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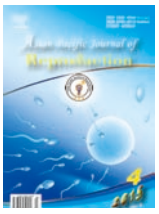

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Association of the patterns of global DNA methylation and expression analysis of DNA methyltransferases in impaired spermatogenic patients

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ABSTRACT

Objective: To analyse global DNA methylation along with DNA methyltransferases (DNMTs) expression at transcript level in patients with impaired spermatogenesis to dissect its role in pathophysiology of human male infertility. **Methods:** The content of global methylated cytosine (mC) was determined using ELISA system (Imprint Methylated DNA Quantification Kit, Sigma-Aldrich) in 31 testicular biopsies showing impaired spermatogenesis and 8 with normal spermatogenesis. Real-time reverse transcription-polymerase chain reaction was done to analyze DNMTs (DNMT1, DNMT3A, DNMT3B and DNMT3l) mRNA levels in biopsies with different testicular phenotypes. **Results:** There was significant increase in levels of global methylation in different impaired testicular phenotypes as compared to normal. Expression analysis revealed significantly increased expression of DNMT1 and its positive correlation with global DNA methylation. **Conclusion:** In conclusion, increased levels of global methylation in impaired cases might be the one of the contributing factors for aberrant gene expression in infertile patients.

1. Introduction

Germ cell development involves demethylation of whole genome and facilitate establishment of new methylation marks[1-3]. Erasure of the parental pattern in the germline is thought to be essential for resetting DNA methylation both to ensure gender specific methylation of imprinted genes as well as to prevent transgenerational inheritance of abnormal DNA methylation patterns [4, 5]. Proper Deoxyribonucleic acid (DNA) methylation is an integral part of spermatogenesis progression[6, 7]. Distinct pattern of methylation was found in testicular germ cells and patterns are established prior to meiosis[8]. Different members of the DNMT family of enzymes act either as de novo DNMTs [*DNMT3A*, 3B, 3L) or as maintenance DNMTs (DNMT1). In male germ cells, the establishment of the paternal imprints involves a factor named BORIS (brother of the regulator of imprinted sites)[9] and the DNA methyltransferases, *DNMT3A*, *DNMT3B* and the closely related DNMT3L[10]. DNMTs are predicted to be needed in mitotic cells for maintenance methylation and for de novo methylation, which is known to occur for imprinted and genome-wide methylation to ensure chromatin integrity[8, 11, 12]. Improper DNA methylation of different genes has been implicated in abnormal semen parameters, as well as several instances of male factor infertility. Hypermethylation of these loci results from the improper erasure of already established methylation marks rather than aberrant de novo methylation following epigenetic reprogramming[13]. Knockout of *DNMT3A* and DNMT3L showed altered expression in impaired spermatogenesis[14]. Accurate establishment of DNA methylation patterns is essential for the development of male germ cells and fertility. In the present study we have analyzed global DNA methylation along with the expression analysis of DNMT1, 3A, 3B and 3L in testicular biopsy tissues of patients having impaired spermatogenesis.

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2. Materials and methods

2.1. Subjects

The study was approved by the Institutional Human Ethics Committee of Faculty of Science, Banaras Hindu University, Varanasi, India. Informed written consent was obtained from every participant. Patients underwent a standardized clinical and laboratory evaluation. Patients with obstructive azoospermia, hypogonadism, hypoandrogenism, chronic diseases and history of pelvic/spinal injuries, karyotype abnormalities and AZF microdeletions were excluded. Thirty nine infertile patients were recruited. As it's difficult to acquire testicular biopsy samples of normal controls of known fertility, patients with obstructive azoospermia were confirmed by histological examination showed normal spermatogenesis were analyzed as controls.

2.2. Histological analysis

Tissue blocks of testicular biopsies obtained from patients were fixed for 24 h with Bouins fixative at room temperature. After dehydration in ascending alcohol concentrations these testicular blocks were embedded in paraffin. Five micrometer sections were mounted onto poly-L-lysine precoated slides. The sections were deparaffined, rehydrated and further stained with the routine histopathology stain Hematoxylin and Eosin (H&E) for light microscope examination.

2.3. Isolation of genomic DNA from tissue sample

Genomic DNA was isolated from wound samples using Phenol-chloroform method. The quality of isolated genomic DNA was observed on 0.8% Agarose gel with Ethidium Bromide (EtBr) by visual estimation.

2.4. Global DNA methylation quantification

Genomic DNA was isolated using standard (Phenol: chloroform: isoamyl) method. The content of Global mC was determined using ELISA (Enzyme-linked immunosorbent assay), system (Imprint Methylated DNA Quantification Kit, Sigma-Aldrich) according to manufacturer's instructions.

2.5. Calculate relative global methylation levels

2.5.1. Single point method (Percent methylation relative to the methylated control DNA)

Average the A450 replicates for the blank, samples and Methylated Control DNA were taken to perform the calculation and obtain the percent methylation of the samples relative to the Methylated Control DNA

[(A450 Average Sample - A450 Average Blank) / (A450 Average Methylated Control DNA - A450 Average Blank)] × 100

2.5.2 Quantitative real-time PCR

Total RNA was isolated from testicular biopsy samples using TRIzol (Sigma) reagent followed by DNaseI (NEB) treatment. cDNA was synthesized according to manufacturer protocol (Applied Biosystem Kit, USA). Quantitative real time PCR analysis of genes DNMT1, DNMT2A, *DNMT3B* and DNMT3L was done using primers described in Table 1. Gene expression data were normalized to the mRNA levels of housekeeping gene GAPDH.

Table 1

Primer used for Real Time PCR analysis.

Genes	Primer sequence	Product size
DNMT1 F	TACCTGGACGACCCTGACCTC	103 bp
DNMT1 R	CGTTGGCATCAAAGATGGACA	
DNMT3A F	TATTGATGAGCGCACAAGAGAGC	111 bp
DNMT3A R	GGGTGTTCCAGGGTAACATTGAG	
DNMT3B F	GGCAAGTTCTCCGAGGTCTCTG	113 bp
DNMT3B R	TGGTACATGGCTTTTCGATAGGA	
DNMT3L F	GGCCCTTCTTCTGGATGTTCGT	94 bp
DNMT3L R	ATGGTGACTGGCTCCATCTCCA	

2.5.3. Statistical analysis of expression data

The data were expressed as mean ± SEM (standard Error Mean). Statistical significance ($P<0.05$) was determined with Student's t test (two-tailed) using Graph Pad Prism5 software. The differences between the control and experimental groups in the relative gene expression were analysed by Kruskal-Wallis (KW) ANOVA, and post hoc analysis was performed by Dunn's test (Kruskal–Wallis is used when the examined groups are of unequal size). The P values that was equal to or lower than 0.05 was considered to be significant.

3. Results

3.1. The patterns of global DNA methylation in different testicular phenotypes

Among the 39 testicular biopsy included, 8 showed normal spermatogenesis (NS) and 31 were with impaired spermatogenesis. Among 31 impaired spermatogenesis cases, 14 were hypospermatogenic [HS]; 5 were maturation arrest [MA] and 12 were Sertoli cell only syndrome [SCOS]. The subjects selected for global methylation analysis were similar in age. The content of mC was determined using ELISA system (Imprint Methylated DNA Quantification Kit, Sigma-Aldrich). The levels of global methylation were calculated and compared relative to the methylated (100%) control DNA provided with the kit. There was significant increase in levels of global methylation in impaired spermatogenic patients compared to controls. Further analysis using KW test along

with Dunn's multiple comparison test were used to compare the methylation in different groups of impaired spermatogenesis (SCOS, MA and HS) with normal spermatogenesis. KW test comparision between different groups showed statistically significant differences (P<0.05) among them (Figure 1). The results of Dunn multiple comparison test revealed statistically significant differences in only between the NS *vs.* SCOS group (P<0.001). Global methylation analysis revealed a significant increase in levels of methylation in cases of impaired spermatogenesis as compared to normal spermatogenesis.

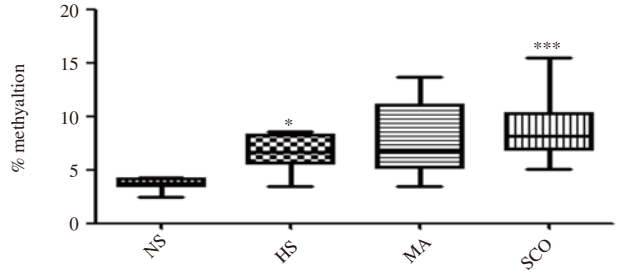

Figure 1. Box plot depicts levels of global methylation in different groups of impaired spermatogenesis (HS, MA and SCOS) *vs.* Normal spermatogenesis.

3.2. m-RNA levels of DNA methyltransferases (DNMTs) in impaired spermatogenesis cases compare to controls

Quantitative real-time PCR assays were carried out to evaluate the mRNA levels of DNMTs. Relative quantification could demonstrate a significant difference for transcripts between the crossing points normalized to the GAPDH gene and relative to the calibrator (CT) of both groups. Quantitative real-time PCR analysis showed significant up-regulation of DNMT1 and *DNMT3A* transcripts whereas *DNMT3B* and DNMT3L do not reach the level of significance. Analysis using KW test along with Dunn's multiple comparison test were used to compare the levels of transcript in different groups of impaired spermatogenesis (NS *vs.* SCOS, MA and HS) with normal spermatogenesis. KW test for DNMT1 gene expression for comparison between different groups showed statistically significant differences (P<0.05) among them (Figure 2). The results of Dunn multiple comparison test revealed statistically significant differences in only between the NS *vs.* SCOS group (P>0.05). *DNMT3A* gene expression for comparison between different groups showed statistically significant differences (P<0.05) among them (Figure 2). The results of multiple comparison test revealed statistically significant differences between (NS *vs.* HS, and NS *vs.* SCOS) group (P>0.05). KW test for *DNMT3B* and DNMT3L genes expression on comparison between different groups did not show significant difference among them (Figure 2C and D).

3.3. Correlation between global DNA methylation and DNMTs expression

We analyzed the associations between the patterns of global DNA

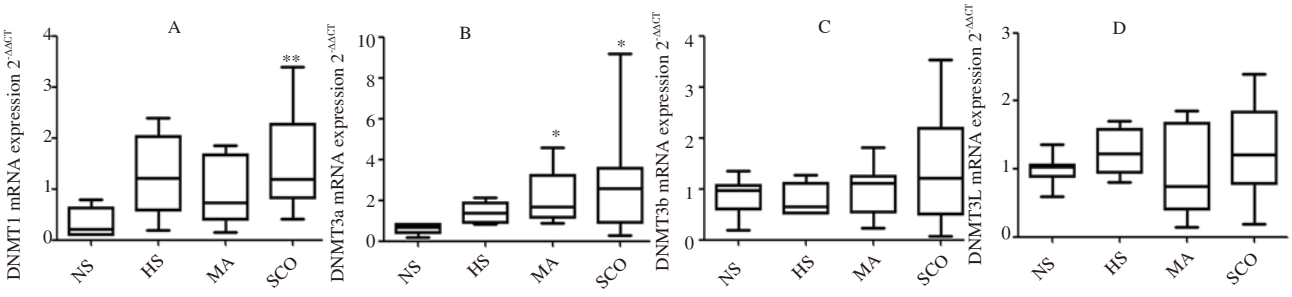

Figure 2. Box plot showing fold change ($2^{-\Delta\Delta Ct}$) in expression through quantitative real time PCR in different phenotypes.

The KW test along with Dunn's multiple comparison test were used to compare the levels of transcript in different groups of impaired spermatogenesis (NS *vs.* SCOS, MA and HS) with normal spermatogenesis. Multiple comparison tests for (A) DNMT1, (B) DNMT3a, (C) DNMT3b and (D) DNMT3l between NS *vs.* HS, MA and SCOS groups.

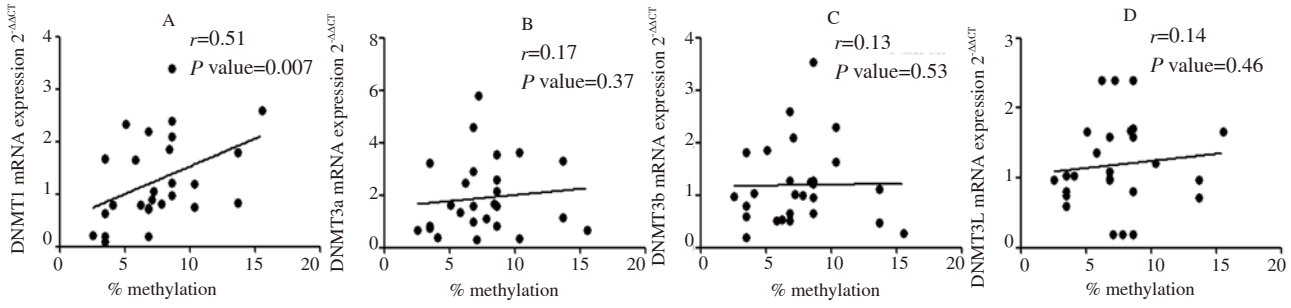

Figure 3. Correlation between % global methylation and normalized levels of DNMTs.

The r-value shows positive correlation between all DNMTs and global DNA methylation. But it reaches the level of significance in DNMT1 correlation and global DNA methylation. Significance level is denoted by*.

methylation and DNMTs mRNA levels using Spearmen correlation (data were not normally distributed). Global methylation showed significant positive correlation with DNMT1 mRNA levels (r=0.41, P<0.05) (Figure 3A). On the other hand there was no correlation with % methylation observed for *DNMT3A*, 3B and 3L levels among different groups (Figure 3 B, C and D). However the levels of *DNMT3A* mRNA were higher in impaired cases as compared to controls.

4. Discussion

The pattern and level of DNA methylation in various cells is determined during development then replicated during cell division. However, methylation patterns can sometimes change in fully differentiated cells. Any change in pattern of DNA methylation in the promoters of mature cells may have pathologic consequences[15, 16]. In the present study we observed increased levels of methylation in impaired cases as compared to controls. Previous studies have shown that hyper-methylation of the promoter region influences the expression of several genes. For instance hyper-methylation in the promoter region of MTHFR (Methylenetetrahydrofolate reductase) which down regulates MTHFR expression and as a consequence reduces its enzymatic activity. In a very recent study it was concluded that hyper-methylation of MTHFR gene promoter in sperm was associated with idiopathic male infertility[17]. The authors demonstrated that the number of patients with hyper-methylation were three times more than that of control individuals[17]. Their hypothesis was supported by the finding that changes in DNA methylation pattern in mouse male germ cells can result in absence of vas deference or azoospermia[18]. The abnormalities of DAZL promoter DNA methylation pattern and its expression are closely associated with spermatogenesis disorders in patients with infertility[19–21]. In addition we analyzed DNMT's levels in impaired cases vs. controls. We observed significantly increased levels of DNMT1 and DNMT3 whereas DNMT 3B and 3L showed similar levels of expression in all groups. Deletion of Dnmt3l in mouse results in a loss of methylation at paternally imprinted regions. Spermatogonia deficient in Dnmt 3a and Dnmt 3b displayed variations in methylation patterns at paternally imprinted regions in animal model[10]. Reduced expression of *DNMT3B* in the germ cells of patients with bilateral spermatogenic arrest in human does not lead to changes in the global methylation status[22]. DNA methyltransferases are expressed throughout human spermatogenesis, possibly maintaining the methylation patterns in order to avoid the transmission of imprinting errors by the male gamete[23].

Therefore maintenance of DNA methylation within the testis is important for normal male fertility. Our data adds to the existing knowledge that correlates aberrant global methylation and altered DNMTs expression association impaired spermatogenesis. To the best of our knowledge this is the first study on global DNA methylation and its correlation with all DNMTs transcripts in impaired spermatogenic cases.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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