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De-Novo Nucleotide Variants of Stem Cell Markers Oct4 and Nanog Increased Risk factor for Development of Cryptorchidism

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Abstract

In human, sex-differentiation is a highly complex phenomenon and if testes fail to descent in scrotum, the clinical condition known as cryptorchidism. Early transcription factors i.e. stem-cell markers Oct4 & Nanog play an important role to maintain pluripotency during embryogenesis. There is lack of genetics and stem cells interaction failed to synchronize that increase risk for the abnormal-differentiation of testes and failed to reach in the scrotum. The etiopathology is still not clear but its believe if fetus is exposed antenatal with teratogen that might be the causative factor for undescended (testes) phenomenon. Present study has been designed to evaluate the frequency of karyotypes, DNA/RNA profiles by using RT-PCR and nucleotide variations using Sangers sequencing. Findings reveal, that the highest frequency (52.0 %) were observed in numerical chromosome variations including loss of Y-chromosomes and chromatid break-point at 4q35 with loss of 7.4 Mb DNA fragment is the novel finding reported first time in the cases of cryptorchidism. PCR analysis showing disappearance of Oct4 band (577bp) and Nanog (151bp), additional band of isoform (100bp). Data was further characterize by Sanger's sequencing after exposure with cyclophosphamide (1.0 ugm/ml) in the proliferating lymphocytes cultures showing single nucleotide substitution i.e. TTT →TAA, TCA →TAA, and GGA→ TGA followed by changes in the respective amino acids-phenylalanine, serine and glycine after decoding and concludes that-1) genetics and stem cells factors together influence abnormal differentiation of testicular tissue during organogenesis. These findings were further support by nucleotide variations after exposure with cyclophosphamide, and 2) nucleotide substitution might have originates truncated proteins that interfere during descent of testis followed by increase "risk factor" for developing cryptorchidism.

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Introduction

Epidemiological study reveals that the frequency of undescended testes to scrotum varies from 1.5 to 3.5 % and remain exist either in abdominal or in inguinal region. Cryptorchidism, the most complex congenital anomalies of infant and may cause by-1) impairment of infertility due to high abdominal temperature (2-3ºC) and effects spermatogenesis, 2) increasing risk for developing testicular cancer (2-3%) and 3) developing torsion [1]. Etiopathology of cryptorchidism is idiopathic, multifactorial and has been be part of a 'testicular dysgenesis syndrome' that reduced semen quality. There are emerging evidences that cryptorchidism is regulated by endocrine dysfunction and insulin like peptide from leydig cells may influences descent of testis [2].

Hormone metabolism is also responsible for poor development of seminiferous tubules and their contents i.e. germ cells and Sertoli cells. Earlier study reveals that that antenatal exposure of cyclophosphamide (CP) cause delayed in descend of testes and significant changes in qualitative and quantitative cellular content of seminiferous tubules, including chromosome aberrations and protein-profile alterations followed by interference in reproductive performance has also been documented in the literature [3-5].

Early transcription factor plays a relevant role during organogenesis after differentiation of primary three germ layers i.e. ectoderm, endoderm and mesoderm during organogenesis. Etiopathology of sex-differentiation and further development of gonads (testis) are still not clear. Molecular biology involves to maintain pluripotentency of stem cells i.e. early transcription factors - Oct4, Nanog3 and Sox4 are responsible for initiation of migration of primordial germ cells (PGC) from secondary yolk sac to mesonephric region and to form gonadal-ridge. Anatomically, this is an undifferentiated stage of gonadal development and suddenly genetic factor become active by unknown signaling to develop testis or male first.

Earlier studies reveals that stem cell significantly associated in the variety of disease like infertility in males and neural tube defects (NTDs) maintaining pluripotency [6,7]. The transabdominal migration of the testis, shows point mutation RXFP2 genes, whereas, the alanine changes to threonine followed by changes in amino acid glycine to glutamic acid. [8,9]. These stem cell marker Oct4 and Nanog are the key regulator during organogenesis to decide the fate of differentiation of germ layers and variations in miRNA expression and DNA copy number variations, if mutation occurs due to antenatal exposure with teratogenic agents.

These agents might have increase "risk factor" for abnormal differentiation and development of male gonads. The over-expression of Nanog promotes pluripotency and self-renewal capacity during organogenesis. Interestingly, changes in expression of Oct4 or Nanog might be lost, results failed to synchronize the pluripotency during differentiation of cells. To elucidate the etiopathology of cryptorchidism, the present study has been designed with the aims to evaluate the followings-1) frequency (%) of karyotypes, 2) gene-expression of Oct4, and Nanog, 3) and nucleotide variations by Sanger's sequencing in the cases of cryptorchidism.

Materials and Methods

Cytogenetics Analysis

Peripheral lymphocyte blood (PLB) samples (2.0 ml) were collected under sterile conditions from clinically diagnosed cases (n=29) of cryptorchidism from the outpatient department (OPD) of Pediatric Surgery and referred to human genetic laboratory, department of pathology/lab medicine, All India Institute of Medical Sciences, Patna, for genetic screening. Present study is approved by Institute Ethical Committee (IEC) and after informed consent from legal guardians, samples were obtained under sterile condition for short term lymphocytes cultures for 72hrs at 37 °C using RPMI-1640 medium, 5.0 % fetal bovine serum, antibiotics (penicillin-streptomycin) and phytohemagglutinin (Gibco, USA).

To arrest the cell-division colchicine was added, two hours prior to harvest the proliferating cells inside

cultures. Pre warmed hypotonic solution (KCl) was used and the cells were fixed in acetic acid and methanol (1:3) mixture. More than ten (n=10) well spread metaphases were selected for karyotyping, after using GTG banding according to ISCN 2016 using applied spectral imaging software system (Genesis, USA) according to routine procedure. *In-vitro* cells (lymphocytes) were exposed with CP (0.01microgram/ml) for 24 hours to study of Oct4 and Nanog gene-expression and sequencing for the evaluation of nucleotide variations in cryptorchidism alone and compare with CP exposure lymphocyte in cryptorchidism cases.

Isolation of DNA, RNA and cDNA Synthesis.

Cultures were harvested, and cells were used for the isolation of genomic DNA using kit protocol (Promega, USA) and for RNA the cells were fixed in TRIzol, quantified by nanodrop spectrophotometer (Thermo Fisher). The cDNA synthesized using Go Script™ reverse transcription system at 25°C for 5 min, 42°C for 1 hour and 70°C for 15 min using (Promega, USA).

Analysis of Oct 4 and Nanog Gene Expression

RT-PCR based analysis were carried out using specific forward and reverse primers for the study of Nanog and Oct4. Total PCR protocol includes 25 µL of reaction volume containing- 1µl of cDNA, 1U Taq polymerase, 5X Green GoTaq reaction buffer, 10mM of each dNTP and 10 pmol of each primer. Amplification was performed for 35 sequential cycles, including 30 sec of denaturation at 94 °C, 1 min of primer annealing at 56 °C and 1 min of extension at 72 °C followed by 5 min final extension at 72 °C for Nanog3 gene forward 5'-CTGTGATTTGTGGGCCTGAA-3' (151 bp) and reverse 5'-TGTTTGCCTTTGGGACTGGT-3' primers, while for Oct 4 forward 5'-GACCATCTGCCGCTTTGAG-3' and reverse 5'-CCCCCTGTCCCCCATTCCTA-3' (577 bp), performed 35 cycles of denaturation- 95°C for 1min, annealing- 57°C for 1 min and elongation 72°C for 1 min followed by final elongation- 72°C for 8 min. Before the first cycle, all samples were incubated for 5 min at 95 °C. PCR products (specific bands) were analyzed on 1.5% agarose gel by electrophoresis in TAE buffer, after stained with ethidium bromide, the bands were visualized and characterized under Gel doc system (Bio Rad, USA).

Analysis of Nucleotide Variations using Sangers Sequencing

PCR product was further characterized for nucleotide Changes by Sanger's sequencing after collection of 20 ng genomic DNA from different experimental groups i.e the cells of the cryptorchidism were exposed with or without cyclophosphamide. The nucleotide sequencing data were evaluated in the form either substitution or insertion and deletions. Further analysis was carried out after decoding for respective amino acids (essential & non-essential) to evaluate the translational event during progression of disease i.e. undescended of male gonads in the fetus.

Results

Figure-1A&B showing a representative case of cryptorchidism, where the testes were failed to reach to scrotum (arrow). Only one cases showing unilateral undescended testis with small penis. The mean age groups of the cryptorchidism cases were eight years to one month with the mean size of the right is 5.5 cm and left testis 4.6 cm. The serum testosterone and dihydrotestosterone level varying 0.65 IU and 36.51 with respect to controls i.e. 10IU and 1.79IU, respectively.

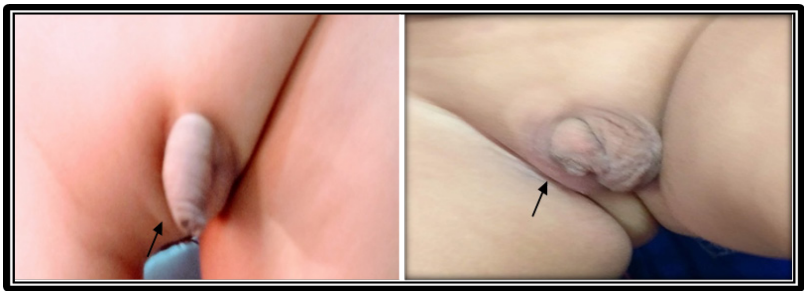

Figure 1A & B: Representative figures of clinical phenotype showing the cases of cryptorchidism cases (arrow head) showing lack of testicles in scrotum

Karyotyping Analysis

Cytogenetics analysis showing the changes in frequency of both structural and numerical chromosome variation as details depicted in table-1. Karyotypes showing the highest frequency (52.00 %) in numerical chromosome with either gain or loss of sex- chromosome such as 47, XY+ trisomy of 12 and 15. The monosomy of chromosome 16, 18 including loss of Y were observed in the karyotypes as shown in table-1. In another case ring chromosome was also observed with chromatid break (4q35), with loss of 7.4Mb DNA fragment (figure-2B), is the most relevant findings in the case of cryptorchidism. Robertsonian translocation (D/D association) was observed in case no-7 between chromosome 15 and 21. Similarly, another (case no.10), karyotype showing 47, XYY with fragile-X (Xq27) break point has not been reported earlier.

Table 1: Karyotypes Showing Structural and Numerical Variation in the Cases of Cryptorchidism.

Patient 1	1.	46, XY
	2.	46, XY
	3.	46, XY
	4.	Chr. Break 4q35 (7.4 Mb)
	5.	46, XY (“Y” Ring)
	6.	45, XY (-Chr. 18)
	7.	45, XO (-Chr. "Y")
Patient 2	1.	46, XY
	2.	49, XY (polyploidy)
	3.	45, XY (-Chr. 16)
	4.	47, XY (Trisomy 15)
Patient 3	1.	45, XY (-Chr. 18)
Patient 4	1.	46, XY
	2.	46, XY with chromatid break and monosomy
Patient 6	1.	46, XY
	2.	46, XY
	3.	46, XY
Patient 7	1.	46, XY
	2.	46, XY
	3.	46, XY
	4.	45, XY (-Chr. 18)
Patient 8	1.	47, XY (+Chr. 12)
	2.	45, XY (Monosomy 21chromosome (Robertsonian translocation)
	3.	46, XY
Patient 9	1.	46, XY
	2.	46, XY
	3.	46, XY
Patient 10	1.	45, XY (-Chr. 22)
	2.	46, XY
	3.	46, XY

Patient 11	1.	47, XYY
	2.	45, XY (-Chr. 18)
	3.	46, XY
	4.	46, XY (Fra. Xq27)

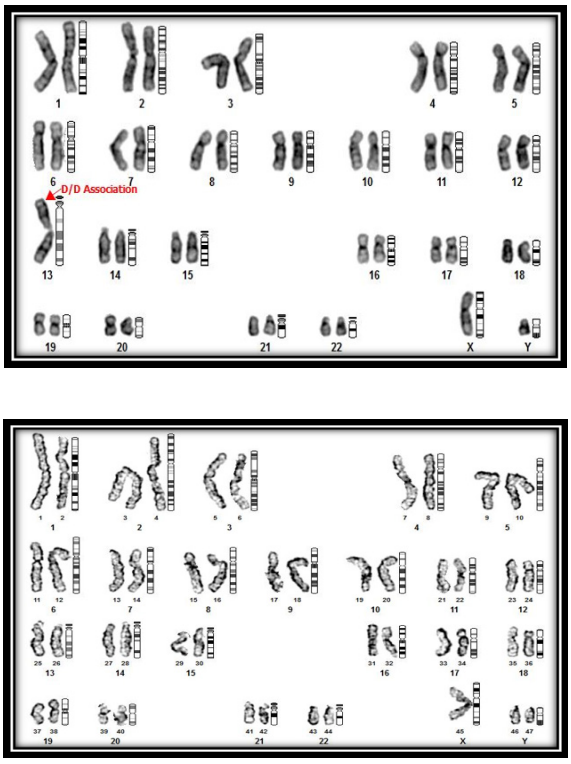

Figure 2 A & B: Representative karyotypes showing numerical (47, XYY) and structural (D/D association) in the cases of cryptorchidism

Molecular Genetic Analysis

RT-PCR based analysis showing the complete disappearance (absent) of Oct 4 (577bp) band on 1.5% agarose gel after staining with ethidium bromide staining, where, the cells were exposed with cyclophosphamide (figure-3A arrow). Nanog (171bp) showing appearance of isoform of 100 bp as shown in figure-3B (arrow). The appearance of isoform of Nanog is a unique phenomenon and its function still not known. Nucleotide sequencing data showing large number of variations like 12 substitutions 3 insertions, and deletion (1) including pretermination codon (TTT →TAA) between case and CP exposed cells (figure-4).

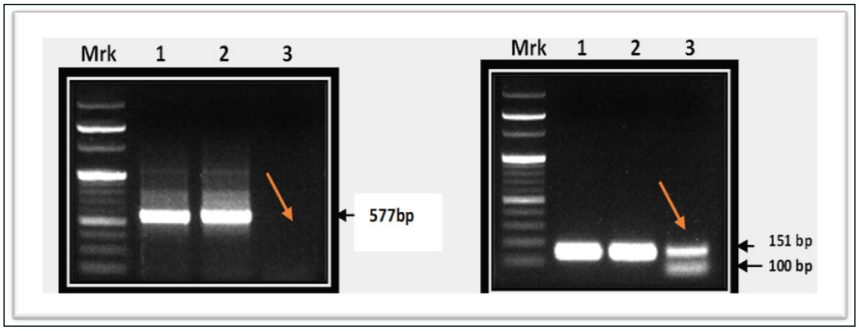

Figure 3 A & B: PCR based analysis of stem cell markers Oct- 4 (fig. 3A) and Nanog (fig. 3B) gene in the cases of cryptorchidism. Fig3A showing disappearance of Oct4 (577 bp) band after exposure with cyclophosphamide and Nanog (151bp) showing isoform of 100bp (fig. 3B lane-3 arrow) with respect to controls (fig. 3B lane 1, 2).

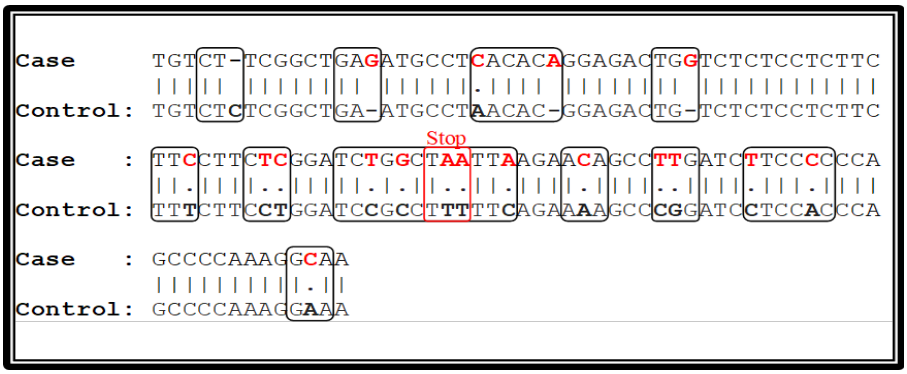

Figure 4: Comparative analysis of nucleotide variation (mutations) between cases (cryptorchidism) exposed with cyclophosphamide showing TCA →TAA (stop codon) and controls

Data was further analyzed after decoding that few nucleotide sequences fail to translates into respective essential amino acid i.e. phenylalanine, resulting might be alters translational events followed by protein-synthesis. Similarly, Nanog also showing variations in nucleotide sequences and again showing stop codon i.e. TCA TAA that fail to translates serine, a non-essential amino acid as depicted in figure-5.

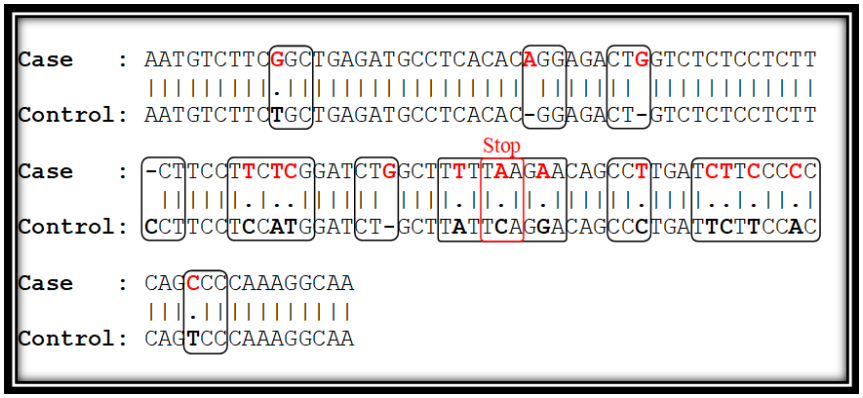

Figure 5: Nanog showing nucleotide variations in the case of cryptorchidism lymphocytes exposed with cyclophosphamide and compared with controls

Discussions

In human, undescended testes in the scrotum is a complex phenomenon due to involvement of genetics and epigenetics factors. The complex chromosome rearrangements (CCRs) play a significant role during organogenesis followed by to increase "risk factors" for the abnormal development of male gonads (testis) followed by delayed in descend of testes to reach in scrotum [10]. However, these chromosome aberrations in new born cases that lead to either unilateral or both the testes fail to reach in scrotum with different clinical conditions warn the severity of the disease that becomes challenge to the clinicians for proper management. Earlier study also showing the similar findings i.e. frequency of numerical variations (>52%) with trisomy -21 in the cases of cryptorchidism, but there is two different clinical condition (congenital heart defects) during organogenesis, suggesting, there is some "common factor" exist in the environment that influencing non-disjunction event of cell- division during organogenesis and leads to "birth defects" [11].

Present study showing interesting findings that numerical chromosome variations showing dominancy in nature over the structural changes suggesting, severity of the disease. The chromosome break point (4q35) with loss of 7.4Mb DNA is a novelfindings and reported first time in the cases of cryptorchidism and authors believe that loss of DNA might have interfere in translational process during development of testis. Human Y-chromosome play an important role for normal development and differentiation of testis, but CCRs including loss of

Y- chromosome or extra copy of Y that leads to develop mosaicism. The appearance of fragile site (fraXq27), in the case of cryptorchidism is another relevant finding, suggesting have a positive link between hormonal dysfunction (sex-hormones) and mental retardation. Earlier study of the same author shows that 13.30 % frequency of centromere breakage were observed with induced common fragile sites expression involving chromosome 2q21,3p14 and 7q11, after in-vitro exposure with CP in Down syndrome patients [12]. Karyotype showing 46XY+18 were observed in more than 60% of cases of cryptorchidism.

The sequencing data is highly relevant to evaluate de novo mutations like substitution, deletion or insertion. In-vitro model, showing variety of nucleotide sequences in the case of cryptorchidism, where the proliferating cells were exposed with CP and unexposed consider as controls showing stop codon i.e. TTT →TAA, fails to translate essential amino acid phenylalanine, suggesting either to inhibit essential protein that required for normal development of testes or truncated protein that might have interfere for cryptorchidism phenomenon. However, findings of the sequencing data is interesting and further required in other labs to confirm the role of pretermination codons in the cases of cryptorchidism. Earlier studies also support our findings to validate that CP cause DNA damage and significant changes in nucleotide sequences followed by change in translational event i.e. proteins-synthesis [13-15].

In human, early transcription factors Oct4, Nanog and Sox4 genes decides the fate of germ layers during normal physiological function and maintaining pluripotency during organogenesis. Interestingly, present study showing the disappearance of Oct4 and isoform of Nanog, suggesting poor signaling for differentiating germinal (gonocytes) or non-germinal (Sertoli) cells inside the somniferous tubules, resulting failure to maintaining pluripotency during ontogenetically development of testes. Earlier study also revels the disappearance of Oct4 in tumor cells, suggesting failure to maintain pluripotency during progression of disease [16,17]. Present study of Nanog showing increase sensitivity either because of appearance of isoform (100bp) and nucleotide variations i.e. deletion or substitution sequences TCA→ TAA and GGA →TGA, that failed to transcribe to translational event with respective essential amino acids (phenylalanine) consider as pretermination codon or stop codons, exploring new insight knowledge of stem cell biology associated risk factor for abnormal differentiation/development followed by interference with reproductive performance during onset of adult life.

Conclusion

Present study concludes that-1) genetics and stem cell factors are responsible for failure of pluripotency during proliferation of both germinal and non-germinal cells. 2) Nucleotide variations in the sequences of Oct4 and Nanog supporting above that data of clinical consequences of undescended and, 3) CP used as an in-vitro model to induce mutations confirming risk factor for the abnormal differentiation and development of testes that leads to develop cryptorchidism.

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Authors' Contributions

AKS is designed and Shalini implemented for data analysis while Amit Sinha helps for clinical diagnosis.

Ethics Approval and Consent to Participate

Study was approved by Institute ethical committee of AIIMS Patna (AIIMS/Pat/IEC/2020/425), and informed consent form was signed by guardian.

Competing Interests

There are no competing interests between the authors

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