MTHFR-C-polyG, 5' – GGCGGGCGGCCGGGAAAAGCTG CGTGATGATGAAATAGG-3'; MTHFR-cf, 5' - TGTCATCCCTATTGGCAGGTTACCCCAAA-3'; MTHFR-cr, 5' - CCATGTCGGTGCATGCCTTCACAAAG-3'.These group of tetra-primer used for ARMS PCR and SYBR Green used as fluorescent dye for *Tm* (melting-point) analysis to evaluate the frequency of mutant allele (CT) of MTHFR gene in heterozygous condition.

RT PCR procedure for MTHFR C677T allele analysis: PCR reaction was carried out in total volume (20µl), containing 10 µl of SYBR Green, (Bio red USA), 0.1 µl of each primer per reaction, 40 ng of genomic DNA was added finally the volume was maintain by sterile distilled water followed by initiation of PCR. Protocol consisted of an initially denaturation step (95 "C for 7 min) followed by amplification and quantification steps repeated for 30 cycles (95"C for 10 s, 60"C for 10 s, 72 s with a single fluorescence measurement at the end of the elongation step at 72° curve analyzed the data and reaction was terminated by cooling to 40°C. Melting curves were constructed by lowering the temperature to 65°C and later increasing the temperature by 0.2"C/s to 98°C to measuring the change fluorescence consistently. *Tm* values were assigned to develop plot generated by the RT-PCR of the negative derivation of fluorescence versus temperature (dF/dT) of the melting curve for amplification products measured at 530 nm. Primers of T plex RT PCR assay were designed for alleles 677C→T, confirmed by BLAST program belong to homeobox region for the study of MTHFR gene sequences CAGTA GGGATAACCGTCCAATGGGGTTTCCCATAAACAGGA ATGAAGTATGAACCCACGCTACCACCAGGATGAACC TGAGGACGTATTGCTGAGTGAAAGAAGTCAG TCGCA GCAGGCCGCACATGGTGTGTTTCA TTTATGTG AACG TCCAGGCAAATCCA CAGACAC AGGTACAGCC ACGT CGGAATTGTTTCATGTCGGTGCATGCCTTCACAA AG GAAAGCGGGTGGGTG GTTGCCTGGGGCCGGGGG CAGGGAGCATGAACTTCCT TCCACACAGGACCCCG CA (18). PCR product was further confirmed by agarose gel (1.5%) electrophoresis and the bands were visualized on Gel Doc system after ethedium bromide staining.

Statistical Analysis: The statistical analysis were carried out in three different hormones - FSH, LH, Testosterone between infertile cases and controls to find out significant difference ($p<0.05$) using chi square test.

RESULTS

Table -1 showing the details statistical analysis, to obtain the mean values of hormone profile changes in three different hormones - FSH, luteinizing LH and testosterone between cases of azoospermia and controls. The highest level was observed in FSH with mean value (21.22 ± 11.71) and calculated value of confidence interval (C.I.) was varying between 13.27-29.17 at 95% O.R 8.39, and the significant differences ($P<0.05$) were observed with respect to control. Figure-1A-C showing significant trend with increasing values of hormone profile changes in LH and testosterone with respect to controls. In the cases of oligozoospermia, LH shows highest mean values (22±4.47) with calculated values of C.I. at 95% varying between 11.1533 - 20.9667 and O.R (2.33) as depicted in table-2 (Figure 2A-C) and statistical analysis again showing highly significant ($p<0.001$)) differences with respect to controls. Cytogenetics study was carried out in infertile cases to evaluate the frequency chromosome aberrations for the confirmation of end point as "genetic marker" during development of high resolution GTG karyotypes. After karyotypic analysis, the significant variation in the frequency of total structural (9.74%) and numerical (65%) chromosome

Table 1. Statistical analysis showing the variation in three different hormones between azoospermic cases and control

Case Vs Control	Mean± S.D	S.E.	O.R	C.I. at 95%	p-value
LH	10.29± 2.97	6.60	1.73	8.2838 - 12.304	0.0125*
FSH	21.22 ± 1.71	40.35	8.39	13.2794 - 29.1746	0.0109*
Testosterones	13.82±3.28	4.60	1.41	11.4736 - 16.1664	0.044*

Chi square test analysis showing significant difference in LH levels (10.0 IU/mL vs. 5.94 IU/mL, p = <0.0012**), FSH level significant (1.22 IU/mL vs. 2.523 IU/mL , P= 0.0109*) , Testosterone level significant (13.82 IU/mL vs. 7.58 IU/mL , P= 0.044*), comported with control group.*Significant difference were observed (P< 0.05) between cases and their respective controls.

Table 2. Statistical analysis showing the variation in three different hormones between controls and oligozoospermic cases

Case vs control	Mean (±SD)	S.E.	C.I. at 95%	O.R.	p-value
Testosterone	5.62 ±1.74	1.943	0.0904 - 9.0496	2.544	0.0465*
LH	22±4.47	2.128	11.1533 - 20.9667	2.335	0.0012**
FSH	8.26±4.69	2.384	8.2435 - 19.2365	0.450	0.0425*

Chi square test analysis showing significant difference in LH levels (22.0 IU/mL vs. 5.94 IU/mL, p = <0.0012**) , FSH level significant (8.26 IU/mL vs. 2.523 IU/mL ,(p= 0.0425*), Testosterone level significant (5.62 IU/mL vs. 9.83 IU/mL, P= 0.0465*) in cases and compared with control group

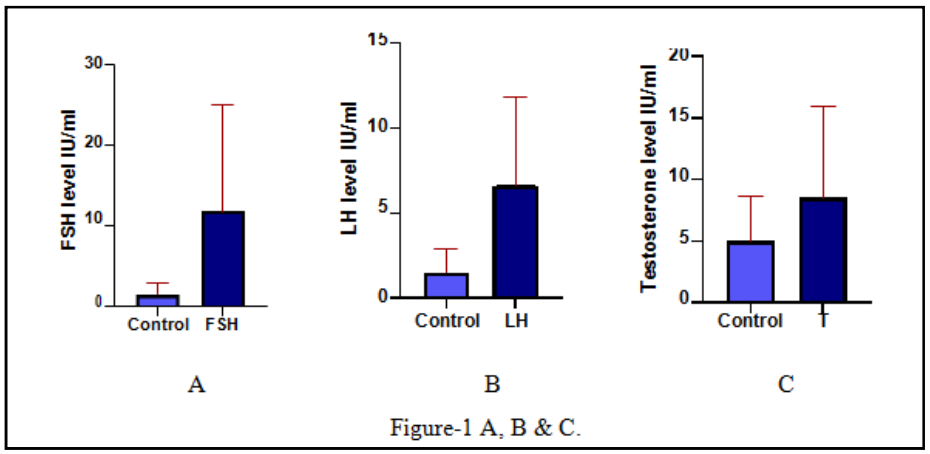

Figure 1 A, B & C. Bar diagram showing the significant variation of three different hormones (follicle stimulating hormone, luteinizing and testosterone) in azoospermic cases with respect to controls

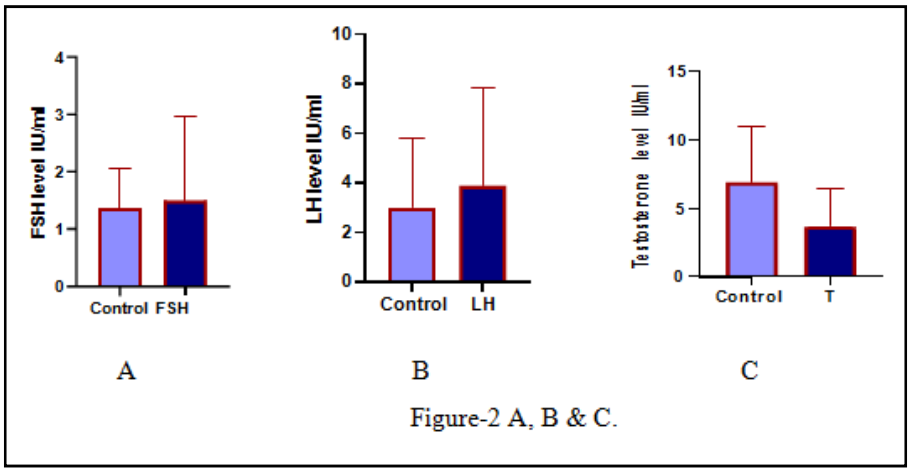

Figure 2 A, B & C. Bar diagram showing the lack of variation between two different hormone i.e. follicular stimulating and luteinizing hormone(fig.2A & B) and seems to be significant difference in testosterone (fig.2c) in oligozoospermia with respect to controls

aberrations were observed. Further analysis were carried out which show the higher frequency were observed in numerical chromosome rather than structural chromosome aberration. The study was further analyzed to evaluate the individual variation in the frequency of structural aberration, which shows the frequency of reciprocal translocation (5.58%) between D and G group chromosome was higher than chromatid break (4.16%) in the karyotypes. Similarly, in numerical chromosome aberration shows the highest frequency (42.6%) in mosaic (46, XY/XYY) karyotypes in the cases of NOA and only five cases (23.84) shows XYY karyotypes.

In the cases of oligozoospermia, there is lack of chromosomal aberrations were observed and consider as normal (46, XY) karyotypes may be due to either less sample size or unknown reason. MTHFR C677T gene polymorphism were carried out to evaluate the frequency between (CC) wild type allele and mutant (CT) allele either in homozygous or heterozygous condition, using ARMS PCR which is highly sensitive and more reliable techniques for SNP analysis (18). The methods employ four set of primers to amplify mutant 'T' alleles (two set of primers – one for identification and other for amplify the mutant amplicons 677CT or 677TT in heterozygous or in homozygous condition, while, two allele were used for wild

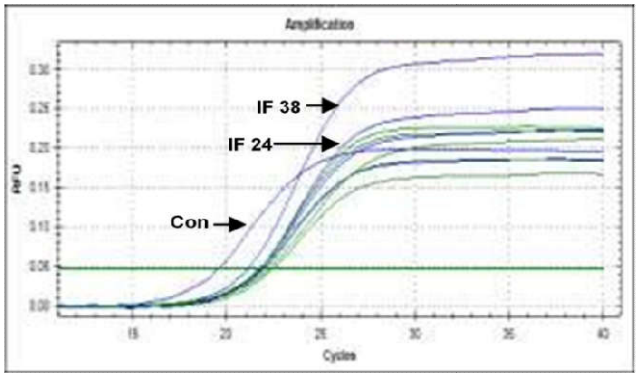

Fig. 3.A

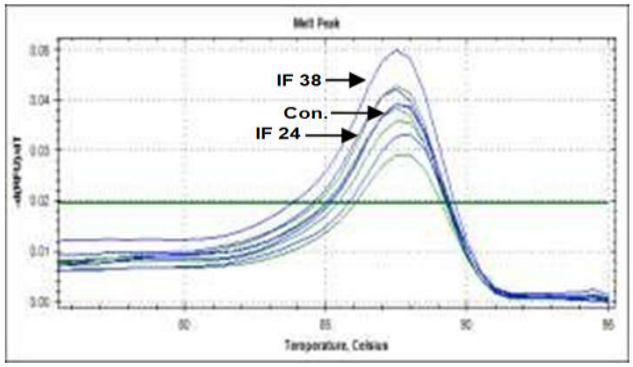

Fig. 3.B

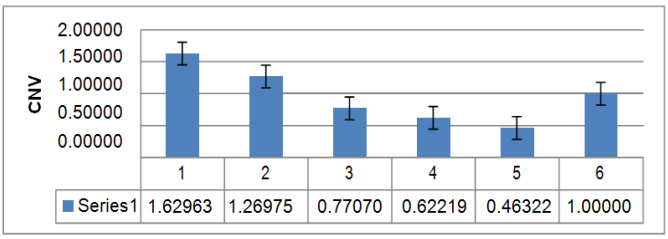

Fig. 3.C

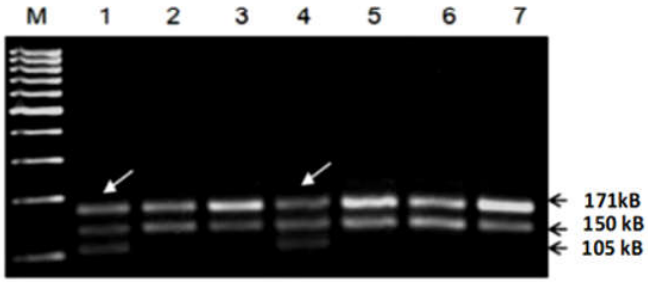

Fig. 3D

Figure. 3 A, B, C & D. RT PCR analysis of MTHFR C677T gene polymorphism in male infertile cases and controls. Fig. 3A showing Ct value of the cases (22 cycles) and control (19 cycles). Melt peak analysis showing shifting of Tm values from 87.0˚C (wild type CC allele) to 87.5˚C in mutated (CT) allele (fig.3B). Bar diagram showing a trend of down regulation of MTHFR gene expression and the copy number variations (CNVs) with respect to control as shown in fig.3C (Bar-1). PCR product further analyzed to confirmed by agarose (2.0 %) gel electrophoresis and specific amplicons of 677CC (wild type) genotype of 171 bp, 150 bp (lane 2,3,5,6 & 7) and 677CT allele (Arrow head) in heterozygous condition (105 bp) as shown in lane1 &4 shown in infertile cases (fig.3D),with controls after staining with ethedium bromide and bands were visualized on Gel Doc System (Bio Rad USA) as shown in fig.3D

type (677CC). The present study shows increase of CT values (fig.3A) and shift of *Tm* value from 87.0℃ for wild type (CC) genotype to 87.5℃ for mutant type (CT) allele in heterozygous condition (n=5) having frequency (15.15%). There is lack of shift in *Tm* value in wild type (CC) allele in (84.8%) in homozygous condition in the cases of NOA (fig.3B). Interestingly, there is lack of mutant allele (TT) in homozygous condition in both NOA and oligozoospermic case. In case of oligospermia there is lack of shift of *Tm* value and only wild type (CC) allele was observed in homozygous condition. *Tm* value are close to one another due use of 677C allele specific primer and addition of a short GC tail (primer), carry the specific genotype either CC or CT amplicons with unique shape of the melting peak as shown in fig. 3B. Bar diagram showing decreasing trend in DNA copy number variations (CNVs) of MTHFR C677T gene in azoospermic cases, when compare to controls (fig. 3C), but this trend is lacking in the cases of oligozoospermia. Statistical analysis showing the significant ($p<0.05$) variations of C.I. at 95% (19.686- 21.593) in azoospermic cases as well as in the cases of oligozoospermia C.I. values vary between 18.98-21.51 at 95% with respect to controls. These findings of RT-PCR products were further confirmed by (1.5%) agarose gel and the appearance of additional amplicons of 105 bp (lane-1 & lane-4), confirming the genetic heterogeneicity of MTHFR 677C→T allele and wild type (677CC) allele shown in lane - 2,3,5,6,7 in NOA cases (figure - 3D). In the present study we did not observe rare mutant genotype (TT) in homozygous condition in both azoospermic and oligozoospermic cases. The individual allele frequency of MTHFR C677T gene were observed using Hardy Weinberg equilibrium showing more than ten times higher value of "C" allele (0.9242) rather than "T" allele (0.0758) but statistical analysis showing ($p=0.221$) lack of significant difference between cases and controls.

DISCUSSION

Human male Infertility is multifactorial disease involving large number of genes regulating spermatogenesis. Environmental factors play an important role to influence hormone regulation and "genetic susptibility" followed by increase of "risk factor" in male infertility. Folate metabolism plays a significant role in DNA methylation during cellular differentiation and proliferation of germ cells (spermatocytes) as well as non germinal cells i.e. Sertoli cells during spermatogenesis. Over the past several years large number of controversial data has been accumulated in two different populations i.e. Asians and Caucasians regarding the mutation frequency of MTHFR C677T alleles in male infertility. In testis, spermatogenesis process is highly sensitive towards the constitutive cell division mitosis followed by meiosis leading towards the proliferation of germ cells till the maturation of single haploid cell is formed - the "sperm". The events of spermatogenesis are highly sensitive towards endocrine regulation and FSH secretion by pituitary that plays an important role for Sertoli cells proliferation. Present study shows significantly elevated level of FSH ($P<0.001$) in the cases of azoospermia and LH levels in oligozoospermia. Such differences in endocrine regulation might be due to different environmental conditions responsible for cellular susceptibility in these different clinically defined cases of male infertility (9,10). The significant variation of FSH, LH and Testosterone in the present study may also justify that genetic imbalance might interfare with spermatogenesis leading to infertility in male (2, 19, 20). However, genetic

heterogenecity of MTHFR C677T has been associated as a "risk factor" for increasing genetic susceptibility other than infertility in different pathological conditions such as aplastic anemia, neural tube defects, cancer and female infertility (21, 22, 23, 24). Therefore, findings from earlier studies on MTHFR gene C677T and A1298C polymorphisms in determining "risk" remains controversial due to variation in the frequency of alleles in male infertility. Environmental factors including dietary factors including folate supplement play an important protective role during testicular differentiation and cellular proliferation and there are also plausible explanatory mechanisms as an epigenetic factor. Hence, folate becomes essential component to maintain genomic stability in differentiating germ cells. There are still lack of factor influencing sequential event of mitosis and meiosis during spermatogenesis but certainly genetic and epigenetic variation influencing folate metabolism. Since, folate is an essential component of DNA methylation and has been associated with male infertility suggesting endocrine dysfunction occurs due heterozygocity of CT allele of MTHFR gene either due increase of mosaicism (46, XY/47, XYY) or unknown environmental factor (25). Our findings also suggest that there is variation between C and T allele frequency in heterozygous condition observed by Hardy Weinberg equilibrium test.

Figure-3C showing significant trend of down regulation of MTHFR C677T allele frequency may be due to changes in the DNA copy number variations (CNVs) followed by increase of genetic susceptibility with respect to controls. However, the synergetic effect underlying the significant relationship between MTHFR C677T and endocrine dysfunction regulating spermatogenesis regulating has been defined by Wallock et al. (26). Similarly, that MTHFR C677T polymorphism has been associated to sperm concentrations in the wild-type (CC) genotype in homozygous condition and intake of folic acid with zinc sulphate in dietary supplement may improve the life infertile patients (27). However, the cellular mechanism that maintain folate equilibrium pool in germ cells during spermatogenesis are still not clear and may required more required more focused on individual genes regulating folate metabolism. Although, present study shows the significant correlation between MTHFR genetic heterogeneity and endocrine dysfunction associated higher risk in male infertility. Earlier study of microdeletion of Y-chromosome is quite relevant to understand the mechanism of spermatogenesis because every individual (infertile proband) belongs to different genetic background and clustering of gene has been identified to play a significant role in three major AZFa, AZFb & AZFc loci coding genes are responsible for germ cell proliferation (1). The quantitative role of genetic abnormalities in men is still unexplained due to the presence of several copies of gene assigned on Yq11.23 (25, 28). 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 morphological features (29, 30). However, *De novo* mutation of Y- chromosome occurs during recombination events of meiosis involving repetitive DNA sequences and might have play an essential role in increasing of infertility in heterozygous condition (29, 30). To add further , authors also hypothesize that the mutated Y-chromosome is either transmitted as whole or a copy from father to proband in familial mode inheritance or fetus might have exposed antenatally with teratogen such as cyclophosphamide during "critical period" of testicular differentiation leading to interfare in spermatogenesis.

Conclusion

Over all, present study concludes that – 1, Genetic heterogenecity of MTHFR C677T gene in heterozygous (CT) conditions and DNA copy number variation is associated with endocrine dysfunction suggesting at higher "risk" of developing infertility in azoospermic cases, 2, karyotypic imbalance leading to increase mosaicism, suggesting increase of Y-chromosome frequency, either due to paternal mode of transmission or may be sporadic in nature. Further, disequilibrium between three different hormones FSH, LH and Testosterone increases genetic susceptibility and makes the study more complex. Hence, the author of this study strongly suggests that male infertile proband required genetic counseling before treatment to assisted reproductive techniques. Further study is required to add more samples size in different research laboratory to reach into final conclusion otherwise the study remain inclusive.

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Additional Information: The authors declare no conflict of interest.

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