

Original Article

Genome-wide DNA methylation and transcriptome changes in *Mycobacterium tuberculosis* with rifampicin and isoniazid resistance

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Abstract: We investigated the genome-wide DNA methylation and transcriptome changes in *M. tuberculosis* with rifampicin or isoniazid resistance. Single-molecule real-time (SMRT) sequencing and microarray technology were performed to expound DNA methylation profiles and differentially expressed genes in rifampicin or isoniazid resistant *M. tuberculosis*. Kyoto Encyclopedia of Genes and Genomes (KEGG) biological pathway analysis and methylated regulatory network analysis were conducted by online forecasting databases. Integrated analysis of DNA methylation and transcriptome revealed that 335 differentially methylated genes (175 hypermethylated and 160 hypomethylated) and 132 significant differentially expressed genes (68 up-regulated and 63 down-regulated) were found to be regulated by both rifampicin and isoniazid in *M. tuberculosis* H37Rv. Correlation analysis showed that differential methylated genes were negatively correlated with their transcriptional levels in rifampicin or isoniazid resistant strains. KEGG pathway analysis indicated that nitrogen metabolism pathway is closely related to differentially methylated genes induced by rifampicin and isoniazid. KEGG also suggested that differentially expressed genes in rifampicin or isoniazid-resistant strains may play different roles in regulating signal transduction events. Furthermore, five differentially methylated candidate genes (Rv0840c, Rv2243, Rv0644c, Rv2386c and Rv1130) in rifampicin resistant strains and three genes (Rv0405, Rv0252 and Rv0908) in isoniazid-resistant strains were verified the existence of protein-protein interaction in STRING database. Integrated DNA methylation and transcriptome analyses provide an epigenetic overview of rifampicin and isoniazid-induced antibiotic resistance in *M. tuberculosis* H37Rv. Several interesting genes and regulatory pathways may provide valuable resources for epigenetic studies in *M. tuberculosis* antibiotic resistance.

Keywords: Epigenetics, DNA methylation, transcriptome, antibiotic resistance, *Mycobacterium tuberculosis*

Introduction

Tuberculosis (TB) is caused by infection with *Mycobacterium tuberculosis* (*M. tuberculosis*), which is a pathogen of tremendous global significance. Almost one-third of the world's population harbors an asymptomatic latent *M. tuberculosis* infection, resulting in nearly 9 million new cases and 1.5 million deaths per year [1]. Fortunately, TB is a curable disease with antibiotics if treated early, but prolonged and improper therapies have led to resistance to most antibiotic agents [2]. With the prevalence of resistant strains, antibiotic therapies are not

always effective [3]. Intrinsic antibiotic resistance mechanisms show that *M. tuberculosis* is able to neutralize antibacterial activity of antibiotics through the cell wall barrier, modification of drug targets, chemical modification of drugs, enzymatic degradation, and other methods [4-7]. However, the precise molecular mechanisms underlying the formation of drug tolerance in *M. tuberculosis* remain largely unknown.

DNA methylation as an important epigenetic modification with pleiotropic roles that include regulating chromosome stability, DNA mismatch repair, transposition and genetic tran-

scription [8-11]. 5-methyl-2'-deoxycytidine (5-MdC) is a principal form of DNA methylation and is predominantly found within the promoter regions of various genes [12]. Cytosine methylation participates in repressing gene expression in higher eukaryotes [12], and a recent report suggests that 5-MdC has regulatory roles in prokaryotes [13]. DNA methylation is regarded as the only known mechanism to achieve epigenetic inheritance in prokaryotes [8]. Accumulating evidence suggests that methylation of ribosomal RNA is widespread in *M. tuberculosis* and is further suggested to maintain its functional integrity and survival [8, 14]. Several reports have raised concern over the correlation between DNA methylation and the mechanisms of drug resistance in *Mycobacterium tuberculosis* complex (MTC) [3, 15, 16]. For example, the methylation pattern produced by Erm(38) in *Mycobacterium smegmatis* (*M. smegmatis*) confers resistance to lincosamides and erythromycin [15]. Methyltransferase Erm(37) confers resistance to the ketolide telithromycin in *M. tuberculosis* [3]. KsgA gene encodes a 16S rRNA adenine dimethyltransferase that methylates A1518 and A1519 of 16S rRNA and plays an important role in intrinsic clarithromycin resistance in *M. tuberculosis* [16]. Erm(37) (also termed ermMT) is present in all members of the MTC and confers macrolide-lincosamide-streptogramin (MLS) resistance by methylation of 23S rRNA [4]. However, until now, a comprehensive analysis of *M. tuberculosis* genomic DNA methylation and transcriptome changes has not been reported.

Rifampicin and isoniazid are the key drugs in current and future TB treatment regimens [17, 18]. *M. tuberculosis* strains resistant to at least isoniazid and rifampicin are defined as multi-drug-resistant tuberculosis (MDR-TB) [19]. At present, resistance to rifampicin and isoniazid remains a major troublesome clinical problem [20, 21]. Numerous studies have shown that gene mutation causes high-level resistance to rifampicin and isoniazid in *M. tuberculosis* strains [2, 22]. However, the correlation between rifampicin and isoniazid resistance and genome-wide DNA methylation and transcriptome changes has rarely been reported in *M. tuberculosis*. In the present study, based on the information obtained from single-molecule real-time (SMRT) sequencing and microarray technology, we investigated the methylation

status and differentially expressed mRNA transcripts, respectively, and performed gene regulatory network analysis, which were able to discover the key genes regulating rifampicin and isoniazid resistance in *M. tuberculosis* H37Rv.

Materials and methods

Bacterial culture and drug exposure

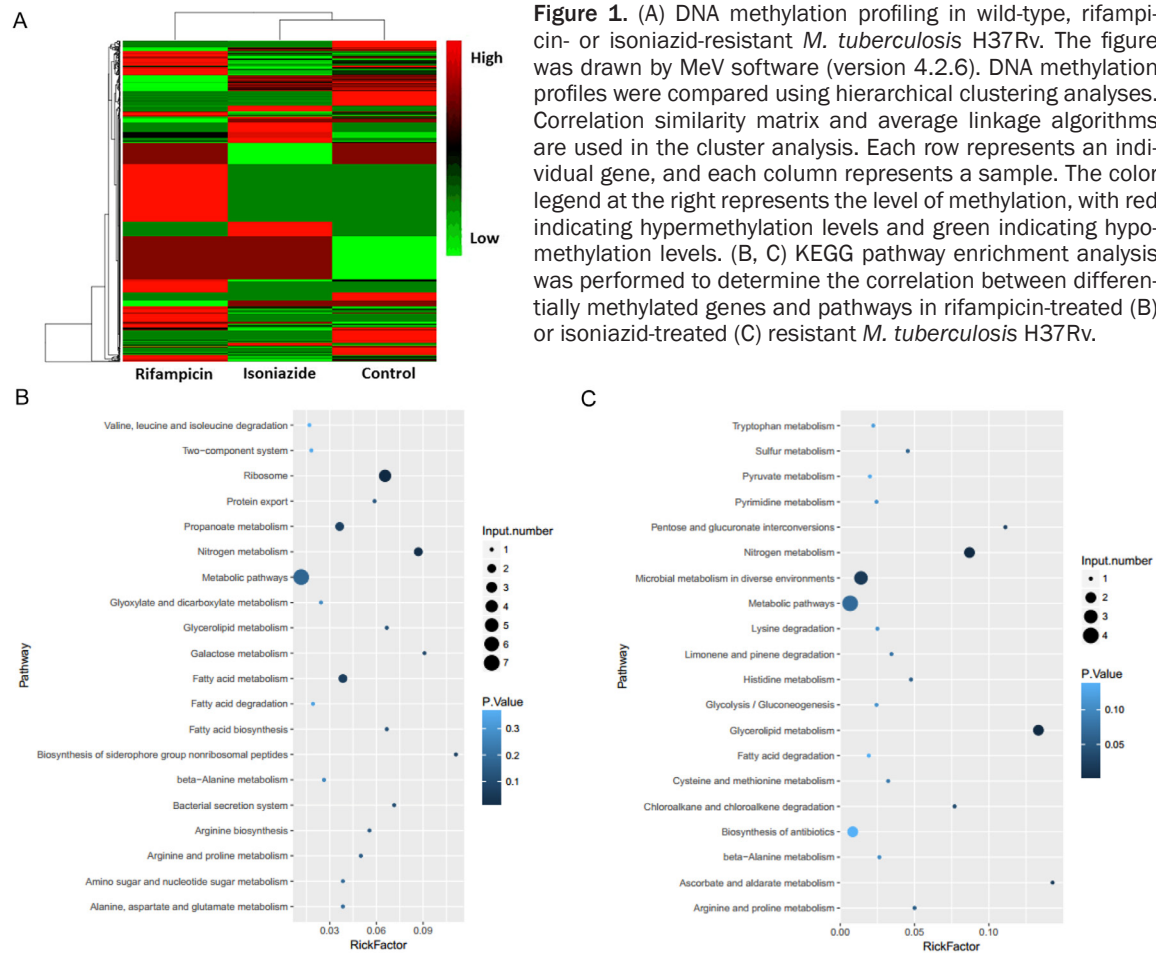
Wild-type *M. tuberculosis* H37Rv strain was obtained from the Sample Bank of the Reference Laboratory of Guang Dong Province. *M. tuberculosis* H37Rv strains were cultured on Löwenstein-Jensen (LJ) medium, a monoclonal was picked and processed by amplification culture named as generation 0 (G0) strains, which were selected for preparing rifampicin or isoniazid-resistant strains. In brief, World Health Organization (WHO) criteria for rifampicin or isoniazid-resistant strains suggested that *M. tuberculosis* H37Rv strains could survive on the rifampicin-(4000 µg/mL) or isoniazid-contained (20 µg/mL) medium, respectively. First, concentration gradients (10^{-4} , 10^{-3} , 10^{-2} , 10^{-1} , 10^0) of rifampicin or isoniazid contained LJ medium were prepared, and G0 strains (1 MCF) were cultured in LJ medium (10^{-4} rifampicin or isoniazid concentration) for approximately 4 weeks named as generation 1 (G1). This step was repeated until the emergence of generation 4 (G4) strains, which met the WHO criteria for rifampicin or isoniazid-resistant strains, and then the resistant strains were verified by the appropriate concentrations of drug used in the drug-susceptibility test. The resistant strains were continuously cultured to generation 7 (G7), and the DNA and RNA were extracted from the resistant strains for methylation and transcriptome analysis, respectively.

Rifampicin and isoniazid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Selection of rifampicin or isoniazid-resistant strains was performed as described previously [23-25].

Single-molecule real-time (SMRT) sequencing

Genomic DNA from the rifampicin or isoniazid-resistant strains was extracted using TIANamp Bacteria Genomic DNA Kit (Tiangen Biotech Co. Ltd., Beijing, China). Pacific Biosciences RSII DNA sequencing system (Pacific Biosciences, Menlo Park, CA, USA) was used as the sequencing platform. A10-kb SMRTbell library was pre-

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pared from sheared genomic DNA (> 5 µg) using a 10-kb template library preparation workflow according to the manufacturer's recommendation, with an additional bead clean-up before primer annealing. The library was bound with P4 polymerase and complexes were loaded on to version V3 SMRT cells. These were sequenced using 165 min programs. Two SMRT cells were used for each strain, yielding output data with average genome coverage of ~100×. Bioinformatic analyses of SMRT sequencing data were performed as described previously [26].

Microarray analysis

RNA was isolated using a FastPrep-24 with a FastRNA Pro Blue Kit (MP Biomedicals, Irvine, CA, USA). To remove residual DNA, RNA samples were treated with RNase-free DNase (Ambion, Austin, TX, USA) and were purified using the RNeasy MiniElute Clean-up Kit

(Qiagen Benelux, Venlo, the Netherlands), all according to the manufacturer's protocol. Each hybridization mixture was made up from 500 ng test (Cy3) and 500 ng common reference (Cy5) materials. Hybridization mixtures were prepared as described in the Agilent Two-Color Microarray-Based Gene Expression Analysis guide version 5.5 (G4140-90050, Agilent Technologies) without the inclusion of the RNA fragmentation mixture. Hybridization samples were loaded onto 8×15 k *M. tuberculosis* microarrays (Design ID: 027543, Agilent Technologies) and hybridized for 17 h at 65°C. Afterward the slides were washed and scanned (20 bit, 5 mm resolution) in an ozone-free room with the Agilent G2505C scanner as described in the Agilent G4140-90050 guide. Data were extracted with Feature Extraction (v10.7.3.1, Agilent Technologies) with the GE2_107_Sep09 protocol for two-color Agilent microarrays. Microarray data processing was performed as described previously [27].

Kyoto Encyclopedia of Genes and Genomes (KEGG) biological pathway analysis

KEGG pathway analysis was performed to determine the involvement of linear transcripts in different biological pathways, as described previously [28]. The KOBAS software was used to test the statistical enrichment of the differentially expressed circRNA-host genes in the KEGG pathways (<http://www.genome.jp/kegg/>) [29].

Protein-protein interaction analyses

Differentially expressed genes combined with differential methylation was considered significant when a *p*-value of less than 0.05. STRING v10.0 (Search Tool for the Retrieval of Interacting Genes; <https://string-db.org/>) was used to examine protein-protein interaction networks [30].

Statistical analysis

Data are presented mean $\pm$ standard deviation (SD) for each group. Inter-group differences were analyzed by one-way analysis of variance, followed by a post hoc Tukey test for multiple comparisons. Spearman's linear regression analysis was used to identify the correlation between differential methylation and gene expression levels. *P* < 0.05 was considered to indicate a statistically significant difference.

Results

*Analysis of DNA methylation profiles between wild-type *M. tuberculosis* H37Rv and strain with rifampicin or isoniazid resistance*

To compare the genome-wide DNA methylation patterns between wild-type *M. tuberculosis* H37Rv and strains with drug resistance, the hierarchical clustering was analyzed according to the DNA methylation status in three different groups, including wild-type *M. tuberculosis* H37Rv, rifampicin-treated group, and isoniazid-treated group. Compared with the wild-type strain, 440 hypermethylated genes and 210 hypomethylated genes had emerged in the rifampicin-resistant strain. Moreover, 285 of these 1691 genes and 283 of these 1691 genes appeared to be hypermethylated and hypomethylated, respectively, in the isoniazid-resistant strain compared with wild-type strain. Among these differentially methylated genes,

hypermethylation in 175 genes and hypomethylation in 160 genes were collectively regulated by rifampicin and isoniazid in *M. tuberculosis* H37Rv, indicating that aberrant hypermethylation of these genes occurred exclusively for rifampicin and isoniazid resistance (**Figure 1A** and [Supplementary Table 1](#)).

*KEGG pathway enrichment analysis for the differentially methylated genes in rifampicin and isoniazid-resistant *M. tuberculosis* H37Rv*

To further analyze the potential functions of the differentially methylated genes, we performed a systematic KEGG pathways analysis. As shown in **Figure 1B** and [Supplementary Table 2](#), ribosome and nitrogen metabolism were significantly enriched between the wild-type *M. tuberculosis* H37Rv and rifampicin-treated group, indicating that the methylated modification of ribosome and nitrogen metabolism pathways may be involved in *M. tuberculosis* H37Rv with rifampicin resistance. In addition, our findings demonstrated that differentially methylated genes in isoniazid resistant *M. tuberculosis* H37Rv were implicated in the following pathways: glycerolipid metabolism, nitrogen metabolism, microbial metabolism in diverse environments, ascorbate and aldarate metabolism, pentose and glucuronate interconversions and chloroalkane and chloroalkene degradation (**Figure 1C** and [Supplementary Table 3](#)). Among these pathways, nitrogen metabolism pathway is response to both rifampicin and isoniazid-induced drugs resistance in *M. tuberculosis* H37Rv. It is possible that nitrogen metabolism pathway may be involved in the regulation of antibiotic resistance mechanisms and can serve as a therapeutic target for drug-resistant TB.

*Comparison of differentially expressed genes in wild-type, rifampicin- or isoniazid-treated *M. tuberculosis* H37Rv*

Genome-wide transcriptome analysis was performed in wild-type, rifampicin- or isoniazid-treated *M. tuberculosis* H37Rv in triplicate. Differentially expressed genes were selected by *P* < 0.01 and Log₂ (fold change) $\geq$ 1 or $\leq$ -1. The results demonstrated that 551 genes were differentially expressed between wild-type and rifampicin-treated *M. tuberculosis* H37Rv, among which 261 genes and 290 genes were up-regulated and down-regulated, respectively (**Figure 2A** and **2B**; [Supplementary Table 4](#)).

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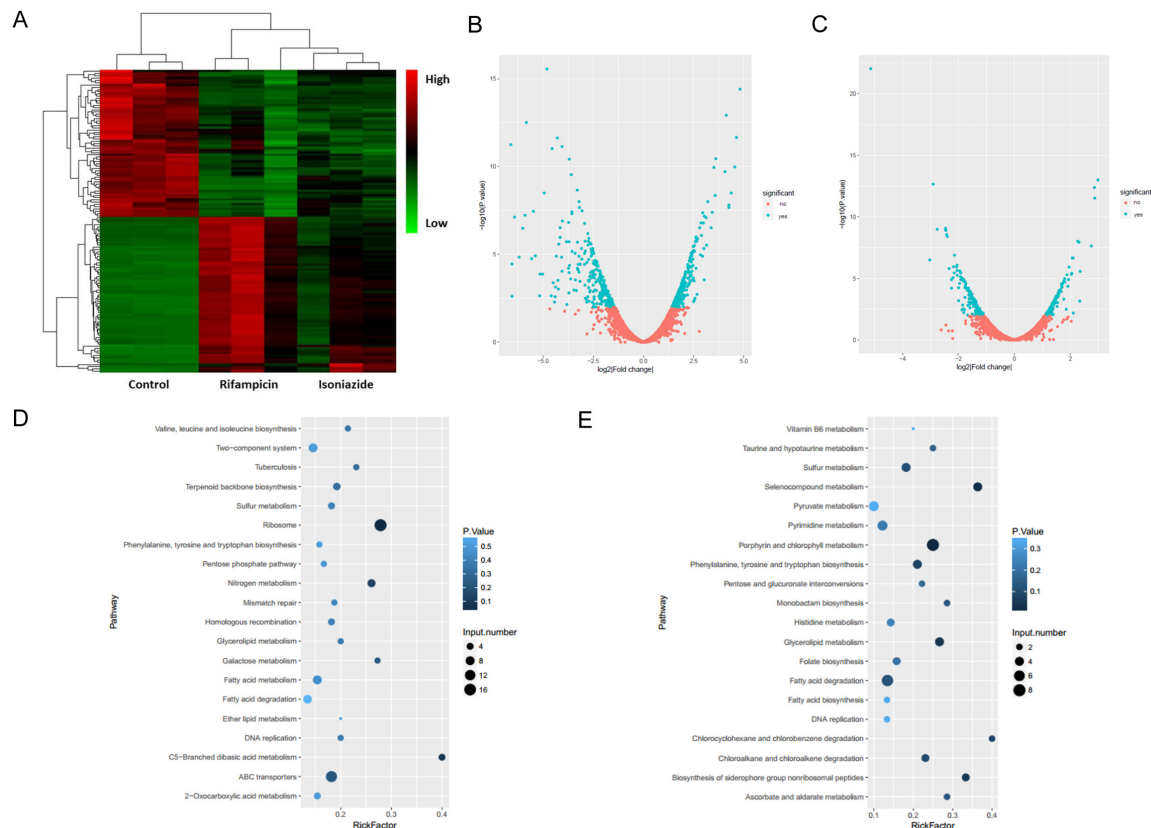


Figure 2. (A) Transcriptome differential expression profiling in wild-type, rifampicin- or isoniazid-treated *M. tuberculosis* H37Rv. The figure was drawn by MeV software (version 4.2.6). Transcriptome differential expression profiles were compared using hierarchical clustering analyses. Correlation similarity matrix and average linkage algorithms are used in the cluster analysis. Each row represents an individual gene, and each column represents a sample (triplicate in each group). The color legend at the right represents the level of methylation, with red indicating high gene expression levels and green indicating low gene expression levels. (B, C) Volcano plot of differentially expressed genes in rifampicin-resistant (B) or isoniazid-resistant (C) *M. tuberculosis* H37Rv compared with wild-type. (D, E) KEGG pathway enrichment analysis for the differentially expressed genes in rifampicin (D)- and isoniazid (E)-resistant *M. tuberculosis* H37Rv.

The expression of 147 genes was up-regulated and the expression of 154 genes was down-regulated in isoniazid-treated group as compared to control group (Figure 2A and 2C; Supplementary Table 4). A comparison of the differentially expressed genes between rifampicin and isoniazid treatment group revealed that the up-regulation of 68 genes and the down-regulation of 63 genes were regulated by both rifampicin and isoniazid-induced drug resistance in *M. tuberculosis* H37Rv.

KEGG pathway enrichment analysis for the differentially expressed genes in rifampicin and isoniazid resistant *M. tuberculosis* H37Rv

KEGG pathway enrichment analysis of the differentially expressed genes between the control group and rifampicin-treated *M. tuberculo-*

sis H37Rv found that a significant number of differentially expressed genes in rifampicin-treated *M. tuberculosis* H37Rv were involved in the ribosome pathway (Figure 2D and Supplementary Table 5). Differentially expressed genes in the isoniazid-treated group compared with control group were most likely to involve porphyrin and chlorophyll metabolism, selenocompound metabolism, glycerolipid metabolism, and biosynthesis of siderophore group nonribosomal peptides pathways (Figure 2E and Supplementary Table 6).

The correlation of gene expression and DNA methylation

DNA methylation is linked to gene silencing and considered to be an important mechanism in the regulation of mRNA transcription [9]. To

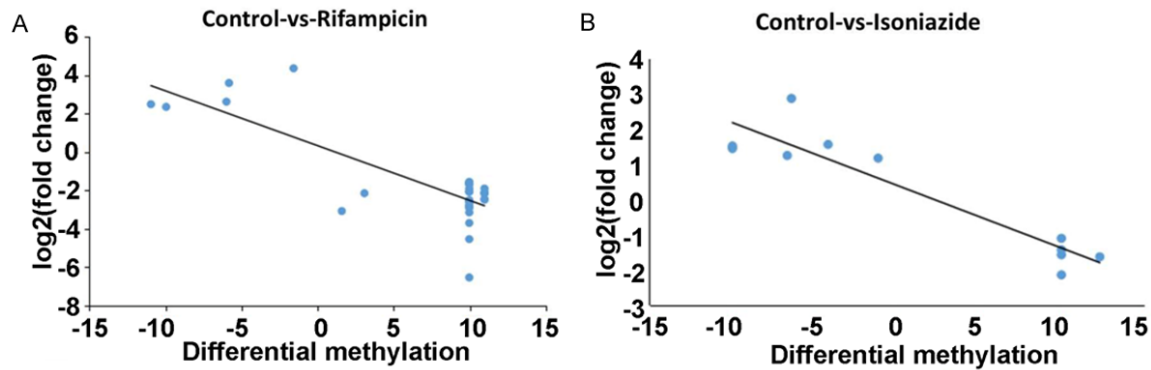


Figure 3. The correlation of gene expression levels and DNA methylation status. Scatterplots of gene expression fold change vs. DNA methylation change in rifampicin-resistant *M. tuberculosis* H37Rv (A). Scatterplots of gene expression fold change vs. DNA methylation change in isoniazid-resistant *M. tuberculosis* H37Rv (B).

examine the relationship between differential methylation and gene expression levels, 31 genes for control/rifampicin cohort and 11 genes for control/isoniazid cohort were selected based on differentially methylated genes combined with significant difference of transcriptional level. Correlation analysis showed that differential methylation of 32 genes was negatively correlated with their transcriptional levels in control/rifampicin cohort (**Figure 3A** and [Supplementary Table 7](#)). A similar result was found in control/isoniazid cohort (**Figure 3B** and [Supplementary Table 7](#)).

Functional protein association networks analyses of differentially methylated candidate genes

Differentially methylated candidate genes were obtained from rifampicin and isoniazid resistant *M. tuberculosis* H37Rv as shown in [Supplementary Table 7](#). On the basis of the protein-protein interaction data from STRING (Search Tool for the Retrieval of Interacting Genes; <https://string-db.org/>), the networks of protein interactions for differentially methylated genes in rifampicin and isoniazid resistant *M. tuberculosis* H37Rv were conducted. As shown in **Figure 4A**, three modules and five nodes (Rv0840c, Rv2243, Rv0644c, Rv2386c and Rv1130) were predicted by STRING in the rifampicin resistant strain. In isoniazid-resistant *M. tuberculosis* H37Rv, three differentially methylated candidate genes (Rv0405, Rv0252 and Rv0908) were verified to lead to the protein-protein interaction in STRING database (**Figure 4B**). These findings suggest that eight

predicted genes may help to evaluate the underlying molecular mechanisms of rifampicin and isoniazid resistance in *M. tuberculosis* H37Rv.

Discussion

In this study, we have characterized the differentially expressed methylation and RNA transcriptome in rifampicin or isoniazid resistant *M. tuberculosis* H37Rv compared with wild-type controls. The results show that rifampicin or isoniazid-resistant *M. tuberculosis* H37Rv has an altered response upon rifampicin or isoniazid exposure. Intriguingly, 335 differentially methylated genes and 132 significant differentially expressed genes were found to be regulated by both rifampicin and isoniazid, indicating that the similar antibiotics resistance mechanisms may be involved in rifampicin and isoniazid-resistant *M. tuberculosis* H37Rv.

A previous study of de Knecht et al. showed that rifampicin can induce a transcriptome response to rifampicin-resistant *M. tuberculosis* H37Rv, suggesting that Rv0559c and Rv0560c are remarkably up-regulated and play a pivotal role in rifampicin resistance of *M. tuberculosis* H37Rv [27]. In the present study, we found that the expression levels of Rv2386c, Rv0840c, and Rv1130 were dramatically increased, and Rv2243 and Rv0644c were markedly decreased in rifampicin-resistant *M. tuberculosis* H37Rv. In contrast, hypermethylation of Rv2243 and Rv0644c and the hypomethylation of Rv2386c, Rv0840c and Rv1130 were detected in rifampicin-resistant *M. tuberculosis* H37Rv.

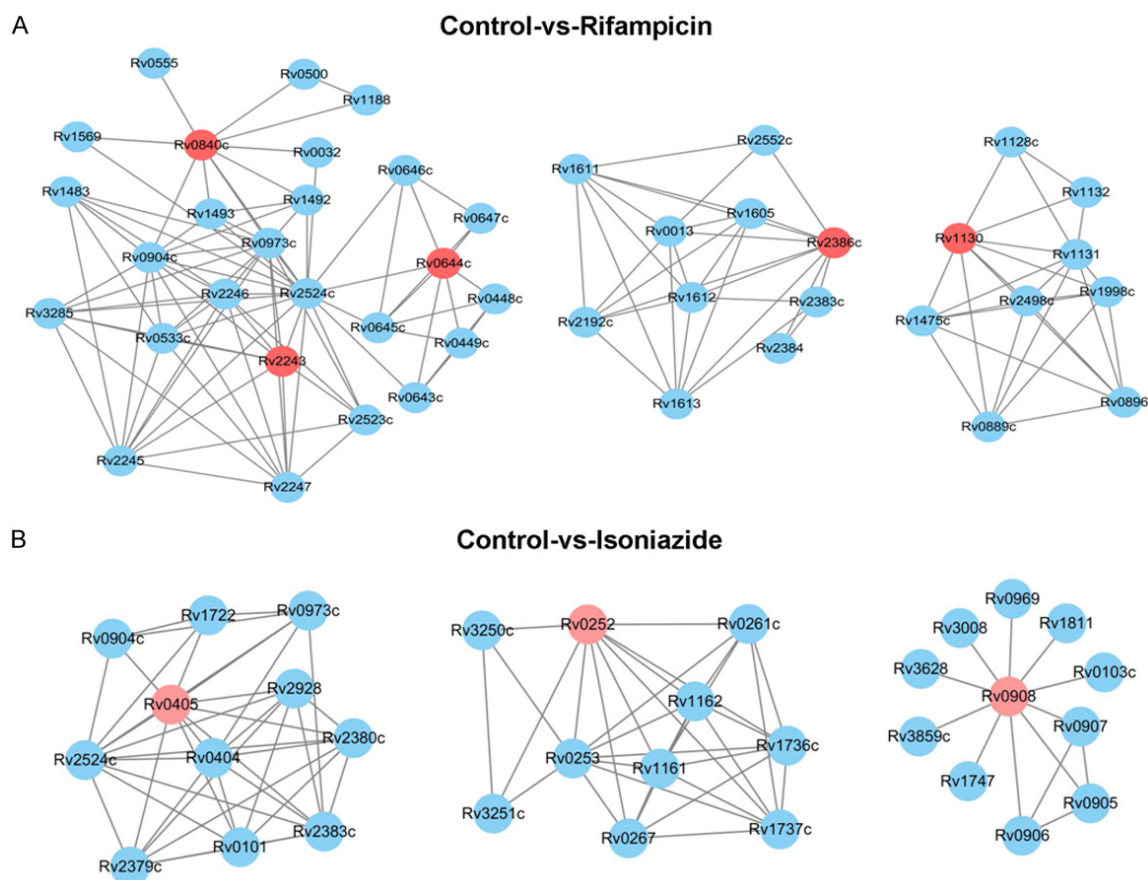


Figure 4. Functional protein association networks analyses of differentially methylated candidate genes. The networks of protein interactions for differentially methylated genes in rifampicin-resistant or isoniazid-resistant *M. tuberculosis* H37Rv were found using STRING 10.0 database.

The Rv2386c gene encodes a protein of the large subunit of anthranilate synthase and shows to be essential for the in vitro growth of *M. tuberculosis* [31]. Our results help to describe the function of Rv2386c, which can enhance rifampicin resistance in *M. tuberculosis* H37Rv through demethylation of Rv2386c in an attempt to increase the transcriptional level of Rv2386c. Rv2243 gene codes malonyl CoA-acyl carrier protein transacylase, which is related to the metabolism and biosynthesis of fatty acid and some amino acids [32]. Rv2243 is found up-regulated after exposure to ATB107, a potent inhibitor of indole-3-glycerol phosphate synthase (IGPS) and inhibition of the growth of drug-resistant *M. tuberculosis* strains as well as *M. tuberculosis* H37Rv, implying that overexpressed Rv2243 may play a crucial role in controlling pathogenic bacterium growth [33]. Our data showed that the expression of Rv2243 was significantly down-regulated, which implied an association with the hyper-

methylation of Rv2243 in rifampicin-resistant *M. tuberculosis* H37Rv. These findings suggest that DNA methylation may negatively regulate gene expression involving in rifampicin-resistant *M. tuberculosis* H37Rv.

M. tuberculosis H37Rv exposure to isoniazid promotes the demethylation of Rv0405, Rv0252 and Rv0908 genes, resulting in a significant up-regulation of gene expression. Rv0405, also known as polyketide synthase 6 (pks6) is related to the synthesis of complex lipids and may be necessary for in vivo pathogenicity [34]. Metal cation transporter ATPase E (CtpE; Rv0908) is essential for growth and maintenance of cell surface properties [35]. Rv0252 also termed as nickel Resistant Bacteria (NiRB) encodes a protein of nitrite reductase large subunit in *M. tuberculosis* H37Rv [36]. However, the function of Rv0252 in isoniazid-resistant *M. tuberculosis* H37Rv had no relevant report. To be sure, our results

show that several interesting genes were associated with isoniazid-induced antibiotic resistance in *M. tuberculosis* H37Rv.

It is interesting to note that the nitrogen metabolism pathway can be regulated by both rifampicin and isoniazid in *M. tuberculosis* H37Rv using KEGG pathway enrichment analysis for the differentially methylated genes. Ingenuity pathway analysis showed that the ribosome pathway is the primarily biological target in rifampicin-induced resistance in *M. tuberculosis* H37Rv. Porphyrin and chlorophyll metabolism are predicted as the major regulatory patterns in the progression of isoniazid-induced antibiotic resistance in *M. tuberculosis* H37Rv. These results implicate that the antibiotic resistance mechanisms in *M. tuberculosis* H37Rv may have inherent differences between rifampicin and isoniazid.

Taken together, we found that a number of molecules are involved in rifampicin- and isoniazid-resistant *M. tuberculosis* H37Rv, including the hypermethylation of Rv2243 and Rv0644c, and the hypomethylation of Rv2386c, Rv0840c, Rv1130, Rv0908, Rv0252 and Rv0405. These findings indicate that methylation-mediated regulatory pathways may contribute to antibiotic resistance mechanisms in *M. tuberculosis* H37Rv. Importantly, examining the roles of individual key gene in rifampicin- and isoniazid-resistant *M. tuberculosis* H37Rv will be the subject of future investigations.

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Disclosure of conflict of interest

None.

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