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Impairment of *Nanog3* Stem Cell Dysregulation Associate with Male Infertility in Human

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Abstract

Stem cells have capacity to differentiate into a variety of cell types. The inner cell mass of epiblast and primordial germ cells (PGCs) are pluripotent in nature. *Nanog3* has been identified as a pluripotent transcription factor belongs to homeobox family of protein required for the survival of primordial germ cell differentiation. Spermatogenesis is associated with highly specialized kind of germ cells (spermatocytes) to form mature single sperm that contribute to the formation of totipotent zygotes any defect in stem cells can lead to infertility. Since, *Nanog* is a regulatory factor hence the present study has been carried out to evaluate a novel role of *Nanog* in male infertility and their association with stem cell dysregulations. Blood samples were collected from the patients of azoospermic (absence of sperm) patients and genomic DNA was isolated subjected to PCR based analysis were carried out using specific forward 5'CTGTGATTTGTGGGCCTGA3' forward and 5'TGTTTGCCTTTGGGACTGGT3' reverse primers of *Nanog3* gene. Interestingly, 8.33% cases revealed a complete disappearance (null) of 151bp length fragment of *Nanog* (deletion), while 25% overexpression (up regulation) when compared with the normal healthy fertile individuals act as controls. Present study concluded that "mutation" of *Nanog3* interfere the process of spermatogenesis either in synergistic manner or with other stem cells (Oct4 or Sox) and increase "risk factor" in male infertility.

Keywords: Stem cells; Azoospermia; *Nanog*3; Male infertility

Introduction

Infertility is defined as the failure of a couple to become pregnant after one year of regular, unprotected, sexual association. Approximately, 15-20% of all cohabiting couples are affected by infertility during their reproductive lives. Infertility is not "just a female problem" as there is a male infertility component in approximately 50% of couples, suggesting that nearly 7.5 to 10% of all men in the reproductive age group are infertile. Variety of factors that includes infections, injury, sexually transmitted diseases, idiopathic-cause, chemotherapy, systemic illnesses, endocrine and familial disorders, genetic and others are associated with infertility. Recent advances have suggested an important role of stem cells in male infertility given its importance in spermatogenesis [1]. Spermatogenesis is a highly complex process where stem cells or spermatogonia, either divide to reproduce themselves for "stem cell" renewal or they divide to produce daughter cells that will later become spermatocytes [2]. Stem cells are responsible for clonogenic proliferation supported by self renewal and differentiation of spermatogonial stem cells (SSCs) during spermatogenesis.

Nanog, a homeodomain containing transcription factor and in combination with Oct4 and Sox2 act as both activator and suppressor of multiple targeted genes responsible for self renewal and differentiation [3]. Although, in human, a verity of stem cell markers have been identified which are required for cell proliferation to maintain pluripotency including Oct4 (octamer-binding transcription factor 4), SRY-related HMG-box (Sox) 2, c-Myc and *Nanog* [4]. These are gene products and acts as transcription factors essential for self-renewal of undifferentiated embryonic stem cells. *Nanog3* is a homeobox transcription factor and a key player to maintain pluripotency i.e., the ability of a cell to differentiate into any fetal or adult cell type [5]. Further, studies have suggested that *Nanog3* is dispensable for the initial differentiation and reprogramming of germ cells. Although, the mutation of *Nanog* fails to explain pluripotency and might be the cause of failure of production of sperms during spermatogenesis. Thus, the study of stem cells in infertile patients becomes imperative to understand the correlation between the genetic components (gene regulation) of stem cell and their correlation with testicular dysgenesis.

Materials and Methods

In the present study, blood (3 ml) from the clinically diagnosed male infertile patients and the healthy controls were collected after written consent. The samples were collected in containing EDTA vials (anticoagulant). The inclusion criteria of the patients based on a clinical diagnosis of sperm count present in semen that confirmed azoospermia. A total of n=12 male infertile patients suffering from azoospermia and n=20 fertile male act as controls (confirmed having normal sperm counts and normal children) were included in the study. The age groups of the patients were between 18-34 years of age. The study was approved by the ethicical committee of the Institute. Genomic DNA was isolated from whole blood using a Bioner kit (Korea) and samples were kept at -20°C until further analysis. PCR analysis was performed for the identification and characterization of *Nanog* using forward 5'-CTGTGATTTGTGGGCCTGAA-3' and reverse-5'-TGTTTGCCTTTGGGACTGGT-3' primers. A total volume of 25 µl containing 50-100 ng DNA, 20 pmol of each primer, 200 µM

of each dNTP with Taq buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 3.0 mM $MgCl_2$, and 3 U Taq polymerase (New England Biolabs). Cycling conditions were 4 min at 94°C for initial denaturation, 56°C/30s of annealing, followed by a final extension of 35 cycles at 72°C for 5 min. PCR products were characterized on 1.5% agarose gel, stained with ethidium bromide and bands were visualized on the Gel Doc system (SR Biosystem).

Results and Discussion

A role for *Nanog* gene was studied in male infertile patients as well as healthy controls. The levels of *Nanog* were studied using specific markers for *Nanog* gene on the basis of the visualization of bands (gene products), their signal intensity to characterize specific amplicons for over expression (up-regulation), regression (down-regulation), and complete disappearance of band (null/deletion). Findings were repeated three times to confirm the genetic diversity (Table 1). Statistical analysis was carried out using the χ^2 test for comparison of controls and infertile male patients to determine the level of significance. Our results indicated a significant change in the levels of *Nanog* among male infertile patients compared to the controls. The complete disappearance (deletion) of band of 151bp of *Nanog3* in male infertile patients with a frequency of 8.33%, seems to be quite interesting has not been reported earlier (Figure 1). Furthermore, frequency of up-regulation of *Nanog* was 25%, whereas down-regulation frequency was 16.66% as compared with the controls. Apparently, our results identifying a dysregulation of *Nanog* in the samples of azoospermic patients in comparison with the normal healthy controls, suggesting a positive association between stem cell gene mutation and infertility. Because *Nanog* expressed during embryonic stem (ES) cells and embryonic germ (EG) cells and loss of *Nanog* results confirmed interference with spermatogenesis. It has been identified that *Nanog* maintains pluripotency [5], hence the present study indicate that loss of *Nanog* (null) in infertile patients, may result in loss of pluripotency results down regulation or up regulation. However our findings similar to the research work published by Zaehres et al., [6] suggesting any defect in stem cell either failed to maintain self renewal during differentiating stage or failure of spermatogenesis as observed in 8.33% cases showing complete loss of *Nanog3*. On the other hand, it has been shown that the over-expression of ESC genes, including *Nanog3* suppresses differentiation of ESCs [7]. Thus, upregulation as well as down regulation of *Nanog* may lead to defect in stem cells (spermatogonia), as a result whole of the spermatogenesis process disorganized leading to infertility. It is interesting to note that *Nanog3* gene regulation was highly variable in nature amongst the population of male infertile patients sperm during spermatogenesis [8] suggesting the mimicry of stem cells may be different due to different cell lineage (spermatogenesis) or interaction with other of stem cells such as Sox 2 or Oct 4. However it has been confirmed with the present study with other group of study suggested that *Nanog3* play an essential role during spermatogenesis [9]. Although, our research group also published similar findings that *Nanog3* dyregulation in neural tube defect (NTD) cases and Acute Myeloid Leukemia [10,11]. Present study is small but promising to characterized and identify an important linkage between infertility and stem cell. However, future studies are required by share large sample size in different ethnic group of infertile cases to confirm cellular genetic heterogenity.

Markers	frequency (%) of stem cell expression		
Expression	Deletion	Up regulation	Down regulation
Nanog (%)	8.33	25.00	16.66

Table 1: Frequency of stem cell (*Nanog*) changes in azoospermic cases.

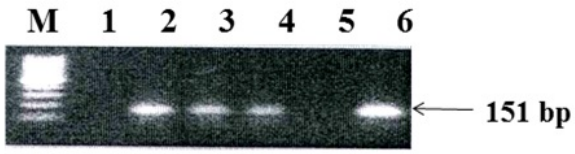

Figure 1: Amplified PCR product of 151bp of *Nanog* analyzed on (1.5%) agarose gel visualized after ethidium bromide stained. Lane M-100 bp marker; Lane 6 is showing up regulation (over expression); lane 1 and 5 showing complete disappearance/deletion of band; lane 3 and 4 have down regulation, while lane 2 showing normal appearance of *Nanog*3. The present study repeated three times to confirm the appearance of bands with respect to controls.

Conclusion

The exact cause of male infertility is still not clear but it is believed to involve multivariate genetic and epigenetic factors. Study of stem cells in infertile male patient's gives new enlightenment for therapeutic in regenerative medicine. The variations of *Nanog3* in the present study are due to (1) heterogeneous (spermatogenesis) group of cell population, (2) severity of disease, and (3) fail to maintain autopluripotency.

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