

# Infection with influenza A viruses causes changes in promoter DNA methylation of inflammatory genes

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**Background** Replication of influenza virus in the host cells results in production of immune mediators like cytokines. Excessive secretion of cytokines (hypercytokinemia) has been observed during highly pathogenic avian influenza virus (HPAI-H5N1) infections resulting in high fatality rates.

**Objective** The exact mechanism of hypercytokinemia during influenza virus infection is still not known completely. As promoter DNA methylation changes are linked with expression changes in genes, we intend to identify whether changes in promoter DNA methylation have any role in expression of cytokines during influenza A virus infection.

**Methods** A panel of 24 cytokine genes and genes known to be involved in inflammatory response were analyzed for their promoter DNA methylation changes during influenza A virus infections. Four different strains of influenza A viruses, viz. H5N1, H1N1, pandemic (2009) H1N1, and a vaccine strain of H5N1, were used for the study.

**Results** We found seven of the total 24 inflammatory genes studied, showing significant changes in their promoter methylation levels in response to virus infection. These genes included

proinflammatory cytokines CXCL14, CCL25, CXCL6, and interleukines IL13, IL17C, IL4R. The changes in DNA methylation levels varied across different strains of influenza viruses depending upon their virulence. Significant promoter hypomethylation in IL17C and IL13 genes was observed in cells infected with HPAI-H5N1 virus compared with other influenza viruses. This decrease in methylation was found to be positively correlating with the increased expression of these genes. Analysis of IL17C promoter region using bisulfite sequencing resulted in identification of a CpG site within Retinoid X receptor-alpha (RXR- $\alpha$ ) transcription factor binding site undergoing demethylation specifically in H5N1-infected cells but not in other influenza-infected cells.

**Conclusion** Thus, the study could demonstrate that changes in promoter methylation in certain specific cytokine genes actually have a possible role in their expression changes during influenza A virus infection.

**Keywords** Cytokines, DNA methylation, host gene, influenza A viruses, pathogenicity, subtypes.

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## Introduction

Influenza A viruses are an important causative agent of respiratory tract infections and diseases. The clinical outcome of influenza virus infection, which includes fever, pneumonia, and even death, is a complex interplay of viral and host factors. Along with the viral factors, host cellular responses also play a significant role in virus pathogenesis. Influenza viruses, which infect the epithelium of the upper and lower respiratory tract after entry through the oral or nasal route, have been shown to cause secretion of many cytokines and chemokines in avian and mammalian hosts.<sup>1–4</sup> The production of cytokines by infected cells, which is caused by viral surface glycoproteins, double-stranded RNA, and intracellular viral proteins, is also dependent on host immune

responses.<sup>4,5</sup> Cytokine-mediated inflammatory responses have been linked to influenza pathogenesis. The mechanism of induction of cytokines by influenza virus is not completely understood but was found to vary depending on the cell type and the strain of influenza virus.<sup>4</sup> Host immune response in the form of excessive secretion of cytokines (hypercytokinemia/cytokine storm) was found to be characteristics of highly pathogenic avian influenza virus HPAI-H5N1 infection and believed to be associated with human mortality.<sup>1,6,7</sup> However, this response was found to vary among different strains of H5N1 viruses.<sup>8,9</sup> Also, influenza viruses of other subtypes like panH1N1 (2009) could induce cytokine production in infected cells comparable to H5N1 viruses.<sup>9</sup> This clearly indicates greater involvement of host cellular factors in responses to influenza virus infection.

In this study, we wanted to investigate whether epigenetic modifications like DNA methylation changes are being involved in the expression of inflammatory genes during influenza virus infection. DNA methylation has been shown to play an important role in regulating gene expression in eukaryotes.<sup>10,11</sup> Modifications at regulatory regions particularly gene promoters correlate well with the transcriptional state of a gene: hyper-methylation represses transcription, while hypo-methylation can lead to increased transcription levels. Gene silencing by means of hypermethylation of tumor suppressor genes is a well-known feature of viruses which cause cancers in human cells.<sup>12,13</sup> Changes in host DNA methylation has been shown to be caused by the viruses which integrate into host genome.<sup>13,14</sup> Viruses such as Epstein–Barr virus (EBV) and human immunodeficiency virus (HIV) remain latent inside the host cells through epigenetic modification of their genome thus mimicking host genome and preventing recognition by host immunosurveillance.<sup>13,15</sup> Also, other viruses epigenetically regulate host gene expression preventing the activation of immune and apoptotic proteins required for inhibiting viral replication in the host cells.

There are limited reports which indicate that such mechanism is being used by those viruses which do not integrate into host genome. Influenza viruses, which replicate as extracellular virion particles, do not integrate into host genome and are not associated with any type of human cancers. But recent studies have shown involvement of epigenetic regulation in the expression of certain cytokine genes during influenza A virus infection.<sup>16–18</sup> The present study that involves promoter DNA methylation analysis of immune genes known to be involved in influenza-mediated inflammatory response in four different strains of influenza virus-infected human cells will provide new insight into the ways viruses interact and modulate host cellular responses.

## Materials and methods

### Viruses and cell line

Influenza A viruses, A/Chicken/India/WB-NIV2664/2008 (H5N1), A/Jalna/NIV9436/2009 (pandemic H1N1), A/NIV/0914864/2009(H1N1), and reverse genetically modified H5N1 vaccine virus A/India/NIV/2006(H5N1)-PR8-IBCDC-RG7 were used for the study as described earlier.<sup>19,20</sup> Human lung epithelial (A549) cells used for virus infection were maintained in Dulbecco's modified Eagle's tissue culture medium (Invitrogen Life Technologies, Carlsbad, CA, USA) containing 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin in tissue culture flasks (Corning, NY, USA) at 37°C in a CO<sub>2</sub> incubator.

### Virus infection

A549 cells at a concentration of  $3 \times 10^6$  cells/ml were infected with the two above-mentioned H5N1 viruses at a

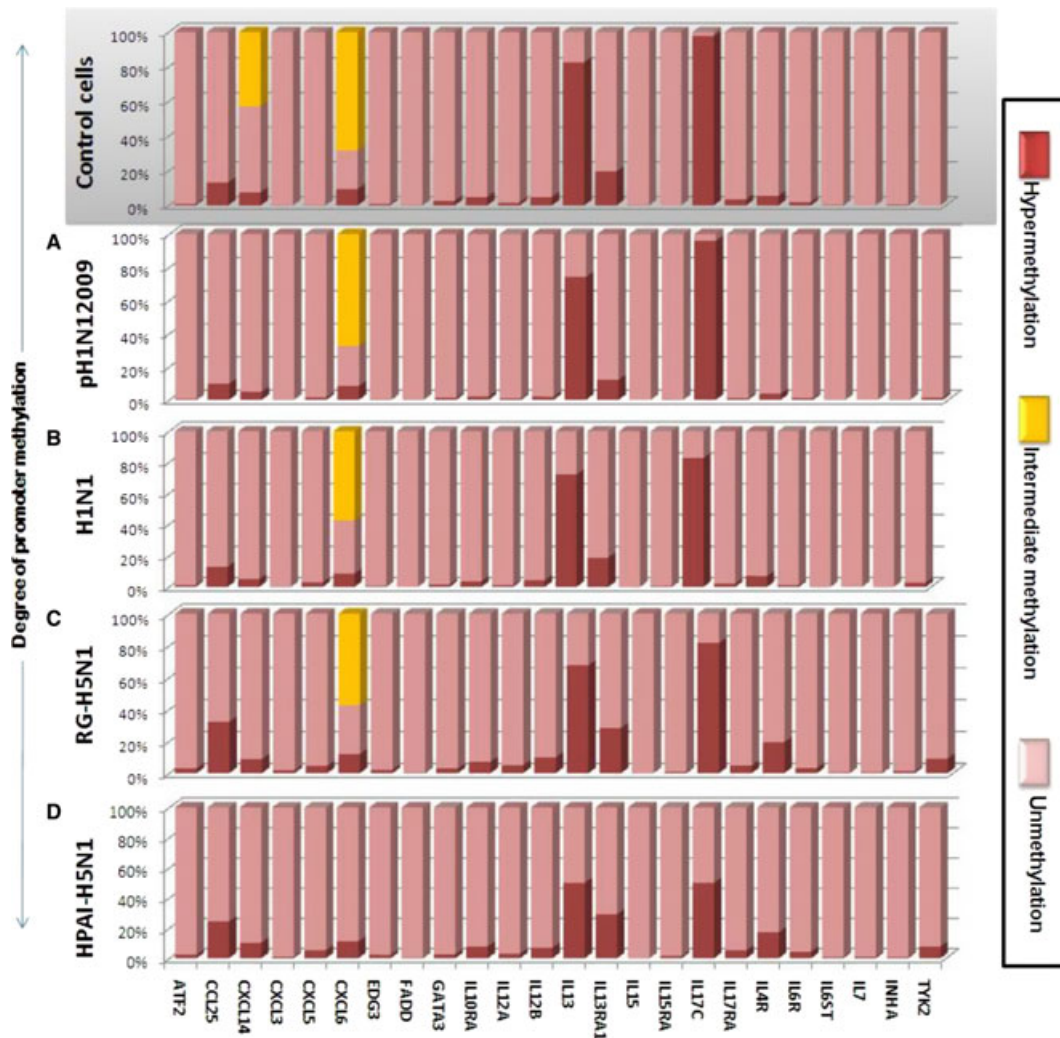
multiplicity of infection (MOI) of 1. After 1 hour, the inoculum was removed; the cells were washed twice with phosphate-buffered saline (PBS) and supplemented with growth media. For each virus, different sets of tissue culture flask were infected and cultures harvested at 16 hours post-infection (hpi) time point. As at around 16 hpi, virus progeny particles get completely assembled inside the cells which can give an increased host response. Hence, analysis was carried out at this time point. Mock-infected cells of the respective time point were taken as controls. Infection of the viruses was performed in BSL-3+ facility.

### Analysis of DNA methylation

Genomic DNA isolated from control and infected cells was analyzed for DNA methylation in the promoter region of 24 genes involved in inflammatory response, using Methyl-Profiler DNA Methylation qPCR Assays according to the supplier's instruction (SABiosciences Corp., Frederick, MD, USA). Briefly, genomic DNA was isolated using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) and treated with RNase to remove potential RNA contamination. For each assay, a total of 1 µg of genomic DNA for each sample was used. The Methyl-Profiler DNA Methylation qPCR Assay is based on the digestion of unmethylated and methylated DNA, using methylation-sensitive and methylation-dependent restriction enzymes. The remaining DNA after digestion is quantified by real-time RT-PCR, using primers that specifically flank the promoter region containing CpG island. For this analysis, the relative concentrations of differentially methylated DNA (specifically hypermethylated, unmethylated, and intermediary methylated DNA) are determined by comparing the amount of each digest with that of a mock digest. For each sample, data are expressed as the sum of the percent hyper, intermediary, and unmethylated DNA. ABI7300 RT-PCR instrument was used to read the plates. Standard  $\Delta\Delta C_t$  method was used for the calculation of the proportion of hypermethylated, intermediately and unmethylated DNA for each gene using the manufacturer supplied Excel macro spreadsheet.

### Bisulfite sequencing

Bisulfite conversion was carried out using EpiTect bisulfite kit (Qiagen) according to the manufacturer's instructions. Briefly, 500 ng of DNA was treated per column, and purified DNA was eluted in 20 µl elution buffer. Purified DNA was used as template for PCRs with following primers for the CpG island of human IL17C gene 5'-GTTGTTT TAGAGTTTGTGGTGTG-3'(sense) and 5'-ATCCAAT CTA AAAACCCAC-3'(antisense) synthesized according to bisulfite-converted DNA sequences for the regions of interest using the Methprimer software.<sup>21</sup> The PCR product was gel-purified and sequenced by conventional Sanger Sequencing.



**Figure 1.** Promoter DNA methylation analysis of inflammatory genes in A549 cells infected with A. Pandemic H1N12009 B. Seasonal H1N1 C. Reverse genetically (RG) modified vaccine strain of H5N1 D. HPAI-H5N1. The analysis was carried out in comparison with control cells (shaded area). The analysis was carried out using a quantitative PCR-based DNA methylation assay. The data are presented as percent methylated DNA ( $n = 3$ ). In each sample, the pink bars indicate no methylation; yellow bars indicate moderate methylation levels; and dark red bars indicate heavy levels of DNA methylation.

### Real-time quantitative reverse-transcription PCR (RT-PCR)

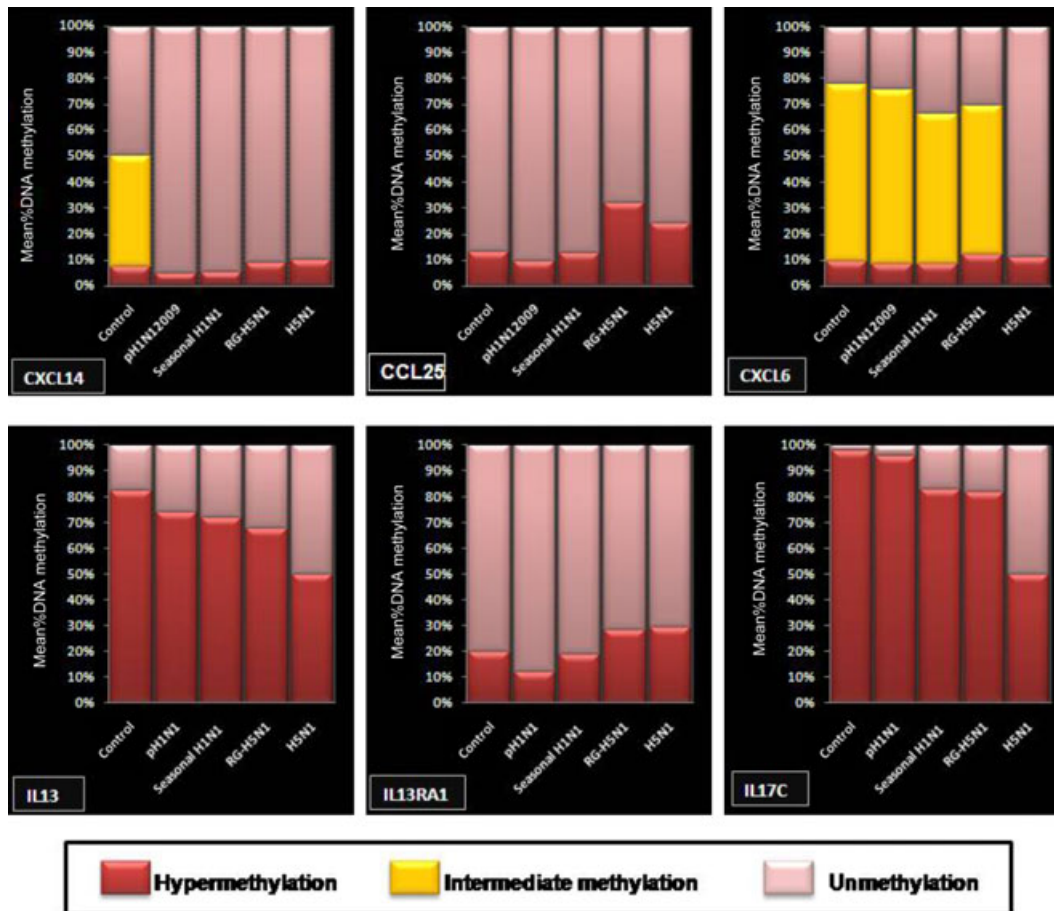
Total RNA extracted from control and infected cells was used for quantitative real-time PCR using Quantitect SYBR green one step RT-PCR kit (Qiagen, Carlsbad, CA, USA). All quantifications [threshold cycle (CT) values] were normalized to that of  $\beta$ -actin and analyzed to determine the relative level of gene expression. The relative fold change was determined using standard  $2^{-\Delta\Delta CT}$  method. The experiments were carried out in triplicates. The RT-PCR were carried out using following gene primers-IL17CFwd 5'-CATCGATACAGCCTCTGCAC-3' IL17CRev 5'-GAGGTGTTGGAGG-CAGAC-3'; IL13Fwd 5'-TGACACGTTGATCAGGGATT-3' IL13Rev 5'-GGTCAACATCACCCAGAACC-3'; CXL6Fwd 5'-GGCAATTTTATGATGCATGG-3' CXCL6Rev 5'-GGGAA

GCAAGTTTGTCTGGA-3';  $\beta$ -actin\_Fwd 5'-CCTTGACATGCCGGAG-3'  $\beta$ -actin\_Rev 5'-GCACAGAGCCTCGCCTT-3'; the primer sequences for DNA methyltransferases are described earlier.<sup>22</sup>

## Results

### Promoter DNA methylation analysis of inflammatory cytokine genes in A549 cells infected with Influenza A viruses

A set of 24 inflammatory genes were analyzed for promoter DNA methylation using methyl-profiler assay. The assay provides gene methylation status as percentage unmethylated (UM), percentage hypermethylated (HM), and intermediate methylated (IM) fraction of input DNA. Unmethylated



**Figure 2.** Selected genes from Figure 1 showing detectable methylation changes in response to influenza virus infection compared with uninfected (control) cells. The data are presented as percent methylated DNA ( $n = 3$ ). In each sample, the pink bars indicate no methylation; yellow bars indicate moderate methylation levels; and dark red bars indicate heavy levels of DNA methylation.

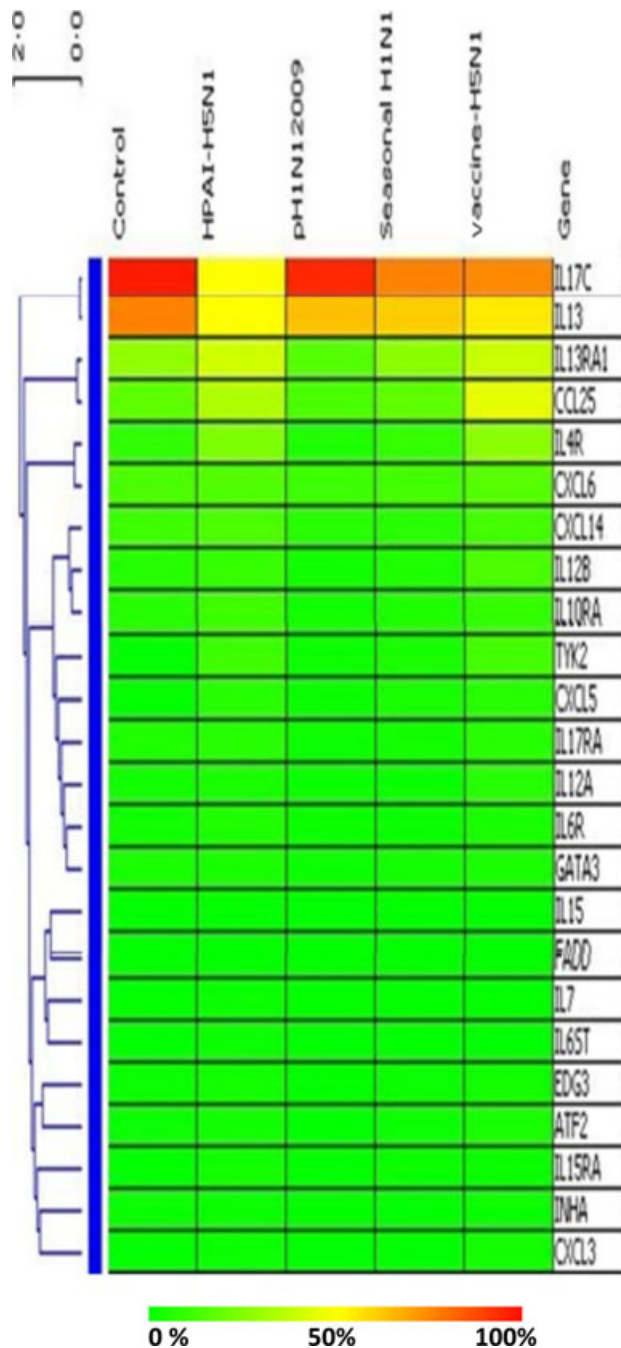
represents the fraction of input genomic DNA containing no methylated CpG sites in the amplified region of a gene. Methylated represents fraction of input genomic DNA containing two (IM) or more methylated (HM) CpG sites in the targeted region of a gene. The level of methylation of each gene was compared between infected and control cells (Figure 1).

The analysis revealed that genes IL13 and IL17C were hypermethylated, whereas genes CXCL6 and CXCL14 were intermediately methylated in control A549 cells. Infection with influenza viruses resulted in decrease in promoter methylation of IL13 and IL17C genes (Figure 2). This decrease in methylation was maximum (50%) in case of cells infected with highly pathogenic H5N1 virus. Cells infected with pH1N12009 showed no significant change in methylation of IL17C but marginal (10%) decrease in IL13 methylation levels (Figure 2). Infection with seasonal H1N1 and vaccine strain of H5N1 resulted in minor (20%) but significant decrease in promoter methylation of these genes

compared with controls (Figure 2). Genes CXCL6 and CXCL14 also showed decrease in methylation, the most prominent being in cells infected with H5N1 virus (80% and 60%, respectively; Figure 2). In contrast, we observed minor increase in the levels of promoter methylation in CCL25 and IL13RA1 genes in cells infected with H5-subtype-specific viruses (30%) compared with H1 viruses (10%; Figure 2). Remaining 17 genes present in the panel of assay did not show any significant methylation change to influenza virus infection (Figure 3).

### Expression analysis of the genes showing significant methylation changes in response to influenza infection

To understand whether differential methylation status of the above-mentioned inflammatory genes caused by influenza virus infection actually correlate with their expression changes, we analyzed the expression of genes showing most drastic increase or decrease in promoter DNA methylation



**Figure 3.** Hierarchical clustering of the 24 inflammatory genes analyzed in this study. The genes are arranged on the basis of the degree of methylation (in percentage) in A549 cells infected with different strains of influenza viruses. Red color indicates 100% methylation, yellow color indicates 50% methylation, orange color indicates 0–40% methylation, and green indicates no methylation.

levels. For this, we selected IL17C, IL13, and CXCL6 genes for the real-time PCR analysis (Figure 4). We found significant up-regulation of these 3 genes in influenza-infected cells, and in accordance with the decrease in

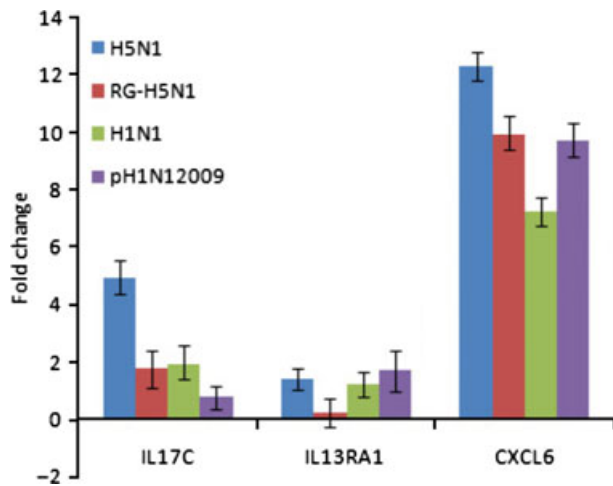
methylation levels, there was corresponding increase in expression levels, the highest being in H5N1-infected cells. CXCL6 gene, an important cytokine known to be involved in inflammatory responses to influenza viruses, was up-regulated by 12-folds compared with controls. In RG-H5N1-infected cells, CXCL6 was up-regulated by ninefolds, while in pH1N12009- and seasonal H1N1-infected cells, it was up-regulated by eight and sixfolds respectively. Also, IL17C gene was significantly up-regulated in H5N1-infected cells, which was approximately twofolds more than other influenza-infected cells (Figure 4).

### Promoter analysis of IL17C gene using bisulfite sequencing

To confirm the observations obtained from methyl-profiler assay and to identify specific sites undergoing demethylation, bisulfite sequencing of the CpG island present in the promoter region of IL17C gene was carried out with the control and influenza virus-infected cells (Figure 5). The CpG island was identified using CpG island finder software (<http://bioinformatics.wistar.upenn.edu/cpg>), which indicated the location of the CpG island 1 kb downstream of transcription start site (TSS). The software did not show precise results for IL13 and CXCL6 promoters. Using specific primers designed using Methprimer (<http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi>) Web-based tool, a region of 200 bp inside the CpG island containing 16 CpG sites was amplified. We found that CpG site at the positions 1254 from TSS was demethylated in all the strains of influenza viruses. However, interestingly, CpG site at the position 1290 was dramatically demethylated exclusively in H5N1-infected A549 cells (Figure 5). The region was found to be highly methylated in control A549 cells. Sequence analysis<sup>23</sup> further revealed that 1254 CpG is located within the NF- $\kappa$ B binding site, whereas 1290 CpG is located in Retinoid X receptor-alpha (RXR- $\alpha$ ) transcription factor (TFs) binding site. These two TFs play important role in influenza infection-mediated activation of signal transduction pathways in host cells.

### Expression analysis of DNA methyltransferases in Influenza A-infected cells

In mammalian cells, DNA methylation is governed by methyltransferases. To determine whether changes in promoter methylation in inflammatory genes are mediated by DNA methyltransferases, we analyzed the expression of DNMT1, DNMT3a, and DNMT3b at mRNA level in control (uninfected) and A549 cells infected with the four different influenza viruses. The transcriptional levels of the three methyltransferases were determined using quantitative real-time RT-PCR (Figure 6). We observed decrease in expression of DNMT3a and DNMT3b as well as DNMT1 with all the influenza viruses except in H5N1-infected cells. However, the



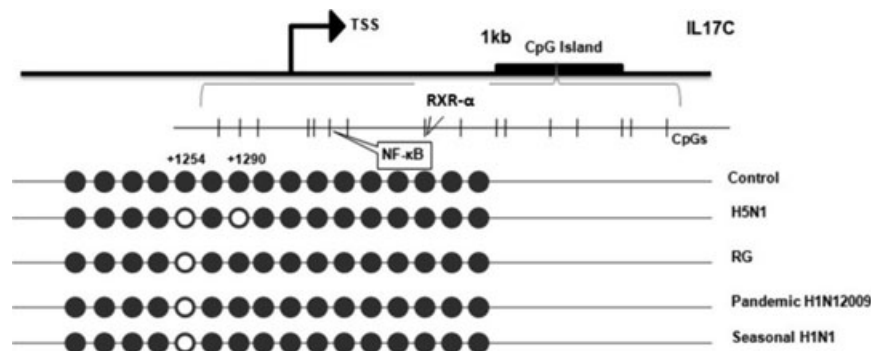
**Figure 4.** Quantitative real-time RT-PCR-based expression analysis of genes showing significant methylation change in response to influenza virus infection. IL17C, IL13, and CXCL6 genes were selected for the analysis on the basis of Figure 2. The PCRs were carried out using RNA isolated from A549 cells infected with HPAI-H5N1, RG-H5N1, seasonal H1N1, and pandemic H1N1 2009 influenza viruses. The experiment was carried out in triplicates. Data are represented as mean  $\pm$  standard deviation. Expression of  $\beta$ -actin was used as internal control.

level of decrease varied depending on the strain and virus pathogenicity.

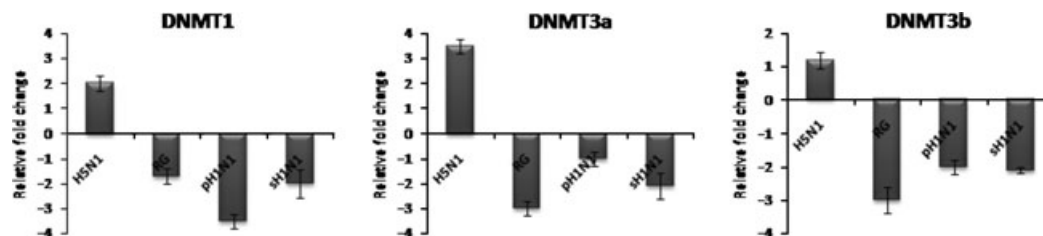
## Discussion

Acute inflammation caused by excessive secretion of cytokine has been observed with influenza virus infection, especially with H5N1 and pathogenic strains of H1N1 viruses. In this study, we provide evidence that change in promoter DNA methylation of inflammatory genes is involved in excessive secretion of cytokines during infection with influenza viruses. Also, the change in methylation level greatly depends on the strain of the influenza virus and its pathogenicity. We observed most significant changes in DNA methylation of inflammatory genes with highly pathogenic-H5N1 influenza viruses.

Our results showed that IL17C and IL13 were the main genes regulated by epigenetic mechanism during influenza virus infection. IL-17C that is a member of the Interleukin-17 family is selectively induced in epithelial cells by inflammatory stimuli.<sup>24</sup> IL-17C functions in an autocrine manner and binds to receptors (IL-17RA and IL-17RE),



**Figure 5.** DNA methylation analysis of IL17C gene promoter region using bisulfate sequencing in A549 cells infected with HPAI-H5N1, RG-H5N1, seasonal H1N1, and pandemic H1N1 2009 influenza viruses. Using specific primers designed for the CpG island present in the IL17C gene promoter, a region of 200 bp containing 16 CpG sites was amplified and sequenced. Filled circles represent methylated CpGs and empty circles represent unmethylated CpGs (16 circles for 16 CpGs). Further analysis revealed that +1254 CpG lies in the binding site of NF- $\kappa$ B and +1290 CpG lies in the binding site of Retinoid X receptor- $\alpha$  (RXR- $\alpha$ ) transcription factors. The +1254 CpG site was found to be demethylated in all the influenza virus-infected cells, while +1290CpG exclusively demethylated in H5N1-infected cells.



**Figure 6.** Real-time PCR-based gene expression analysis of DNMT genes in A549 cells infected with influenza viruses. The experiment was repeated thrice, and data representation of mean  $\pm$  standard deviation. Expression of  $\beta$ -actin was used as internal control.

which are preferentially expressed on tissue epithelial cells. IL-17C plays an important role in stimulating epithelial inflammatory responses, including the expression of pro-inflammatory cytokines and chemokines. It also plays a significant role in activation of adaptive immune responses to viral infection.<sup>24</sup> IL13 has also been shown to cause influenza virus-mediated lung inflammation and allergic response.<sup>25</sup> In our study, we found that the IL17C and IL13 promoter are methylated and transcriptionally inactivated in uninfected cells. Infection with influenza viruses resulted in decrease in promoter methylation of these genes which was found to be virus strain specific. For example, the decrease in methylation level in pH1N12009 infected was not significant than that of the control cells; however, it decreased by 20% in case of seasonal H1N1- and RG-H5N1-infected cells. But decrease in the level of promoter DNA methylation was most prominent and significant in H5N1 (50%)-infected cells, clearly indicating the role of virus pathogenicity in these epigenetic modifications. This result was further verified in bisulfite analysis of IL17C promoter region, which resulted in identification of CpG sites which were undergoing demethylation specifically in H5N1-infected cells. This demethylation was not observed in other influenza virus-infected cells.

Further analysis of this region revealed that CpG site specifically undergoing demethylation is a binding site for RXR- $\alpha$  transcription factor. RXR- $\alpha$  has been shown to play a significant role in regulating expression of cytokines involved in inflammatory response. Binding of RXR- $\alpha$  to the promoter region has been reported to be essential for the transcription of CCL6 and CCL9 chemokines.<sup>26</sup> Loss of methylation at that CpG site as observed in our study might facilitate binding of RXR- $\alpha$  at the promoter region of cytokines causing their increased expression in H5N1-infected cells.

Influenza virus infection-mediated promoter DNA methylation changes in inflammatory genes have been reported earlier.<sup>16,18</sup> It has been shown that aberrant DNA methylation changes in the IL32 promoter region resulted in its transcriptional activation in response to influenza virus infection.<sup>16</sup> Also, specific CpG demethylation at CREB1 binding region was important for the regulation of COX2 gene expression during influenza virus infection.<sup>18</sup> We also observed that the binding site of NF- $\kappa$ B transcription factor at the promoter region of IL17C gene was demethylated in all the influenza virus-infected cells. Transcription factor NF- $\kappa$ B has been shown to play important role in influenza virus-mediated host immune responses.<sup>4,27</sup> Our result further signifies the involvement of NF- $\kappa$ B in host responses to influenza virus infection which is not dependent on the virulence or pathogenicity of the virus. This indicates that activation of NF- $\kappa$ B is a generalized response to influenza virus infection.

To understand the mechanism further, we analyzed the expression of the 3 methyltransferases (DNMT1, DNMT3a, and DNMT3b) in the influenza virus-infected cells to investigate the involvement of DNA methyltransferases in the epigenetic regulation of inflammatory genes in response to influenza infection. Gene expression analysis showed that all the three types of DNA methyltransferases were affected by influenza virus infection indicating their involvement in the promoter methylation changes in inflammatory genes. We observed down-regulation Dnmt3a and Dnmt3b genes in all the influenza virus infections except for H5N1-infected cells. This observation was in accordance with earlier studies where decrease in the expression of DNMT3a and DNMT3b was observed in cells infected with H3N2 influenza A virus.<sup>18</sup> However, the increased expression or up-regulation of DNA methyltransferases in H5N1-infected cells indicates a strain- and subtype-specific host cellular response and needs further studies.

Overall, this study provides evidence that infection with influenza viruses can cause epigenetic changes like DNA methylation. This mechanism is being used to regulate the expression of host inflammatory genes and thus can play an important role in regulating host immune responses against influenza viruses. However, detailed understanding of this needs further investigation.

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