Original Article

The methylation state of *VDR* gene in pulmonary tuberculosis patients

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Background: Our previous study suggested that the methylation of vitamin D receptor (*VDR*) gene affected its expression and the defense against tuberculosis (TB) infection *in vitro*. In this study, we further investigated the methylation level of *VDR* gene in pulmonary TB patients.

Methods: The consecutive TB patients who were admitted into our department from January 2013 to January 2015 were recruited. The potential methylation sites analyzed in this study included 16 CpG sites. The amplification of bisulfite modified genomic DNA was ligated to pUC18-T vector and the positive clone of blue-white selection was sequenced to analyze the methylation state of *VDR* gene.

Results: This study had samples of 27 TB patients and 30 healthy controls. TB patients were in the hyper-methylation state compared to the healthy controls. The significant differences between TB patients and healthy controls were detected in 7 of these 16 CpG sites. The mRNA expression of AKT, GSK3β and FOXO1 decreased in TB patients compared to that of healthy controls.

Conclusions: Our study contributes to supplying more evidences that the methylation level of *VDR* gene affects the progression of TB.

Keywords: Tuberculosis (TB); vitamin D receptor (*VDR*) gene; methylation

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Introduction

Tuberculosis (TB) is the second leading cause of death in infectious diseases around the world. According to "Global tuberculosis report 2015" published by WHO, there were 9.6 million new infections and 0.48 million multi-drug resistant (MDR) TB in 2014. Mycobacterium TB complex (MTBC) is the main pathogen of TB (1).

Any of the organisms in the MTBC, which includes Mycobacterium TB, Mycobacterium africanum, Mycobacterium bovis and Mycobacterium microti, can cause TB. Although the consistency on genome sequence among MTBC is over 99%, each lineage is associated with different virulence. The virulence of different strains might be regulated by DNA methylation according to the whole-genome study (2). Furthermore, several genes which were associated with TB susceptibility, like NRAMP1 (3) and IFNG (4), have been subjected to epigenetic modification.

Epigenetics refers to the mechanism which result in gene expression changes without DNA sequence changes. The frequent modification includes DNA methylation and acetylation (5). In infectious diseases, DNA methylation in enhancers plays a critical role in regulating

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the transcriptional response to infection, even in non-proliferating cells (6). The pathogenic bacteria infected human dendritic cells (DCs) immediately caused the active demethylation at thousands of loci. Although more and more studies were reported, epigenetics of genes associated with TB susceptibility remain largely untapped field.

Vitamin D receptor (VDR) is a ligand-dependent transcription factor which mediated the activation of $1,25(OH)_2D_3$ (7). It predominantly located in the nuclei of target cells. Upon the binding of vitamin D, VDR forms a heterodimer with retinoid x-receptor (RXR). VDR complex regulates the activity of target molecules through binding to the specific vitamin D response elements (VDRE). *VDR* gene is one of the key components in the host defense against TB (8,9). The polymorphism of *VDR* gene contributes to the risk of TB. Our previous study suggested that the methylation of *VDR* gene affected its expression and the defense against TB infection *in vivo* (10). In this study, we further investigated the methylation level of *VDR* gene in TB patients and analyzed the correlation between the methylation level of *VDR* gene and patient phenotype. Our data confirmed the correlation between methylation of *VDR* gene and the occurrence of TB.

Methods

Ethical approval

The protocol was approved by the Institutional Review Board (IRB) of Shanghai Pulmonary Hospital (Tongji University) (No. 2014-016). All participants signed the written informed consent.

Patients and specimen collection

The consecutive TB patients who were admitted into our department from January 2013 to January 2015 were recruited. The diagnosis of TB was based on the "Diagnostic criteria for tuberculosis (WS288-2008)" which was made by National Health and Family Planning Commission of the PRC and the Xpert MTB/RIF test which was recommended by the World Health Organization. Patients with chronic obstructive pulmonary diseases, asthma, lung cancer, diabetes and hypertension were excluded. The inclusive criteria of healthy controls included: negative on TB detection of chest X-ray, peripheral blood and sputum; no consanguinity with TB patients; no history of chronic obstructive pulmonary diseases, asthma, lung cancer, diabetes and hypertension. The clinical and pathological data of participants which was recorded twice by two physicians respectively to ensure the accuracy were collected for analysis.

Methylation state analysis

The potential methylation sites analyzed in this study were "TTTTTGTGTTTTTTTTTTATTTTCGTGTTTA TAGATCGTTTTGGGGTGTAGGACGTCGCGTTG ATTGAGGTTATTTAGGATCGTTTGTTTAATACA CTGCAGACGTACATCCGCTGCCGCCACCCGCCC CCGGGCAGCCACCTGCTCTATGCCAAGATGATC CAGAAGCTAGCCGACCTGCGCAGCCTCAATGAG GAGCACTCCAAGCAGTACCGCTGCCTCTCCTTC CAGCCTGAGTGCAGCATGAAGCTAACGCCCCTT GTGCTCG". There were 16 CpG sites in this sequence. The genomic DNA went through bisulfite modification with the CpGenome DNA Modification Kit (Intergen Company, Purchase, NY, USA). After PCR amplification, purification, the fragment was ligated to pUC18-T vector. The positive clone of blue-white selection was sequenced.

QPCR

Total RNA was isolated for real-time PCR analysis to measure mRNA levels of the *AKT*, *GSK3β* and *FOXO1* genes. Data shown are the relative abundance of the indicated mRNA normalized to that of *GAPDH*.

Statistical analysis

χ^2 tests were performed to analyze the association between the methylation level of *VDR* gene and other clinicopathological data. All data were analyzed through the SPSS package. P values <0.05 were considered statistically significant.

Results

Clinicopathological characteristics

During our investigation, 27 TB patients met the inclusive standards. All of them are Han people. The mean age of them was 41.36 years old. Of these patients, 18 (66.67%) were male. Most patients had never smoked. Age- and gender-matched healthy controls (30 controls) were also recruited.

The methylation state of VDR gene

We analyzed the methylation state of *VDR* gene through

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direct sequencing. The fragment (269 bp) of *VDR* gene which contains 16 CpG sites were sequenced. In general, TB patients were in the hyper-methylation state compared to the healthy controls. The significant differences between TB patients and healthy controls were detected in 7 of these 16 CpG sites (P<0.05) (*Figure 1*).

Correlation between methylation state of VDR gene and activity of AKT signal pathway

Having established *VDR* gene was in hyper-methylation level in TB patients, we next analyzed the correlation between methylation state of *VDR* gene and the mRNA expression of key components in AKT signaling pathway. The mRNA expression of *AKT*, *GSK3β* and *FOXO1* decreased in TB patients compared to that of healthy controls (*Figure 2*).

Discussion

The aberrant methylation was correlated with several diseases including autoimmunity diseases (11), diabetes, and cancer. In infectious diseases, a growing number of studies have shown that DNA methylation plays a critical role in regulating the differentiation of immune cells and the expression of cytokines (12-15). The methylation level of several genes, like IL-4, IL-10, IFN-γ and TNF-α had great changes with infection of bacterial and virus (16-18). In inflammatory bowel disease (IBD), the methylation state of *IFNG* might regulate the secretion of cytokines. The methylation level of lamina propria T lymphocyte was significantly decreased in IBD patients, while the mRNA level of *IFNG* gene was increased 3 times (19). In this study, our data suggested that the *VDR* gene were in hypermethylation in TB patients compared to healthy controls. The mRNA expression of key components in AKT signaling pathway decreased in TB patients. Combined with our previous study which suggested that the methylation changes of *VDR* gene also affected the expression of IL-1β, IL-6 and TNF-α (10), these studies contributed more evidences that the methylation level of *VDR* gene might affect the progression of TB.

Currently, TB is diagnosed by chest X-ray, direct staining or culture of the sputum samples and nucleic acid amplification techniques. However, all of them suffer from their own limitations. The image of chest X-ray usually confused with previous TB infection or inflammation of lung. DNA methylation state of *VDR* genes can be directly detected in peripheral blood which provides an efficient method for diagnosis. This needs to be further studied in the future.

DNA methylation regulated the expression of target molecules through directly interfere the combination of transcription factors or binding with the methyl-CpG binding domain protein (MBD) (20). The expression not only changes in time, but also in location and manner. In TB, no evidence showed MBD was involved in the changes resulting from methylation modification of *VDR*. We assumed that *VDR* might bind to corresponding

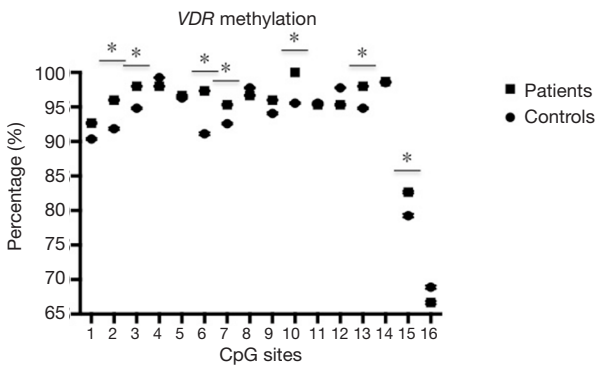

Figure 1 The methylation state of *VDR* gene in TB patients and healthy controls. VDR, vitamin D receptor; TB, tuberculosis.

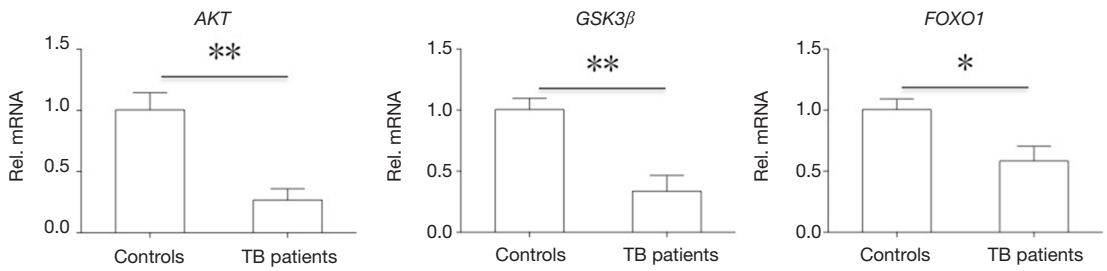

Figure 2 The mRNA expression level of *AKT*, *GSK3β* and *FOXO1* gene.

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components directly to regulate the gene expression.

As DNA methylation is closely related to several diseases, it has the potential to be an ideal target for the treatment of these diseases. Furthermore, it already has been put into practice. Azacitidine, the first approved DNA methylation inhibitor by the US Food and Drug Administration (FDA), is experiencing phase III clinical trial for treatment of myelodysplastic syndrome. In TB, the research has just begun. But it might contribute to provide a new solution in diagnosis and treatment.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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