Impact of the Y-chromosome gene on SOX9 stem cell expression in non-obstructive azoospermic cases

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ABSTRACT. Sex differentiation in males is a highly complex phenomenon based on synergistic interactions between genes and their coded proteins. The Sox9 gene plays a critical role in embryonic development, cell lineages, and stem cell maintenance during testicular development. Sox9 gene expression is associated with sex reversal, with a 46, XX karyotype in males and regulating normal functioning of Sertoli cells during spermatogenesis. Clinically diagnosed infertile males (n = 40) with non-obstructive azoospermia (NOA) with respective controls (n = 38) were included in the study. Blood samples were collected and after isolation of genomic DNA, the expression of the Sox9 gene (DNA copy number variations) was measured by fluorescence based quantitative PCR, deletion of AZFc locus determined by STS markers, karyotype analysis and SRY using GTG banding and FISH techniques, respectively. Cytogenetics findings revealed that more than 40% of cases had mosaicism with 46, XY/47, XYY karyotypes and a high frequency of deletion of AZFc (>70%) regions as compared to AZFb (>20%) using STS markers in the cases of NOA. The mean relative fluorescence values

of Sox9 gene expression differed significantly between cases (0.32) and controls (0.87) ($P < 0.001$) with a confidence interval at 95% of 0.0310 - 0.787 in the cases of NOA. SRY + ve cases had higher Rfv of Sox9 gene expression when compared to SRY- ve cases. There was a positive correlation between stem cell Sox9 gene expression, mosaicism and AZFc region affecting a non-disjunction event during spermatogenesis and fertility.

Key words: Sox9 gene; SRY; AZFc; Karyotype; Infertility

INTRODUCTION

Human sex differentiation and determination involve highly complex mechanisms; Y chromosome genes are responsible for testicular development and spermatogenesis (Berta et al., 1990; Koopman et al., 1991; Foresta et al., 2001). The Sox9 gene plays a critical role during sexual differentiation. The Sox9 gene is mapped on chromosome 17q24.3-q25.1 belongs to the family of Sry related genes and encodes a protein consist of 80 amino acids of HMG domains of DNA binding motif act as a transcription factor (Foster et al., 1994; Grosschedl et al., 1994). The mutation of Sox9 gene causes haploinsufficiency and XY sex reversal with infants requiring genetic analysis due to abnormal external genitalia for confirmation of diagnosis and future management (treatment). Sox9 gene mutation is involved in substitution of either chromosomal translocation or frame shift mutation at nucleotide position 1095G→AT, which alters a C- terminal transcription factor rich in serine, proline and glutamine residues indicating dominant mode of inheritance (Foster 1996; Sudbeck et al., 1996; Hsiao et al., 2006).

In humans, the most common karyotypic variation either is the translocation between X-Y chromosomes or XXY/XX mosaicism (de la Chapelle et al., 1990) associated with male infertility. The primary role of Sox9 gene is sex differentiation; in males its expression is up regulated during testicular development but absent in female gonads (Morais da Silva et al., 1996). The Sox9 gene is involved in regulation Sertoli cell - specific expression of anti Mullerian hormone for regression of ducts and male phenotypic development (de Santa Barbara et al., 1998). Sox9 gene copy number variation is responsible for sex reversal in the 46,XX karyotype (Imataka and Arisaka, 2012). We evaluated Sox9 gene expression (DNA CNVs), microdeletion of the Y chromosome, correlations with karyotypic variations and the etiopathology of male infertility.

MATERIAL AND METHODS

Clinically diagnosed infertile cases with non-obstructive azoospermia (NOA; n = 40) and aged matched controls (n = 38) referred to Department of Pathology/Lab Medicine at All India Institute of Medical Sciences-Patna (AIIMS-Patna) for genetic analysis were included in the study. The study was approved by Institutional Ethical Committee (IEC). Blood samples (2.0 mL) were collected after written informed consent from the infertile males that were categorized on the basis of semen analysis (WHO, 1992) and couples with one or more children were included as controls. Inclusion criteria were no history of childhood disease, radiation exposure or prescription of continuous drug treatment, and the

mean age of patients was 35.4 years (age group range 21-45 years). Serum samples were used for hormone profile (testosterone/luteinizing/follicle stimulating hormone) analysis using standard routine laboratory methods (ELISA) with specific antibodies. Genomic DNA was isolated for the study of Sox9 gene expression, microdeletion of Y-chromosome and chromosomal analysis in the cases and controls.

Karyotypes

Blood samples were collected under sterile conditions and short term lymphocyte cultures were set up for 72 h at 37°C, using RPMI 1640 media with 5% FBS and Phyto - M. Cultures were harvested after addition of colchicine prior 2 h to arrest the dividing cells at metaphase of the cell- cycle, KCL solution was used as hypotonic and cells were fixed in methanol - acetic acid mixture. The karyotypes were prepared according to the recommendations of the International System for Chromosome Nomenclature (ISCN, 2013) using applied spectral imaging software (Saxena and Kumar, 2019).

Isolation and quantitative analysis of Genomic DNA

Genomic DNA was extracted according to standard procedures from blood samples according to the manufacturer's recommendations (Promega kit, USA). DNA contents were measured by Nanodrop spectrophotometer (Thermo USA) and stored at -70°C till further analysis.

Microdeletion of Y- chromosome analysis using RT- PCR.

PCR was performed to analyze the microdeletion in the AZF region of the Y chromosome using specific sequences tagged sites (STS markers Sy254 and Sy267) primers F⁵GGG TGT TAC CAG AAG CTT GCA AA R GAA CCG TAT CTA CCA AAG CAG C³ and F⁵GAA TGT GTA TTC AAG GAC TTC TCG and R' TAC TTC CCT CGG GGC CTC T³, respectively of AZFc region. PCR amplification was performed in a 25µL reaction volume containing 10x Tris (pH 8.4), 50mM KCl, 25mM MgCl₂, 2.5mM dNTP, 10pM of oligonucleotides primers, 50-100 ng DNA and 1U Taq DNA polymerase and PCR condition are 95°C - 5 min (94°C - 30 s, 56°C - 45 s, 72°C - 45 s) X 35 cycles, 72°C - 5 min for SY 254 (350bp) and 95°C - 50 (94°C - 30 s, 56°C - 45 s, 72°C - 45 s) X 35 cycles, 72°C - 5 min for SY 267(102) bp, respectively.

SOX9 gene expression using RT PCR based analysis.

The copy number variation (CNV) of Sox9 gene was analyzed with quantitative PCR using SYBER green as fluorescent dye after selecting two sets of specific primer sequences forward CGGGTGGCTCTAAGGTG, reverse TTGTGCAAGTGCGGGTA (Set A) and forward AGAGGAAGCCGAGTGGT reverse GGCGGGACGGAGATAG (Set B), of 823 bp and 416 bp, respectively.

The housekeeping GAPDH was used as a reference internal gene with sequences forward-TGAAGGTCGGAGTCAACGGATTTG and reverse CATGTGGGCCATGAGGTCCACCAC. PCR amplification was performed in a 25µL

reaction volume containing 10x Tris (pH 8.4), 50mM KCl, 25mM MgCl₂, 2.5mM dNTP, 10pM of oligonucleotides primers, 50-100 ng DNA and 1U Taq DNA polymerase and PCR condition were 95°C - 4 min (95°C - 4 min (95°C - 30 s, 57°C - 30 s, 72°C - 30 s) x 40 cycles, 72°C - 8 min. The PCR products (bands) were further analyzed on 1.5% agarose gel and the bands were visualized on a Gel Doc system (Bio Rad, USA), after ethidium bromide staining.

RESULTS

The study of Sox9 gene expression (copy number variations) was carried out in the cases of NOA by evaluating RT PCR based relative fluorescence values. The mean Ct values of Sox9 were 23.76 as compared to GAPDH (18.12) as shown in Figure 1A.

Figure 1. A-D. Concentration of gradient curve and melting curve of fluorescence quantitative PCR in the cases of non-obstructive azoospermia (NOA). Amplification curve represents Sox9 gene expression (arrow mark) and GAPDH gene (arrow mark) (Figure 1A). Melting curve of Sox9 gene represented (arrow), while GAPDH gene used as internal references as shown by arrow in Figure 1B. Bar diagram showing down regulation of stem cell Sox9 gene expression (Lanes 7 & 8) in terms of relative fluorescence unit (Rfu) in the cases as compared with controls (Figure 1C). The same PCR product was further confirmed by agarose gel electrophoresis (1.5%) and bands were characterized & visualized on a Gel Doc System after staining with ethidium bromide as shown in Figure 1D.

Similarly, the mean T_m values of Sox9 were higher at 93.00 when compared to GAPDH (87.50) act as internal reference gene of homeobox region for controls (Figure 1B). Apparently, bar diagram showing that more than 56% cases have higher expression Sox9 gene (Figure 1C) as compared to controls. The mean values varied between cases (0.32) of NOA then controls (0.87), and statistical analysis showing a significant difference ($P < 0.001$) with variation of confidence interval (C.I.) at 95% 0.0310 - 0.787 using a Student-t test. The same RT PCR products were further analyzed using a 1.5% agarose gel and intensity of bands were visualized on Gel Doc system after ethidium bromide staining showing differential expression of Sox9 gene in different cases of NOA. The case numbers 7 and 8 showed down-regulation of Sox9 gene (arrow mark) as compared to control groups as shown in Figure 1D. The expression of Sox9 gene was also evaluated in the case of SRY (-ve) with 46, XY karyotype, gave a 0.406 value of Rfu and the higher values (0.636) was observed in SRY+ve in the cases on NOA. Interestingly, there was a lack AZFc & AZFb mutation observed in SRY+ve cases.

The cytogenetic analysis showed a high frequency (>40%) of mosaicism i.e. 46, XY/47, XYY observed after karyotyping in the cases of NOA. Besides, the variation of sex-chromosome anomalies, autosomal variation was also observed; i.e. one case showing the formation of ring chromosome -13. The findings of microdeletion of euchromatic region of Y-chromosome revealed again a high frequency of mutation (>60%) of AZFc locus SY267 and SY350 as compared to AZFb region (>20%) in the cases of NOA.

DISCUSSION

Sox9 gene plays an important role during sex determination in 46, XY individuals. The mutation of Sox9 gene is responsible for the cases of sex reversal due to increased gene expression and the corresponding protein (Kwok et al., 1995; Jordan et al., 2001). Cytogenetic finding reveals *de-novo* duplication of Y-chromosomes and to increase mosaicism (46,XY/47,XYY). A significant correlation was observed between microdeletion of euchromatic regions of Y-chromosome and mosaicism. More than 15% of cases of male infertility show an extra copy of Y-chromosomes, leading to variation in karyotypes 47, XXY (aneuploidy) or 47,XYY chromosomes with different mode of inheritance in the male proband (Robinson and Jacobs, 1999; Athalye et al., 2004; Ross et al., 2012). The Y-chromosome and its genes are responsible for normal testicular differentiation, and mutation of its content by either losing or gaining sequences through various actions including recombination mutation, insertion and transposition leading to interference in spermatogenesis (Robinson and Jacobs, 1999; Goncalves et al., 2017). Many studies have documented the frequency of chromosomal aberrations from 16% in male infertility (Mol et al., 2018; Laan, 2019; Oud et al., 2019), but our study also shows the variations of extra copy of Y- chromosome leading to develop mosaicism (46,XY/ 47, XYY) in more than 50% cases. Probably, this occurs due to meiotic non- disjunction event of X chromosome or anaphase lag of chromosome from a normal 46,XY or 47,XXY zygote, suggesting loss of meiotic cells at the pachytene stage, which increases the risk of developing aneuploid sperm having a extra copy of the Y chromosome. These chromosomal imbalances are responsible for failure of the spermatogenic process; the small testis becomes a causative factor for NOA cases and clinically diagnosed as Klinefelter syndrome. Interestingly, we found a high

frequency (40%) of mosaicism with 46XY/47, XYY karyotype; which differs from the findings from South India, where a very low frequency (0.5%) was reported (Saxena and Kumar, 2019). There are possible mechanisms by which these conditions might occur and there is precedence in other organisms for susceptibility the accident of non - disjunction event either in one of the two meiotic divisions during gametogenesis in one of the parents. These genetic factors occur probably due to non - disjunction events during cellular differentiation at meiosis either in zygotene or pachytene stage, leading to an extra copy of the Y chromosome resulting in unhealthy sperm and mosaicism in post meiotic events.

Hence, between a positive correlation between Sox9 gene expression and genetic variations, i.e. either an extra copy of the Y chromosome or microdeletion of a euchromatic region (*AZFc* deletion), resulting in a non-disjunction event at meiotic II developing mosaicism 46,XY/47,XYY in karyotypes. The development of mosaicism with increasing number of Y chromosome has also been reported by Clementini et al. (2005) and Mierla et al. (2014).

The present study reveals significant findings, with more than 56% cases showing down-regulation of Sox9 gene expression due to frameshift mutation might have responsible for the removal of three amino acid glutamine -proline - alanine motif from C terminus in heterozygous condition suggesting that an extra dose of Sox9 is required for normal functioning of testis (Foster et al., 1994; Wagner et al., 1994; Huang et al., 1999).

Sox9 gene map on chromosome 17, is an important member of Sox stem cell family and expressed in multiple tissue since it is testicular determinant and multiple copies has been reported during the formation of gonadal ridge and responsible for sex reversal cases i.e. 46,XY female and 46,XX Male (Georg et al., 2010). In the present study we have observe the higher expression of SRY+ve cases might be due to the microdeletion of Y- chromosome as also observed in the case of female sex reversal (Salehi et al., 2006). Sox9 gene is also associated with hypothalamic and pituitary-gonadal access and effected phenotypes and varies spermatogenic dysfunction leading to gonadal dysgenesis and development of sex reversal cases besides initiation of congenital anomalies such as absence of vas deferens premature ovarian failure has also been reported by (Jedidi et al., 2018).

In Human and mice, the Sox9 gene (stem cell) expression is essential for normal function of Sertoli cell differentiation (Foster et al. 1994; Chaboissier et al. 2004) and mutation leads to affected in SRY expression and triggering abnormal sexual differentiation. Present study based on genetic data, where more than 56% cases of NOA shows down regulation of Sox9 gene expression indicating either loss of precursors of Sertoli cells or loss of cell - population inside the seminiferous tubules during spermatogenesis. Although, the upregulation of Sox9 gene has also been reported by Lan et al. (2013). However, the mechanism by which SRY action affecting Sox9 stem cell gene expression in abnormal sexual development of gonad leading to infertility in human is not yet understood. Further, present findings indicate role of Sox9 in the cases of NOA as compared to controls in the population of Bihar region,

however, further large scale studies needs to be performed in different groups to identify its wider association with male infertility in different ethnic groups.

CONCLUSIONS

Sox9 is expressed in various kind of tissue during early embryogenesis, lineages commitment and stem cell maintenance. SRY belong to a high mobility group (HMG)-box DNA binding motif of the Sox family of transcription factors. We found a significant positive correlation of Sox9 gene expression (copy number variation) with SRY, similar to the microdeletion of Y-chromosome and mosaicism (46,XY/47,XYY). Our hypothesis is based on the above genetic findings to explore the etiopathology of infertility - firstly, SRY expression is modulated either by Sox9 gene or mutation of the AZFc locus or due to mosaicism leading to a non-disjunction event at meiosis II, either alone or in synergistic manner as predicted in Figure 2. Secondly, the increased mosaicism suggests a extra copy of Y-chromosome, which may be due to a non-disjunction event with an extra dose of heterochromatin causing disequilibrium between euchromatin and heterochromatin in the recipient proliferating germ cells. Thirdly we have to find “causative factors” responsible for unequal cell-division during spermatogenesis, and lastly a possible interaction between the sex-chromosome genes and their linkage with autosomes for normal functioning of the events during spermatogenesis. Sox9 acts as transcription factor of stem cells and in the future could be used as a “biological marker” as a source of regenerative medicine to resolve the problem of infertility. Furthermore, the characterization of Sox9 gene mutation should be performed in other laboratories to increase the population size involving different ethnic groups to make the study more applicable.

Figure 2. Hypothesis showing the etiopathology of genetic interaction between Stem cell Sox9 gene, mosaicism and deletion of AZFc region either alone or in a synergistic manner in the cases of infertility.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHORS CONTRIBUTIONS

AKS, A, RK, AK, CKS and MT are responsible for genetics analysis and development of the manuscript, MA made the clinical diagnosis of the patients.

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