

Original Research Article

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Expression Analysis of DNA Methyltransferases among Children Infected with *Mycobacterium tuberculosis*

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ABSTRACT

DNA Methylation plays a significant role in the prompt functioning of the immune system during the pathogenesis of *Mycobacterium tuberculosis* among paediatric population. Methylation is catalysed and regulated by DNA Methyltransferases (DNMTs). DNMT1, DNMT3A and DNMT3B are the essential DNMTs involved in the active transfer of Methyl group to the DNA. Thus, the upregulated DNMTs could pave way for hypermethylation of salient immune molecules and thereby transcriptionally inactivating the immune molecules involved in eliciting primary immune response. This study was aimed to determine the expression levels of DNA Methyltransferases namely DNMT1, DNMT3A and DNMT3B among children infected with *M.tb* and compared with healthy children as controls. A total of 60 cases and 60 controls were recruited to estimate and compare the expression levels of DNMTs. The expression of the mRNAs coding DNMT1, DNMT3A and DNMT3B were analysed by Real-time Quantitative PCR assay. Comparatively among children infected with *M.tb*, the expression of DNMT1, DNMT3A and DNMT3B were upregulated [DNMT1 - 0.72(IQR 0.27-1.0); DNMT3A- 0.52(IQR 0.42-.76)& DNMT3B-0.85(IQR 0.74-0.97)] while healthy controls showed downregulated pattern of expression[DNMT1-0.24 (IQR 0.20-.49); DNMT3A- 0.22 (IQR 0.14-.43)& DNMT3B- 0.37(IQR0.30-0.74)]. Thus, the upregulated pattern of DNMTs expression among cases would have assisted pathogenesis by deregulating the transcriptional machinery of host immune system through DNA methylation based Epigenetic modulation.

Keywords

DNA Methylation,
DNA
Methyltransferases,
DNMT in TB,
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Introduction

DNA methylation is the widely studied mechanism in epigenetics, which was first identified in DNA of calf thymus by Hotchkiss (Bestor, 2000). DNA methylation plays a significant role in several biological

processes like gene expression, organization of chromatin, chromosomal integrity, silencing of harmful sequences, genomic imprinting, homologous recombination(Jones and Wolffe, 1999; Migeon, 1994; Tate and Bird, 1993). On the other hand,the role of DNA methylation gains attention in the

pathogenesis of many infectious diseases as the phenomenon has a wide role in prompt functioning of the immune system and alteration of somatic cells, thus taking part in the aetiology of several diseases. DNA methylation prevents the activation of exogenous DNA in animals and plants, which restricts transgene expression that can lead to tumorigenesis (Cameron *et al.*, 1999). DNA methylation and the associated histone changes play vital role in transcriptional silencing and hetero-chromatin modelling, which alter the gene expression (Colot and Rossignol, 1999). It also maintains cellular activities and alterations in the methylation signature leading to autoimmune diseases and cancer (Richardson, 2002).

DNA methylation is the covalent addition of a methyl group (CH₃) to the 5th carbon of a cytosine base that is followed by guanine base (CpG), which is catalyzed by the enzymes, DNA methyltransferases (DNMTs). The methyl group is donated by S-adenosyl methionine (SAM), which is converted to S-adenosyl homocysteine (SAH). This will then be converted back to SAM through a folic acid and cobalamin dependent pathway. CpG methylation is a carrier of epigenetic signature that plays a vital role in modelling and functioning of the chromatin. DNA methylation acts in two ways either it can inhibit gene expression directly or it increases the chance of mutation in an affected gene (Wajed *et al.*, 2001).

DNA methyltransferases are the enzymes that bind to the DNA and cause the covalent modification of cytosine base for the transfer of methyl group from SAM to C5 of cytosine. Four DNMTs that are distinct in structure and function have been identified till date: DNMT1, DNMT2, DNMT3A and DNMT3B (Okano *et al.*, 1999; Xie and He, 1999; Yoder, 1998). Based on the biological functions,

DNMTs can be classified into two groups:

De novo methyltransferases - DNMT3A and DNMT3B

Maintenance methyltransferases - DNMT1 and DNMT2

The first eukaryotic DNA methyltransferase to be purified and cloned was DNMT1. Loss of DNMT1 leads to deletion of methylation signature found to be a causative risk factor in cancers (Mizuno *et al.*, 2001). DNMT3 family (DNMT3A and DNMT3B) introduces the de novo CpG methylation pattern in early embryonic stage, during organism development, and tumorigenesis while DNMT1 maintains the methylation signature during DNA replication. DNMT3 family includes three methyltransferases: DNMT3A, DNMT3B and DNMT3L. DNMT3L expression was found in germ cells lacking methyltransferase activity but acts as regulator of other DNMT3 proteins (Liang *et al.*, 2002). DNMT3A & 3B also play significant role in methylation maintenance by re-establishing methyl groups that are lost because of DNMT1 (Hu *et al.*, 2008). In several congenital malformations and other diseases, DNMT3 was found to be defective (Tao *et al.*, 2008).

Similar to DNMT3L, DNMT2 also lacks DNA methylation activity but recently it was shown that DNMT2 catalyses tRNA methylation (Goll *et al.*, 2006).

Numerous studies on cancer epigenetics have documented that increase in expression of DNMT1, DNMT3A and DNMT3B are associated with disease onset and disease severity (Cannuyer *et al.*, 2015; Girault and Bieche, 2003; Subramaniam *et al.*, 2014; Zhang and Xu, 2017). Particularly up regulated DNMT1 is a well-defined characteristic in most of the cancers and

several results further speculated the association of the upregulated DNMT1 in gastric cancer and colorectal cancer tissues (Etoh *et al.*, 2004; Kanai *et al.*, 2001; Toyota *et al.*, 1999). Uropathogenic *E. coli* (UPEC) infection has showed upregulated expression of DNMT1 in human uroepithelial cells. This upregulated DNMT1 had facilitated susceptibility in the host by epigenetically downregulating CDKN2A (p16INK4A) pathway (Tolg *et al.*, 2011; Tolg and Bägli, 2012). Madeddu *et al.*, described in a study that in patients with chronic Hepatitis B virus infection the duration of viral therapy has been influenced by the over expression of DNMT1 as it deregulates pathways of cyclin dependent kinase-CDK4/6-pRb-E2F1 and p38mAPK in acquiring immunity (Madeddu *et al.*, 2017). Another study by Fu *et al.*, proved that hepatitis B virus X protein enhances upregulation of DNA Methyltransferases 3A and 3B (Fu *et al.*, 2016). In addition, studies on hepato carcinoma cell lines infected with HCV showed that DNMTs were highly manipulated by the organism to thrive in the host and when the DNMTs were down regulated, severely impaired HCV infection at sub- genomic level were noticed. Interestingly it had also been proved that there was a significant reduction in HCV infection with the introduction of DNMT inhibitors. In addition, reports of many studies on epigenetic reprogramming of host immune genes in bacterial and viral pathogenesis were evident to state the crucial role played by DNMTs in deregulating pathways involved in acquiring immunity (Al Akeel, 2013; Paschos and Allday, 2010). Thus, the study was aimed to determine the association of DNMTs expression and *M.tb* disease pathogenesis in Paediatric Tuberculosis.

Materials and Methods

A total of 60 children with active TB disease (Cases) and 60 healthy children (Controls)

were recruited for the study. Children aged between $>2 \leq 14$ yrs of both genders were involved in the study. The study children were matched with age and gender between the cases and controls. Participants were explained with the purpose of the study and written informed consent/assent was obtained. Venous blood samples were collected from all the cases and controls.

Extraction of Total RNA

An equal volume of TRIzol reagent and whole blood was added to a tube (TRIzol 500 μ L + Blood 500 μ L) and mixed vigorously. Subsequently, 1.5 mL of chloroform was added to the tube and mixed vigorously for 15 sec and kept at room temperature for 5 mins. Then the mixture was centrifuged at 10,000 rpm for 15min at 4°C.

The colourless upper transparent layer was then transferred to a fresh tube and 0.5mL of isopropanol was added and gently the content was mixed. The mixture was incubated at room temperature for 5 mins and centrifuged at 10,000 rpm for 10 min at 4°C.

The supernatant was discarded and 1mL of 70% ethanol was added to the pellet to wash the cellular components/debris. Following the addition of ethanol, the tubes were centrifuged at 14,000 rpm for 5 mins at 4°C. The supernatant was discarded and the washing step was repeated again. The tubes were centrifuged at 14,000 rpm for 5 mins at 4°C. The supernatant was removed and the tubes were allowed to dry in a dry bath to get rid of the residual alcohol.

Finally, RNA containing pellet was suspended in 30-50 μ L of RNase-free water (Clontech Laboratories, Inc, CA, USA) in a water bath at 55° C for 5 min. The suspended RNA was readily used for further analyses.

DNaseI Treatment – Removal of Genomic DNA

Extracted total RNA was subjected to removal of genomic DNA contamination to aid effective conversion of RNA to cDNA.

The procedure was carried out in a RNase free tube as follows,

RNA	- 1 µg
10X Reaction Buffer with MgCl ₂	- 1 µL
DNase I, RNase-free	- 1 µL
Water, nuclease-free	- 1 MI

The tube was mixed gently and incubated at 37°C for 30 min.

1µL 50mM EDTA was added to the content to stop the reaction and followed by incubation at 65°C for 10min.

The prepared RNA was used as a template for reverse transcriptase procedure.

Quality and quantity analysis of Total RNA

The extracted RNA was further checked for its quality to carry out downstream analyses. The purity and concentration of the extracted RNA were measured in NanoDrop spectrophotometer (ThermoFisher Scientific, MA, USA). The absorbance at 260/280 ratio ranging from 1.7-2.0 was considered as good quality RNA. All the extracted RNA samples were subjected to quality analysis before further experiments

Complimentary DNA (cDNA) - First Strand cDNA Synthesis

Complementary DNA (cDNA) was synthesized from one microgram (1µg) of total RNA by using the RevertAid H Minus First Strand cDNA synthesis kit (ThermoFisher Scientific, USA).

All the kit components were thawed into room temperature and mixed by a brief

centrifugation and placed in ice throughout the procedure.

As in the order listed below the kit components were added sequentially in a sterile, nuclease-free tube.

Template RNA (total RNA)	- 2-3µL (1µg)
Primer - oligo (dT) ₁₈ Primer	- 1 µL
Water, nuclease-free (to make-up)	- 8-9 µL
5X Reaction Buffer	- 4 µL
RiboLock RNase Inhibitor (20 U/µL)	- 1 µL
10 mM dNTP	- 2 µL
RevertAid H Minus M-MuLV Reverse Transcriptase	- 1µL
	(200 U/µL)
Total volume	- 20 µL

The content was mixed gently and briefly centrifuged. Then the tubes were incubated for 60 min at 42°C.

Finally, to terminate the reaction, the tubes were incubated at 70°C for 5 min.

The converted cDNA was stored at -40°C for further qRT-PCR analysis.

qRT-PCR analysis of candidate genes

The expression profile of DNA methyltransferase genes was analyzed in a total of 60 TB cases and 60 healthy children. The amplification of both housekeeping and target genes from cDNA was performed in a total of 20 µL of reaction volume.

The preparation of qRT-PCR reaction mixture as follows,

Template cDNA (1:10 dilution)	- 2 µL
Gene-specific Primer (200 nM)	
Forward Primer	- 0.4 µL
Reverse Primer	- 0.4 µL
Takara SYBR master mix (TB Green Premix Ex Taq II Tli	
RNase H Plus, Takara Bio Inc., Japan)	- 10 µL
Water, nuclease-free (Clontech Laboratories, Inc, CA, USA)	- 7.2 µL
Total volume	- 20 µL

The qRT-PCR amplification reaction was performed on CFX96 Real-Time PCR

Detection System (Bio-Rad, USA) with the cycling conditions as follows,

Initial denaturation - 95°C for 30 sec
 Denaturation - 95°C for 5 sec
 Annealing - 60°C for 30 sec

Repeat 2nd and 3rd step for 40 cycles.

Beta-2 microglobulin ($\beta 2M$) was used as a housekeeping gene to normalize the Ct (cycle threshold) value in both cases and control samples.

Melt-curve analysis was performed to ensure accurate amplification and to avoid unspecific amplification; the products were melted from 60°C to 90°C at a rise of 0.2°C/min. The melting temperature (T_m) of the amplicons were observed using fluorescence intensity (-dF/dT) in Real-Time PCR detection System. PCR assays were performed in duplicates, and the reproducibility of the SYBR green assay was assessed by running control samples independently. To determine the relative quantification of a target gene and reference gene, the qRT-PCR data were analyzed in comparison to healthy controls using the comparative 2^{- $\Delta\Delta CT$} method.

Table.1 Primer for Housekeeping Gene

S.No	Gene Symbol	qRT-PCR primers (5' - 3')	Tm
1	$\beta 2M$	ATGAGTATGCCTGCCGTGTG	60
		CCAAATGCGGCATCTTCAAAC	

Table.2 Primers of DNA Methyltransferases

S.No	Gene Symbol	qRT-PCR primers (5' - 3')	Tm
1	DNMT1	ACCAAGCAGGCATCTCTGAC	56
		ACCAGCTTCAGCAGGATGTT	
2	DNMT3A	GGGACGTCCGCAGCGTCACAC	60
		CAGGGTTGGACTCGAGAAATCGC	
3	DNMT3B	AGCAGTACCCCTACGCCCCG	64
		CTGGACCCACCGCATGCCAG	

Results and Discussion

Expression pattern of DNA methyltransferases (DNMTs) among cases and controls

DNA methyltransferases genes DNMT1, DNMT3A and DNMT 3B expression pattern was studied among all the cases and controls. The median difference in expression these genes were compared and a significant difference between cases and controls were observed. The results showed upregulation of

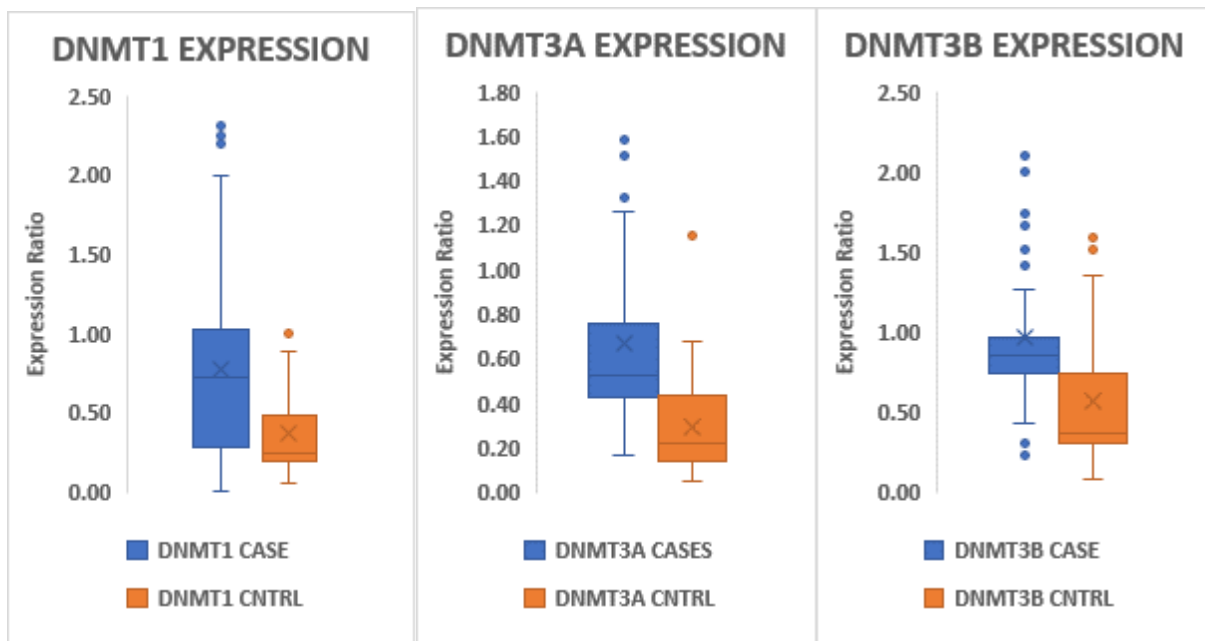
DNMT1, DNMT3A and DNMT 3B genes in children with active TB disease.

Comparatively among children infected with *M.tb*, the expression of DNMT1, DNMT3A and DNMT3B were upregulated [DNMT1 - 0.72(IQR 0.27-1.0); DNMT3A- 0.52(IQR 0.42-.76) & DNMT3B-0.85(IQR 0.74-0.97)] while healthy controls showed downregulated pattern of expression [DNMT1-0.24 (IQR 0.20-.49); DNMT3A- 0.22 (IQR 0.14-.43)& DNMT3B- 0.37(IQR0.30-0.74)] (Fig. 1).

Table.3 Expression analysis of DNMTs among cases and controls

Gene	Cases	Control	P value
DNMT1	0.72 (IQR 0.27-1.0)	0.24 (IQR 0.20-.49)	0.0001
DNMT3A	0.52(IQR 0.42-.76)	0.22 (IQR 0.14-.43)	0.0001
DNMT3B	0.85(IQR 0.74-0.97)	0.37(IQR0.30-0.74)	0.0001

Fig.1 Box plot showing expression ratio of DNMTs among cases and controls



DNA methyltransferases are the enzymes that catalyses the transfer of methyl group to specific CpG sites in DNA. Predominantly this process was carried out by DNMT1 gene while DNMT3A and DNMT3B are known to carry out *de novo* methylation. Upregulation of these genes is associated with the developmental abnormalities, immunodeficiency, onset of cancers etc. The expression of DNMTs in children infected with TB could affect the pattern of methylation of salient immune genes which would in turn affect the expression of these genes and eventually impact the prognosis of the disease. DNA methyltransferases DNMT1, DNMT3A, and DNMT3B holding a

pivotal role in establishing DNA methylation was observed to be significantly upregulated in cases than healthy controls. From this finding it can be inferred that there might be an association between the upregulated DNMTs and aberrant methylation pattern of immune genes as to aid progression of disease in cases.

Infection induced deregulation of DNMTs were recorded from many study findings to state that the molecules secreted by the organism as a part of thriving mechanism or the organism per se could act as a potential epimutagen to enhance pathogenesis. Uropathogenic *E.coli* (UPEC) infection has

showed upregulated expression of DNMT1 in human uroepithelial cells. This upregulated DNMT1 had facilitated susceptibility in the host by epigenetically downregulating CDKN2A (p16INK4A) pathway (Tolg *et al.*, 2011; Tolg and Bägli, 2012). Among patients with chronic Hepatitis B virus infection the duration of viral therapy has been influenced by the overexpression of DNMT1 as it deregulates pathways of cyclin dependent kinase-CDK4/6-pRb-E2F1 and p38mAPK in acquiring immunity (Madeddu *et al.*, 2017). Fu *et al.*, proved that hepatitis B virus X protein enhances upregulation of DNA Methyltransferases 3A and 3B (Fu *et al.*, 2016). In addition, studies on hepatocarcinoma cell lines infected with HCV showed that DNMTs were highly manipulated by the organism to thrive in the host and when the DNMTs were downregulated, severely impaired HCV infection at sub-genomic level were noticed. Interestingly it had also been proved that there was a significant reduction in HCV infection with the introduction of DNMT inhibitors (Chen *et al.*, 2013). These studies further support our finding that upregulated DNMTs might have played crucial role in establishing infection.

The LANA protein expressed in Kaposi's sarcoma-associated herpesvirus was shown to interact with cellular DNMTs and facilitated *de novo* DNA methylation in a study on KSHV infected cell lines. This study further sets example of *de novo* DNMT activity recruited by viral protein to establish promotor specific epigenetic DNA modification (Shamay *et al.*, 2006). Human papillomavirus (Protein-E7) and adenovirus (protein-E1A) infection showed a direct association in stimulating DNMTs upregulation during pathogenesis (Burgers *et al.*, 2007). In addition, reports of many studies on epigenetic reprogramming of host immune genes in bacterial and viral pathogenesis were evident to state the crucial role played by

DNMTs in deregulating pathways involved in acquiring immunity (Al Akeel, 2013; Paschos and Allday, 2010) (Table 1–3).

The present study findings with upregulated pattern of DNMTs (DNMT1, DNMT3A and DNMT3B) in children infected with tuberculosis match with the existing evidences. As quoted earlier upregulated DNMTs might have been promoted by microbial components to aid pathogenesis and to induce immunosuppressives in the host. The enhanced expression level of the members in DNMTs (DNMT1, DNMT3A and DNMT3B) is therefore inferred that it might be responsible for promotor specific epigenetic DNA modification and overall increased methylation level in the immune genes. Thus, functional pathways are further required to be explored to trace the involvement of DNMTs recruitment in hampering/ deregulating the immune molecules in the progression of active disease may lead to new preventative strategies in controlling TB.

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