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A comparison of Rv0559c and Rv0560c expression in drug-resistant *Mycobacterium tuberculosis* in response to first-line antituberculosis drugs

Ratikorn Gamngoen[a], Chanyanuch Putim[a], Parichat Salee[b], Ponrut Phunpae[a], Bordin Butr-Indr[a,*]

[a] Division of Clinical Microbiology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, 50200, Thailand
[b] Division of Infectious Diseases, Department of Medicine, Faculty of Medicine, Chiang Mai University, Chiang Mai, 50200, Thailand

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ABSTRACT

Drug resistance to *Mycobacterium tuberculosis* is a major health problem worldwide. *Mycobacterium tuberculosis* can progress to be mono-drug resistant or multi-drug resistant by improper treatment. The chemical stress of *M. tuberculosis* was performed in this study. Rv0559c is an unknown secreted protein. Rv0560c is a putative benzoquinone methyltransferase of *M. tuberculosis* cell. Rv0559c gene is located downstream of Rv0560c gene. Both genes respond to salicylate stress. Drug susceptible, isoniazid resistant, rifampicin resistant and multi-drug resistant phenotypes of *M. tuberculosis* clinical isolates were used to determine the expression of Rv0559c and Rv0560c by qRT-PCR. In all of mycobacteria strains there was up-regulation in both genes when stressed with isoniazid. This study determined the expression of both genes, which may play important roles in the drug resistance mechanism of mycobacteria.

1. Introduction

Tuberculosis (TB) is a major health problem worldwide, with *Mycobacterium tuberculosis* (*M. tuberculosis*) complex being the most common causative agent [1]. In 2014, an estimated 9.6 million people were new TB patients, of which 0.4 million were HIV positive, and 1.5 million patients died from the disease. One third of the world's population is currently infected with TB, especially in developing countries, and it is a leading cause of death among HIV-infected patients. Inappropriate or incorrect use of anti-TB drugs can cause drug resistance. Also, delayed treatment caused by insufficient sensitivity and specificity in identifying the mycobacteria leads to MDR-TB [2], which is defined as resistance to two important and effective first-line anti-TB drugs; i.e. isoniazid (INH) and rifampicin (RMP). Mutation on the drug target site or efflux pumps can cause drug-resistant *M. tuberculosis*. Drug resistance cannot be predicted by its association with a specific gene mutation, thus suggesting that other mechanisms are involved [3]. *M. tuberculosis* is an intracellular pathogen, and during infection, the mycobacteria are believed to be exposed to adverse conditions such as hypoxia, nitric oxide, and iron starvation. When bacteria encounter the condition of low iron in macrophage infection, they produce iron-sequestering siderophores in order to maintain cellular functions [4,5]. Mycobacteria produce siderophores (mycobactin) that are essential for infection of and survival in the macrophage [6]. Expression of the genes required for mycobactin synthesis is controlled by the regulator of iron homeostasis; IdeR [7,8]. Mycobactin biosynthesis involves the conversion of isochorismate into salicylate by the enzyme, *MbtI*, and mycobacteria accumulate salicylate under iron-depleted conditions [9,10]. Salicylate stimulates oxygen consumption, induces multiple drug resistance in *M. tuberculosis* and also induces multiple antibiotic resistant (mar) phenotype in *Escherichia coli* [11,12]. The stress conditions induce drug resistance in *M. tuberculosis* [11]. Rv0559c and Rv0560c genes have the same operon and form a small gene cluster in *M. tuberculosis*. Rv0559c encodes nonessential exported protein found in culture filtrate, membrane and whole-cell lysate. It also has an open reading frame located downstream of Rv0560c [13], which is benzoquinone methyltransferase (coenzyme Q) in the biosynthesis of isoprenoid compound, associated with electron transport [14]. These genes are up-regulated in progeny RMP-resistant strains [15] and also can be induced by salicylate [16]. The study of de Knegt et al. used *M. tuberculosis* H37Rv wild type strain (drug susceptible) and a progeny H37Rv strain with a H526Y mutation in the *rpoB* gene related to rifampicin-resistant strains to perform gene expression when exposed to rifampicin drug [15]. These genes clusters were altered in RMP-resistant mutant H37Rv when compared to RMP-susceptible wild type H37Rv [15]. Meanwhile, Rv0560c associated with intermediary

Abbreviations: INH-R, isoniazid-resistant strain; MDR, multidrug-resistant strain; RMP-R, rifampicin-resistant strain; RT, room temperature; qRT-PCR, real-time quantitative reverse transcription polymerase chain reaction

* Corresponding author. Division of Clinical Microbiology, Department of Medical Technology Faculty of Associated Medical Sciences, Chiang Mai University, 110 Intawaroroj Rd., Sripoom, Chiang Mai, 50200, Thailand.
E-mail addresses: royter99@gmail.com, bordin.b@cmu.ac.th (B. Butr-Indr).

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metabolism and respiration is also up-regulated under iron-limited and anaerobic conditions that contain salicylate [7,17]. The Rv0560c inactivates pyrido-benzimidazole (14) which has inhibitory activity against DprE1 that is involved in arabinogalactan synthesis of *M. tuberculosis* [18]. DprE1 is a key precursor of the cell wall arabinan synthesis system. This study was interested in the chemical stress of Rv0559c and Rv0560c in drug-resistant *M. tuberculosis*, caused by first line anti-TB drugs. The aim of this study was to determine the expression of Rv0559c and Rv0560c in response to first-line anti-TB drugs in *M. tuberculosis* clinical isolates. Quantitative real-time polymerase chain reaction (RT-PCR) was used to determine the gene expression.

2. Materials and methods

2.1. Bacterial strain

Mycobacterium tuberculosis H37Rv (H37Rv), INH-resistant *M. tuberculosis* clinical isolate I0001 (INH-R), RMP-resistant *M. tuberculosis* clinical isolate R0001 (RMP-R) and multidrug-resistant *M. tuberculosis* clinical isolate 38537 (MDR) were cultured in LÖwenstein-Jensen medium (LJ medium) (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) at 35 °C for 4 weeks. The mycobacterial strains were obtained from the TB/HIV Research Foundation (Chiang Rai, Thailand). Drug-susceptibility was performed in all of the isolates by the microscopic observation drug susceptibility (MODS) assay.

Forty-five clinical *M. tuberculosis* isolates were obtained from the TB/HIV Research Foundation (Chiang Rai, Thailand) and Office of Disease Prevention and Control, 1 (Chiang Mai, Thailand). Clinical isolates of *M. tuberculosis* comprised 7 strains of drug-susceptible, 19 strains of isoniazid-resistant (INH-R), 4 strains of rifampicin-resistant (RMP-R) and 15 strains of MDR *M. tuberculosis* (MDR). The isolate profiles of drug susceptibility were evaluated by BACTEC MGIT 960 cultures.

2.2. Drug preparation

Isoniazid (Fluka, Sigma-Aldrich, St. Louis, MO) and rifampicin (Applichem, Darmstadt, Germany) were prepared at 1 mg/mL as a stocking concentration, sterilized with 0.2 μm nylon membrane filters and dissolved in sterile distilled water and dimethylformamide, respectively (Applichem, Darmstadt, Germany), and kept at −20 °C before use.

2.3. Determination of MIC

The drug susceptibility test was carried out according to instructions of the Clinical and Laboratory Standards Institute M24-A2 by using the agar proportion method.

2.4. Bacterial culture and drug exposed conditions

Each mycobacterium was cultured in a 50 mL centrifuge tube, containing 20 mL of Middlebrook 7H9, 0.05% (v/v) tween 20, 10% (v/v) OADC and 0.5% (v/v) glycerol at 35 °C for 6 days, under shaking conditions of 120 rpm. After day 6, 10 mL of bacterial cells was adjusted to Mc. Farland No. 1 (containing approximately 3×10^7 CFU/mL) with Middlebrook 7H9. Isoniazid and rifampicin were added to the adjusted mycobacteria [19].

1) The 1/4 × MIC and 1 × MIC of isoniazid or rifampicin were added to H37Rv, INH-R, RMP-R and MDR *M. tuberculosis*, and mixed and incubated at 35 °C for 30 min and 12 h [15].
2) Isoniazid and rifampicin were used at a C_{Max} concentration of 6 μg/mL and 24 μg/mL, respectively [20]. These drug concentrations were added to H37Rv, INH-R, RMP-R and MDR *M. tuberculosis*, and mixed and incubated at 35 °C for 30 min and 12 h.
3) Isoniazid C_{Max} concentration of 6 μg/mL [20] was added to the forty-five clinical *M. tuberculosis* isolates at 35 °C for 30 min.

The treated cells were pelleted by centrifugation at 5000 × *g* and 4 °C for 20 min (Allegra® X-15R, Beckman Coulter, California) and the supernatant was discarded. The pellets were washed twice with sterile phosphate buffered solution (PBS) and centrifuged at 5000 × *g* at 4 °C for 10 min.

2.5. RNA extraction

One mL of TRIzol® reagent (Ambion, Life Technologies, California) was added to the pellets. The suspension was transferred to a 2 mL screw cap microcentrifuge tube containing approximately 400 μL of 0.1 mm-size zirconia-silica bead (BioSpec, Bartlesville, OK). Mycobacterium cells were disrupted by using a BeadBug™ Microtube Homogenizer (Benchmark Scientific, USA) three; set at 4000 rpm for 45 s, and incubated at room temperature (RT) for 5 min. Phenol-chloroform extraction method was used. The amount of RNA was determined by measuring the optical density of 260 and 280 nm. Reverse transcriptase was performed with 250 ng of RNA of each sample using a ReverTra Ace® qPCR RT Master Mix (Toyobo, Japan).

2.6. qRT-PCR analysis

Real time PCR was performed to quantify the expression of Rv0559c and Rv0560c by using SensiFAST™ SYBR No-ROX Kit (Bioline, USA) in the Light Cycler 480 II real-time PCR system (Roche Applied Science, Indianapolis, USA). Primer sequences are shown in Table 1. The fold change in the expression of genes was calculated by the $2^{-\Delta\Delta CT}$ method [21]. Rv2703c (SigA) is a house-keeping gene [22,23].

2.7. Statistical analysis

The results were represented by the mean values of two independent experiments run in triplicate ± SD values. The results were compared by One way ANOVA using GraphPad Prism 7 software. A two-tailed p-value < 0.05 was considered statistically significant.

3. Results

The MIC for treating *M. tuberculosis* cells was determined in order to study gene expression, with the agar proportion method used for the determination of the MIC of isoniazid and rifampicin against drug-susceptible (H37Rv) and drug-resistant *M. tuberculosis* (INH-R, RMP-R and MDR). The MIC values of isoniazid and rifampicin against H37Rv, INH-R, RMP-R and MDR *M. tuberculosis* were 0.976 μg/mL and 0.488 μg/mL, 3.90 μg/mL and 0.244 μg/mL, 0.244 μg/mL and 62.5 μg/mL, and 62.5 μg/mL and 125 μg/mL, respectively. The MIC results were used for treating mycobacteria to determine the expression of Rv0559c and Rv0560c.

The relative expression of Rv0559c and Rv0560c was determined in treated mycobacteria. Four strains of mycobacteria were exposed to various conditions. The relative fold-changes were compared to a control of untreated mycobacteria. The results are presented in Figs. 1 and 2.

Rv0559c and Rv0560c were up-regulated in all of the *M. tuberculosis*

Table 1
Primer sequences.

Primers	Forward primer	Reverse primer
Rv0559c	CGCCCACATGCTCAGGGCAG	GGCGTTGACGGCGATGCGTA
Rv0560c	GTGTCCCCGTTCGGCCAGTG	GGAGGGAGTCCGACCGCCAT
Rv2703c	GTCGGAGGCCCTGCGTCAAG	AGGCCAGCCTCGATCCGCTT