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Screening mutations in drug-resistant *Mycobacterium tuberculosis* strains in Yunnan, China

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ABSTRACT

Drug-resistant tuberculosis (DR-TB), especially multidrug-resistant tuberculosis (MDR-TB), is a serious medical and societal problem in China. The purpose of this study was to evaluate the mutation characteristics of drug-resistant *Mycobacterium tuberculosis* (*M. tuberculosis*) isolates in Yunnan, China. Drug susceptibility testing (DST) was performed in 523 clinical *M. tuberculosis* isolates. Six drug resistance genes (*katG*, *inhA*, *rpoB*, *rpsL*, *embB*, and *pncA*) were selected to screen for mutations. In total, 54 clinical *M. tuberculosis* strains were identified as drug-resistant by DST, including 18 single drug-resistant (SDR) strains and 36 multidrug-resistant (MDR) strains. Twenty-four types of mutations in five genes (excluding the *inhA* gene) were screened in forty-one strains. Six novel mutations were identified in this study, including three missense mutations (p.S302R in *katG*, p.D78G in *embB*, and p.M1I in *pncA*), two frameshift mutations (408 ins A and 538-580 del in *pncA*), and one mutation in a control region (−6 C>T located upstream of *rpsL*). The mutation frequencies in the hotspot mutation regions in the *katG*, *rpoB*, *rpsL*, *embB*, and *pncA* genes were 92.5%, 44.4%, 54.2%, 52.6%, and 37.5%, respectively. The mutation spectra and frequencies seemed somewhat unique in the Yunnan DR-TB strains.

Introduction

Although antibiotics to cure tuberculosis (TB) have been available for several decades, TB remains a serious societal problem. Approximately one-third of the global population is infected with *Mycobacterium tuberculosis* (*M. tuberculosis*). In 2014, 9.6 million new TB cases occurred, of which 480,000 were multidrug-resistant tuberculosis (MDR-TB). More than half of MDR-TB (54%) cases occur in India, China, and the Russian Federation. China belongs to the twenty-two countries with the highest TB and MDR-TB burdens [1]. The early diagnosis of MDR-TB patients is necessary to improve the treatment effect and increase the survival rate.

Rifampin (RIF) and isoniazid (INH) are the two main first-line anti-TB drugs. *M. tuberculosis* strains resistant to INH and RIF are termed MDR-TB strains. Three other drugs [pyrazinamide (PZA), ethambutol (EMB), and streptomycin (SM)] also serve as first-line drugs for patients with TB. Clinical circumstances and genetic mutations are the two main reasons for the evolution of drug-resistant *M. tuberculosis* strains. Inadequate and delayed therapy, co-infection with other microorganisms, and overuse of antibiotics can prolong the treatment progress and increase the mutation rate of *M. tuberculosis*, leading to the emergence of drug-resistant strains [2,3]. Some mutations have been identified as drug-resistant factors in *M. tuberculosis* strains, including the popular candidate genes and hotspot mutations that have been identified in these strains [2]. For example, mutations at codons 315 and 463 in the *katG* gene are two common mutations worldwide [17,18].

Hotspot mutations are similar in different countries or regions, but the mutation frequency is somewhat different [4–6]. Some novel mutations have been reported in various populations. Therefore, the characteristics of mutations in clinical drug-resistant *M. tuberculosis* strains from different regions should be investigated. In this study, six first-line drug-resistant hotspot genes were screened in single- or multidrug-resistant TB strains from Yunnan, and six novel mutations were identified.

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Materials and methods

M. tuberculosis *strain collection and drug susceptibility test*

A total of 523 clinical *M. tuberculosis* strains were collected and cultured in the Third People's Hospital of Kunming, which is an infectious disease hospital in Yunnan Province, China. All of the patients were diagnosed with pulmonary TB with initial infection and recruited by doctors from September 2013 to August 2015. All 523 clinical strains were cultured in Lowenstein Jensen (LJ) medium for further use and identified as TB-positive. Drug susceptibility testing (DST) for first-line drugs was performed according to the international recommendations [i.e., INH (0.2 mg/L), RIF (40 mg/L), SM (4.0 mg/L), EMB (2.0 mg/L), and PZA (100 mg/L)] using the indirect proportion method in LJ medium. The wild type H37Rv strain (No. ATCC 27294, China) was included for quality control. Written informed consent conforming to the tenets of the Declaration of Helsinki was obtained from each participant prior to the study. This study was approved by the institutional review board of Kunming University of Science and Technology.

DNA extraction, PCR amplification, sequencing and DNA sequence analysis

Genomic DNA was extracted from clinical *M. tuberculosis* isolates using the G^+ Bacteria Genomic DNA Kit (ZomanBio, China) according to the manufacturer's instructions. Partial regions of the candidate genes (*katG, inhA, rpoB, rpsL, embB*, and *pncA*) were amplified and sequenced using the primers listed in Table S1 (in the online version at DOI: 10.1016/j.jiph.2017.04.008). The screened regions of each gene contained hotspot mutations. For example, the screened region of the *katG* gene contained mutations at codons 315 and 463. PCR was performed in a 50-μL total reaction volume containing 5 μL of 10× PCR Buffer, 1.25 unit of rTaq (Takara, Japan), 1.5 mM Mg^{2+}, 200 μM of each dNTP, 0.3 μM of each primer, and 30 ng of genomic DNA. The following PCR conditions were used: one cycle of 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 50 or 55 °C for 30 s, and 72 °C for 60 or 90 s; and one extension cycle at 72 °C for 7 min. The PCR products were sequenced, and the sequences were compared to the *M. tuberculosis* H37Rv sequence (GenBank accession number NC_000962.3) to identify mutations.

Evolutionary conservation analysis

We searched all mutations identified in this study in the TB Drug Resistance and Mutation database (http://www.tbdreamdb.com) to investigate whether the mutations were novel. Evolutionary conservation of novel mutations was performed in nine *Mycobacterium* species (*Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium asiaticum*, *Mycobacterium avium*, *Mycobacterium bovis*, *Mycobacterium canettii*, *Mycobacterium gastri*, *Mycobacterium kansasii*, *and Mycobacterium simiae*). All sequences were retrieved from GenBank. Sequences ALF06744.1, KBI31267.1, WP_036359980.1, WP_019733604.1, CAA58266.1, WP_015290248.1, WP_036416255.1, WP_023364424.1, and WP_044506092.1 were used to analyze the evolutionary conservation of the *katG* gene. Sequences WP_057341722.1, WP_049958459.1, WP_051545718.1, WP_062908845.1, ALE45293.1, WP_044081909.1, WP_036411168.1, WP_063468152.1, and WP_061557600.1 were used to analyze the evolutionary conservation of the *embB* gene. Sequences AAV33179.1, WP_031668781.1, WP_036361058.1, WP_023865880.1, AGZ02575.1, WP_015293384.1, WP_036420013.1, WP_063466482.1, and WP_061555917.1 were used to analyze the evolutionary conservation of the *pncA* gene.

Protein structure prediction

The sequencing data from each strain were compared with the sequence of the standard H37Rv strain. The Swiss-Pdb Viewer (http://spdbv.vital-it.ch/) software was used to predict whether the novel mutations could change the protein's local structure.

Results

Drug susceptibility testing results

Among the 523 clinical strains, 54 strains were verified as drug-resistant, including 18 single drug-resistant (SDR) strains and 36 MDR-TB strains. In this study, the resistance rate of the isolates was 10.33% (54/523). The MDR rate was 6.88% (36/523) in the total TB strains and 66.67% (36/54) in the drug-resistant strains, respectively. Most of the SDR strains (44.44%, 8/18) expressed INH resistance. The rates of single RIF, EMB, PZA, and SM resistance were 22.22%, 5.56%, 5.56%, and 22.22% in the SDR TB strains, respectively (Table 1). A total of 17 of the 36 MDR strains (47.22%) were resistant to more than four drugs (Table 1).

Mutation frequencies in drug resistance candidate genes

The target genes were amplified and sequenced from *M. tuberculosis* isolates resistant to INH, RIF, EMB, PZA, and SM. No mutations in the screening regions of the six genes were identified in 13 (24.07%) of the 54 drug-resistant *M. tuberculosis* isolates. Furthermore, no mutations were found in the *inhA* gene in the other 41 drug-resistant *M. tuberculosis* strains. Although only hotspot mutation regions of the candidate drug resistance genes were screened, approximately 75.93% (41/54) of the drug-resistant strains (including 13 SDR strains and 28 MDR strains) contained at least one mutation. A total of 40, 36, 24, 19, and 16 INH-, RIF-, SM-, EMB-, and PZA-resistant strains were identified, respectively. The mutation rates were 92.5% (37/40), 44.4% (16/36), 54.2% (13/24), 52.6% (10/19), and 37.5% (6/16) for the *katG, rpoB, rpsL, embB,* and *pncA* genes, respectively. Among the 28 MDR strains, 10 strains contained at least four mutations (Table S2 in the online version at DOI: 10.1016/j.jiph.2017.04.008). The dual mutation rate (i.e., two mutations present in one gene) was 36.6% (15/41), and the co-existence of mutations in codons 315 (p.S315T) and 463 (p.R463L) in the *katG* gene was the most common (73.3%, 11/15) in the 15 drug-resistant isolates with dual mutations. Moreover, the co-existence of six mutations was identified in a single clinical *M. tuberculosis* isolate (Table S2 in the online version at DOI: 10.1016/j.jiph.2017.04.008).

Mutation spectra of the drug resistance genes

Although some common mutations were identified as the main mutations in our isolates, six novel mutations were also found in this study. No mutation was found in the *inhA* gene in the 40 INH-resistant strains. For the *katG* gene, two hotspot mutations at codons 315 (p.S315T) and 463 (p.R463L) were present in 15 (37.5%, 15/40) and 32 (80%, 32/40) clinical isolates, respectively. The co-occurrence rate of these two codons was 27.5% (11/40) in the INH-resistant strains. One reported mutation at codon 300 (p.W300G) was identified in one clinical strain (Table 2). One novel mutation at codon 302 (p.S302R) of the *katG* gene was also identified (Fig. 1).

Four types of mutations were found in the RIF-resistant clinical strains. Mutations at codons 526 (p.H526N and p.H526Y) and 531 (p.S531L) were common. Mutation p.S531L was found in 14 of the 17 RIF-resistant strains (Table 2). Similarly, one rare mutation at codon 615 (p.V615 M) was identified in a MDR strain (Table 2). In total, 14 mutations were found in the *rpsL* gene of the SM-resistant

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