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Research Article

Genetic interaction between Methylenetetrahydrofolate Reductase C677T Gene Polymorphisms and stem cell associated "Risk Factor" in male infertility

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Abstract: Male infertility is the major multifaceted disorder with several genetic and environmental factors that contribute to the impairment of spermatogenesis.Epidemiological studies reveals that genetic and environmental factors are responsible for the development of male infertility. During spermatogenesis large numbers of extrinsic and intrinsic factors are responsible for the formation of sperm. Later these factors characterize as " MTHFR and stem cells gene" , both are associated independantly or dependantly in the maintainance of pluripotency during differentiation of germ cell.The aim of the study was to examine the association between MTHFR C677T and Nanog3 , Oct4 & Sox2 gene (s) in etiopathology of male infertility. PCR and RFLP based DNA analysis with selected specific forward/reverse primers. Interestingly, the highest frequency (0.6%) of CT assosited with the high frequency (0.96%) of over-expression for Nanog3observed in male infertility. However, MTHFR mutation is directly linked to the genetict diversity of stem cell gene in the male infertility
Keywords: MTHFR, Stem cell; Oct4; Nanog3; Sox2 and male infertility.

INTRODUCTION

Male infertility is characterized by genetic damage in the germ line is responsible for impairment of the male reproductive system. It may be defined as conception is not completed after one year of unprotected intercourse [1]. The epidemiological study suggests that prevalence was reported by WHO in 1987 i.e., 10-15% while approximately, 70% and 30% male infertility affected due to etiological and unknown factors [2].Microdeletions in the AZF (azoospermia factors) of the Y chromosome show the variations in the sperm counts of different populations whereas the interaction between "gene – gene" and "gene – environment" (nutrient foliate) are more prominent for individual susceptibility which influence the spermatogenesis [3]. However, the genetic damage appears in the male germ cells that are directly linked to pluripotency, folate metabolism and also cause cancer in the offspring as well as in the next generations. The origin of pluripotent cells is straightly associated to spermatogenesis; the production of sperm cells and affected from neonatal and adult human testis [4-7].

Folate is essential compound for all the cellular activity and also important for reproductive sysytem. The 5,10- methylenetertahydrofolate reductase (MTHFR) is a major circulating form of folate in blood. MTHFR (methylenetetrahydrofolate reductase) regulate folic acid metabolism through conversion of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. Mutation in MTHFR which is a thermolabile gene, consist of a cytosine (C) to thymidine (T) substitute at nucleotide position 677, leads to the exchange highly conserved alanine to valine resulting decline folate supply. The genotypic variants of MTHFR C -T lead to increase homocysteine subsequently,lead to hyperhomocysteinemia, are associated to cause abnormal spermatogenesis and infertility, [8-12] however, MTHFR play important role in the spermatogenesis .

Oct4, Nanog3 , Sox2 gene and a number of other gene are associated with pluripotency of cell [13]. However, the proper functional activity of these genes are still unclear in germ cell differentiation but the report have been showed the Oct4 and Nanog are involve in the implication of germ cell survival and differentiation [14-15]. Spermatogenesis is a multifarious regulated process in which a germ-line stem cells develops in the form of spermatozoa called spermatogonial stem cells (SSCs) which found in the basal compartment of the seminiferous epithelium. SSC have self-renewal property and ensures the maintenance of the stem cell pool and generate the large number of germ cell. Consequently, a equilibrium is essential for normal spermatogenesis and fertility, between SSC self-renewal and differentiation in the adult testis.Hence, In the present article we have hypothesied how the folate is essential for the maintance of germ-line stem cell and what the impact of their present or absecnce in the male reporductive system [16]. However, we have focused over the interaction which exist between MTHFR C677T gene and different three stem cell marker (Oct4,Nanog3 and Sox2) in the impairment of spermatogenesis lead to male infertilty.

MATERIALS AND METHODS

Collection of blood sample

Blood samples (1.0ml) of azoospermia patient (n=12) were collected for molecular genetic analysis from the O.P.D of S.S Hospital, I.M.S, B.H.U, SRM Hospital and Genome Foundation with different age groups-21 to 65yrs along with their respective controls (n=20), after written consent of patients/attendant. The criteria for inclusion of an individual were based on clinically diagnosed azoospermia patient. This study was approved by ethical committee of the Institute and samples were kept at -20°C, till further study.

Isolation of DNA and PCR based RFLP analysis

Genomic DNA was isolated from the whole blood using Bioner Kit (Korea). The details of forward & reverse primers for MTHFR C677T, Nanog3, Oct4, Sox2 and their sequences were documented in (Table 1). We have developed PCR specific strategies in total volume of 25 µl contain 50-100 ng of DNA , 20 pmole of each primer, 200 µM of each dNTPs mix with Taq buffer (10 mM Tris HCl pH 8.3, 50 mM KCl), 3.0 mM MgCl2 and 3 unit of Taq polymerase (New England Biolab).

RFLP analysis was carried out for the study of MTHFR genotype variants. PCR product (6 µl) were digested at 37ºC for 3hr. in reaction volume of 25 µl containing 1U of Hinf-I restriction enzyme (New England, Biolabs) respectively and NEB buffer (2.5 µl). The digested product of RFLP was separated on 3% agarose gel respectivly, stained with Et.Br bands were visualized and characterized on Gel Doc system (SR Biosystem).

Table 1: MTHFR C677T and stem cell markers with their specific primer sequences used for PCR strategy in male infertility

Stem cell markers	Sequences (forward & reverse)	Base pair (bp)	Annealing Temperature	References
MTHFR C677T F C677T R	5'-TGA AGG AGA AGG TGT CTG CGG GA-3' 5'-TGA GAG TGG GGT GCA GGG AGC TT-3	195	58 ºC/1min	[17]
Nanog3 F Nanog3 R	5'-CTGTGATTTGTGGGCCTG AA-3' 5'TGTTTGCCTTTGGGACTGGT-3'	151	56 ºC/30sec	[18]
Oct4 F Oct4 R	5'-GACCATCTGCCGCTTTGAG-3' 5'CCCCCTGTCCCCCATTCCTA-3'	577	60 ºC/1min	[19]
Sox2 F Sox2R	5'-GGCAGCTACAGCATGATGC-3' 5'-TCGGACTTGACCACCGAAC-3'	236	60 ºC/30sec	[20]

Statistical analysis

The significance ($p<0.05$) differences between cases and controls group were evaluated using x2 test. Statistical analysis was further carried out to compare the expected and observed value by using - ratio was computed at 95% confidence interval to evaluate the "risk factor" between cases and controls.

RESULT

Evaluation of MTHFR C677T gene polymorphism in male infertility:

The statistical analysis were carried out showed significant ($p<0.05$) differences with respect to controls using chi square test. Table-2 depicted the details findings of RFLP analysis of MTHFRC677T gene polymorphism in infertility men cases and their respected controls. The present study shows variable frequency of genotypic variants i.e. CC genotype in (0.48%) (wild type) and CT genotype in (0.60%) heterozygote condition , where as in controls the genotype frequency varies in wild homozygous condition 3.60% (CC) and 0.20% in heterozygous (CT). The mutant homozygous TT (rare type) genotype was 0.36% present in cases and in controls 0.20%. The frequency of heterozygote CT genotype increase significantly ($p<0.010$) as compared to controls when analyzed statistically using chi square test.

Nanog3, Oct4 and Sox2 gene regulation in male infertility and controls:

We observed that stem cell markers play a significant role in the screeing of mutational spectra in male infertiliy. Our findings were quite considerable regarding to stem cell markes involved in the regulation of spermatogenesis. Our observations were based on the appearance of bands and their signal intensity as overexpression, down-regulation and complete disappearance of band (null) in patients, when compare to the same way in controls. The findings of present study were repeated three times to confirm the genetic diversity of stem cell as documented in Table 3.

The over expression , down-regulation and complete disappearance were evaluated for Nanog3 (151bp) .The variable frequency of over expression (0.96%), down regulation (0.36%) and complete disappearance (0.0%) in Nanog3 were observed, which showed significant decreasing trend of gene mutation in patients,OR at 95%C.I. showed highly significant differences ($P < 0.001$) in overexpression by using the chi-square test between cases and controls. Similarly, we observed the overexpression and down-regulation (regression) of Oct4 gene (577 bp) in male infertility cases and controls but Oct4 gene mutation shown the

highest frequencies in down-regulation (0.72%%) as compare overexpression (0.48%) and complete disappearance (0.12%%).OR and 95% C.I. also showed the significant value of overexpression : OR = 9.5(95% C.I. 0.7-63.5) and down regulation5.6 (0.8-43.17) with P = 0.03in both regulation. Sox2 muation we observed that result was quit be closed to the finding of Nanog3 in male infertility. The overexpression (0.48%) was little higher than down regulation (0.36%) whereas complete disappearance was not find as like Nanog3.OR = 9.5(95% C.I. 0.7-63.5) in overexpression5.6 (0.8-43.17) with P = 0.03, as despied in table -3.

Table 2: MTHFR C677T gene showing variable frequency of genotypes between homozygous and heterozygous condition and their individual's allele frequency in infertile male cases with their respective controls.

Genotype	Cases	Controls	O.R at 95% C.I cases vs. controls	p-value
	% frequency			
CC	4(0.48)	18(3.6)	0.056(0.00-0.47)	0.001**
CT	5(0.6)	1(0.2)	13.37(1.1-369.3)	0.010*
TT	3(0.36)	1(0.2)	6.3(0.4-183.9)	0.098
C	0.5	0.9	NaN	NaN
T	0.4	0.07	NaN	NaN

* Significant difference (p<0.05) were observed using chi square test between cases and controls. ** highly significant, NaN= Not Observed

Table 3: Stem cell markers showing variable (%) frequency and O.R. at 95% C.I. between infertile male cases and controls

Types of Stem cell markers	Case	Control	O.R at 95% C.I. cases vs. control	p-value
	% frequency			
Nanog3				
Up regulation	8(0.96)	2(0.4)	13.37(1.1-69.3)	0.001**
Down regulation	3(0.36)	1(0.2)	6.3(0.4-183.9) NaN	0.09
Null/Absent	NaN	NaN		NaN
Oct4				
Up regulation	4(0.48)	1(0.2)	9.5(0.7-63.5)	0.03*
Down regulation	6(0.72)	3(0.6)	5.6(0.8-43.17)	0.03*
Null/Absent	1(0.12)	0(0.0)	inf(0.09-inf)	0.30
Sox2				
Up regulation	4(0.48)	1(0.2)	9.5(0.7-63.5)	0.03*
Down regulation	3(0.36)	1(0.2)	6.3(0.46-183.9)	0.09
Null/Absent	NaN	NaN	NaN	NaN

*Statistical analysis showing significant differences p< 0.05 using chi square test, ** highly significant, NaN= Not Observed

DISSCSSION

Male infertility is a heterogeneous disease because several genetic and environmental factors are invovled in the impairment of spermatogenesis. In present study we evaluated four different genes (MTHFR,Nanog3,Oct4 & Sox2) with important roles in the spermatogenesis. We compared the distributions between MTHFR C677T, Nanog3,Oct4 and Sox2 mutation among 12 men diagnosed with defect spermatogenic process and 20 controls whose normal fertil individuals. Male infertility have complicated aetiology influenced by interaction of "gene-gene" and "gene-environment" (nutrient). Several study have shown the different folate related genes are significantly associated with risk of development of male infertility . Although enzyme involved in the folate dependant homocystine pathway are strongly implicate spermatogensis, because several candidates, wide ethnic variation & population diversity have difference in allele frequency of their polymorphic variation,therefore, environmental factors are also responsible for male infertility. 5'-10 MTHFR is specifically involved in the folate metabolism and it is strongly associated with increased to risk for male infertility . MTHFR C677T homozygous for the thermoability of enzyme is susceptible to infertility risk [17-18] because gene could eithe inhibit DNA replication or fail to faciliate DNA repair mechanism. In this study we observed that deficiency of folate impairs the activity of enzyme which involved in the folate metabolism and instantaneously, increases the level of homocystine and decreases the formation of sperm as compare to normal individuals. Thus, elevated homocystine are independent nutitional factor for

infertility [17]. The present study have shown the significant difference between heterozygous CT of male infertility and normal controls whereas rare homozygous TT are non significantly associated with risk of infertility due to small sample size . However, allele T have high prevalence as compared to C in patient,therefore,allele T are risk of male infertility either independently or dependently.

We were also observe the genetic diversity of stem cell and their relation with MTHFR gene mutation or nutritional deficency . We perceived a quite interesting result with variable expression of stem cell markers in clinically diagnosed male infertility, which has not been still documented,this first time evaluated by author. The specification of cell lineages in the developing germ cell is thought to be regulated by either extrinsic or intrinsic factors. In human, Oct4, Nanog3 and Sox2 genes are the key regulators of transcriptional activity during organogenesis [18-22]. The sequential changes in the expression (over-expression or down-regulation or complete disappearance) of stem cell gene are able to maintaining pluripotency in the reproductive organ ; maybe lead to modify the whole system of discrepancy of developing germ cell during spermatogenesis. The present finding of over-expression in Nanog3, Sox2 & Oct4 has been observed in male infertility with significant difference between cases and controls and suggesting this is due to lack of nutrition or mutation in MTHFR gene. We also observed these transcriptional factor were down regulated or deletion (complete disappearance) results in obstruction of development of germ cell and lead to defect in spermatogenic condition. Interestingly, we observed when Nanog 3 & Sox2 ,over expressed Oct4 was downregulated with significant difference between cases and controls (p=0.03) and except Nanog3 & Sox2, only Oct4 have shown deletion. In the present study the high frequency of heretozyogous condition (CT) assosited with modify expression of stem cell markers in the impairment of spermatogenesis.Finally, it is clear from the present study that MTHFR gene is associated with stem cell markers, which play key role in the determinantion of self-renewal of germ cell including transcriptional regulation in the spermatogenesis.

CONCLUSION

Present case study is quite interesting because folate deficiency or unknown enviromental factor have linkege with the stem cell gene which able to maintance of germ cell, development and formation of sperm but he mode of inheritance is not yet clear because lack of complete family data but authors hypothesize that may either due to segregation of alleles or incomplete dominance with peniterance of gene in proband. The study has an important implications on the assessment of potential “risk factor” either due to folate deficient diet (nutritional factor) and genetic heterogeneity of stem cell.Hence, logically hypothesize that mutation in MTHFR and genetic diversity of stem cell markers associated with the development of defect spermatogenic or impairment of permatogenesis.

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