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# Genotypic Analysis of Multidrug-Resistant *Mycobacterium tuberculosis* Isolates Recovered From Central China

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*DNA sequencing analysis was used to investigate genetic alterations in the* rpoB, katG, *and* inhA *regulatory region and* embB *in 66* Mycobacterium tuberculosis *isolates recovered from Central China. Of the 36 multidrug-resistant isolates, 33 (92%) had mutations in the amplified region of* rpoB. *The most frequent mutation (58%, 19/36) was S531L (TCG → TTG). At least one mutation was found in the* katG *and* inhA *regulatory region in 83% (30/36) of the multidrug-resistant isolates, and mutations at* katG *codon 315 were identified in 78% (28/36). Alterations at* embB*306 may not confer resistance to EMB, and* embB*306 mutants were more frequently accompanied by* rpoB *mutations (100%, 16/16) than by* katG *315 mutations (75%, 12/16). Our results show that geographic variation in the molecular genetic mechanism is responsible for drug resistance in multidrug-resistant* M. tuberculosis. *This observation will facilitate the development of a rapid molecular drug resistance screening approach for drug-resistant* M. tuberculosis.

**KEY WORDS:** *Mycobacterium tuberculosis*; multidrug resistant; gene; mutation; sequence.

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## INTRODUCTION

Tuberculosis (TB) is the most common cause of death due to a single infectious agent—it is responsible for approximately two million deaths annually around the world. The global burden of tuberculosis remains enormous, especially in South and East Asia, sub-Saharan Africa, and eastern Europe (Dye *et al*., 1999). Moreover, 7.6% of the new cases are resistant to multiple drugs, or MDR-TB, which is caused by *M. tuberculosis* isolates that are resistant to, at least, rifampin (RIF) and isoniazid (INH). Although most of the MDR-TB is curable, it requires long-term chemotherapy. Furthermore, some patients have persistently active disease that is refractory to multidrug therapy. Some studies show cure rates for MDR-TB that remain well below those for drug-susceptible TB (Goble *et al*., 1993; Suarez *et al*., 2002). Inadequate chemotherapeutic regimens and poor compliance are proposed to be the major factors in the emergence of MDR-TB. Thus, control of tuberculosis caused by drug-resistant *M. tuberculosis* has become an urgent public health problem in many regions of the world, particularly in developing countries.

Rapid and reliable diagnosis is essential in the management of MDR-TB, not only to optimize treatment but also to prevent transmission. Traditional drug resistance testing for pulmonary TB, however, is still based on isolation of strains on culture media, which takes 4 to 6 weeks, because *M. tuberculosis* is a slow-growing organism. Thus, clinical treatment is prescribed empirically. Understanding the molecular genetic basis of drug resistance might help in the establishment of a novel method for rapid detection of drug-resistant *M. tuberculosis* strains. In contrast to most bacteria, *M. tuberculosis* does not acquire drug resistance through horizontal transfer of resistance-bearing genetic elements. Rather, resistance originates from mutations (caused by nucleotide substitutions, insertions, or deletions) in specific resistance-determining regions of the genetic targets (or their promoters) or by activating enzymes of anti-TB chemotherapeutic agents (Ramaswamy and Musser, 1998). In nearly 95% of rifampin-resistant strains, some mutations can be detected by analyzing a certain part of the RNA polymerase $\beta$-subunit gene, *rpoB* (Ramaswamy and Musser, 1998). In contrast, resistance to INH is associated with a variety of mutations affecting one or more genes. INH is a prodrug that requires activation by the catalase-peroxidase KatG. The resulting active principle, still unknown, inhibits the activity of the InhA enzyme that belongs to a type II fatty acid elongation system, together with KasA/KasB and MabA. Several studies have revealed that resistance to INH is due to mutations in the catalase-peroxidase gene (*katG*) or *inhA*. Mutations in *katG* are responsible for 60–70% of INH-resistant strains (Ramaswamy and Musser, 1998; Zhang *et al*., 2000; Herrera *et al*., 2004), and 20 to 35% of INH-resistant strains contain mutations in the *inhA* regulatory region (Banerjee *et al*., 1994). Ethambutol (EMB) is also a first-line anti-TB drug. *embB* Met306 is located in a cytoplasmic loop that forms an EMB

resistance determining region (Telenti *et al*., 1997). Nearly 50% of EMB resistant strains are involved in mutations at *embB* codon 306 (Rinder *et al*., 2001).

The previous studies have supplied a database of molecular mutations of multidrug-resistant *M. tuberculosis* isolates from different countries or areas. Disequilibrium has been reported, however, in the distribution of resistance mutations from different regions, or different genotype strains isolated from TB patients (Rinder *et al*., 1997; Hillemann *et al*., 2005). The aim of this study was to identify resistance-related mutations in the MDR-TB isolates recovered from the World Health Organization (WHO) drug resistance surveillance program in Henan, Central China. All MDR-TB strains and statistics were obtained according to the principles recommended in the guidelines of the WHO and the International Union Against TB and Lung Disease. Thus, information gained by genotypic analysis of drug-resistant isolates helps not only to identify genetic markers in *M. tuberculosis* strains unique to a particular geographic niche but also to develop some novel molecular screening methods for rapid detection of MDR-TB isolates.

## MATERIALS AND METHODS

We randomly selected 36 multidrug-resistant *M. tuberculosis* isolates and 30 susceptible *M. tuberculosis* isolates from WHO drug resistance surveillance in Henan, China. All 66 resistant isolates and susceptible strains were identified by biochemical tests. Drug susceptibility was tested using the proportion method with INH (0.2 $\mu$g mL$^{-1}$), RIF (40 $\mu$g mL$^{-1}$), and EMB (2 $\mu$g mL$^{-1}$). Resistance to any of the four drugs tested was defined as 1% or more bacterial growth on drug-containing medium compared to a control medium.

Genomic DNA was extracted as previously described by Peng *et al*. (2000). Sequences of *rpoB* (resistance determining hot spot region), *katG* (codon 315), *inhA* (regulatory region), and *embB* were obtained from GenBank (Table I). Four pairs of primers were designed using Primer Premier 5.0 and Oligo 6 software. The uniqueness of the sequences of the primers designed was analyzed with the Blast search (http://www.ncbi.nlm.nih.gov).

PCR amplification was performed with a Perkin-Elmer model 9600 thermal cycler. Ten microliters of extracted DNA was added to a 50 $\mu$L reaction mixture containing 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin, 0.6 $\mu$M (each) primer, 200 $\mu$M (each) deoxynucleotide triphosphates, and 1.5 U *Taq* polymerase (Promega, Shanghai), which then underwent an initial denaturation step at 95°C for 5 min before 35 cycles at 95°C for 1 min, 56°C for 1 min, and 72°C for 1 min, and then a final step at 72°C for 5 min. Efficient PCR amplifications were confirmed by electrophoresis on a 1.5% (w/v) agarose gel, followed by ethidium bromide staining.

PCR products from the *rpoB*, *katG*, *inhA* regulatory region, and *embB* in 36 multidrug-resistant *M. tuberculosis* isolates and 30 susceptible *M. tuberculosis*

**Table I.** Primers Used for PCR Amplification of *rpoB, katG*, *inhA* Regulatory Regions, and *embB*

| Acc. no. (Genbank) | Primer | Primer sequence (5′ to 3′) | PCR product size (bp) |
|---|---|---|---|
| L27989 | *rpoB* -F | 5′-CGATCACACCGCAGACGTTG-3′ | 311 |
| | *rpoB* -R | 5′-CGTTTCGATGAACCCGAAC-3′ | |
| X68081 | *katG*-F | 5′-GCTCGGCGATGAGCGTTAC-3′ | 409 |
| | *katG*-R | 5′-CTCGTAGCCGTACAGGATCTCG-3′ | |
| U41388 | *inhA*-F | 5′-TCGCAGCCACGTTACGCTC-3′ | 175 |
| | *inhA*-R | 5′-CCAGCCGCTGTGCGATC-3′ | |
| U68480 | *embB*-F | 5′-GCAAGCTGGCGCACCTTCAC-3′ | 243 |
| | *embB*-R | 5′-GCGCATCCACAGACTGGCGTC-3′ | |

isolates were sequenced. The amplicons were purified over a QIAquick PCR purification kit (Qiagen, Fremont, CA) according to the manufacturer's protocol. Sequence analysis was determined on both strands by direct sequencing of the PCR products on an automated model 377 DNA sequencer (Applied Biosystems, Foster City, CA ) with fluorescence-labeled dideoxynucleotide terminators (ABI Prism Big Dye terminator cycle sequencing ready reaction kit; Applied Biosystems).

## RESULTS

The drug resistance phenotype for each of the 36 multidrug-resistant *M. tuberculosis* isolates is shown in Table II. All of the strains were resistant to at least RIF and INH, and the drug resistance information for ethambutol EMB is also presented. The 30 susceptible *M. tuberculosis* isolates were susceptible to all three drugs, including RIF, INH, and EMB.

DNA sequencing of the 30 susceptible strains detected no mutations in the *rpoB, katG, inhA* regulatory region, and *embB*. Of the 36 MDR-TB isolates studied, 33 (92%) had mutations in the amplified region of *rpoB* (Table II). Three isolates (8%) carried no mutations in the amplified region. All of the 33 mutated isolates carried mutations in the hot-spot region. Overall, 12 different missense mutations, involving 13 kinds of amino acids, were identified. The most frequent mutation was S531L (TCG → TTG), found in 19 of 33 mutated isolates (58%). Another kind of codon 531 mutation was S531W (TCG → TGG), identified in one multidrug-resistant isolate (3%). Mutations affecting codon 526 (5 isolates, 15%) contained H526Y (CAC → TAC), H526D (CAC → GAC), and H526R (CAC → CGC). Three isolates (9%) exhibited codon 516 mutations, including D516V (GAC → GTC), D516G (GAC → GGC), and D516Y (GAC → TAC). Two isolates (6%) showed the codon 533 mutation L533P (CTG → CCG). One (3%) had the codon 513 mutation Q513K (CAA → AAA). In addition, two (6%) were found to carry double mutations: one carried L511P (CTG → CCG) and

**Table II.** Phenotypic and Genotypic Drug Resistance in Multidrug-Resistant *Mycobacterium tuberculosis* Isolates

| Strain no. | Resistance pattern[a] | Changes | | | |
|---|---|---|---|---|---|
| | | *rpoB*[b] | *katG* | *inhA* | *embB* |
| HS023 | R, I, E | S531L, TCG → TTG | WT[c] | WT | M306V, ATG → GTG |
| HS036 | R, I, E | WT | S315T, AGC → ACC | WT | WT |
| HS076 | R, I | L533P, CTG → CCG | S315T, AGC → ACC | WT | WT |
| HS091 | R, I | S531L, TCG → TTG | S315N, AGC → ACC | WT | WT |
| HS122 | R, I, E | S531L, TCG → TTG | S315T, AGC → AAC | WT | M306I, ATG → ATA |
| HS146 | R, I, E | S531L, TCG → TTG | WT | WT | M306I, ATG → ATA |
| HS155 | R, I, E | S531L, TCG → TTG | S315T, AGC → ACC | WT | M306V, ATG → GTG |
| HS177 | R, I | S531L, TCG → TTG | S315T, AGC → ACC | WT | WT |
| HS193 | R, I | H526Y, CAC → TAC | WT | WT | WT |
| HS221 | R, I, E | S531W, TCG → TGG | S315T, AGC → ACC | WT | M306V, ATG → GTG |
| HS230 | R, I, E | S531L, TCG → TTG | S315T, AGC → ACC | WT | M306V, ATG → GTG |
| HS258 | R, I, E | H526R, CAC → CGC | S315T, AGC → ACC | WT | WT |
| HS267 | R, I | S531L, TCG → TTG | WT | WT | M306I, ATG → ATT |
| HS279 | R, I, E | H526D, CAC → TAC | S315T, AGC → ACC | −15, C → T | WT |
| HS285 | R, I | S531L, TCG → TTG | S315T, AGC → ACC | WT | WT |
| HS291 | R, I | D516V, GAC → GTC | WT | WT | M306V, ATG → GTG |
| HS311 | R, I, E | S531L, TCG → TTG | S315T, AGC → ACC | WT | M306V, ATG → GTG |
| HS329 | R, I, E | S531L, TCG → TTG | S315T, AGC → ACC | WT | WT |
| HS331 | R, I, E | WT | WT | WT | WT |
| HS341 | R, I | Q513K, CAA → AAA | S315T, AGC → ACC | WT | WT |
| HS358 | R, I, E | L533P, CTG → CCG | S315T, AGC → ACC | WT | M306I, ATG → ATA |
| HS394 | R, I, E | H526Y, CAC → TAC | S315T, AGC → ACC | WT | M306V, ATG → GTG |
| HS434 | R, I | H526D, CAC → TAC | S315T, AGC → ACC | WT | WT |
| HS476 | R, I, E | S531L, TCG → TTG | WT | −15, C → T | WT |
| HS490 | R, I, E | D516Y, GAC → TAC | S315T, AGC → ACC | WT | M306V, ATG → GTG |
| HS553 | R, I, E | L511, H526Q[d] | S315T, AGC → ACC | WT | M306V, ATG → GTG |
| HS561 | R, I | S531L, TCG → TTG | S315T, AGC → ACC | WT | WT |
| HS590 | R, I, E | S531L, TCG → TTG | S315T, AGC → ACC | WT | M306I, ATG → ATT |
| HS622 | R, I | L511R, D516Y[e] | S315N, AGC → ACC | WT | WT |

**Table II.** Continued

| Strain no. | Resistance pattern[a] | Changes | | | |
|---|---|---|---|---|---|
| | | *rpoB*[b] | *katG* | *inhA* | *embB* |
| HS697 | R, I, E | S531L, TCG → TTG | WT | − 15, C → T | WT |
| HS722 | R, I, E | S531L, TCG → TTG | S315T, AGC → ACA | WT | M306L, ATG → CTG |
| HS786 | R, I | WT | S315T, AGC → AAC | WT | WT |
| HS843 | R, I | S531L, TCG → TTG | 315T, AGC → ACC | WT | WT |
| HS874 | R, I, E | S531L, TCG → TTG | S315T, AGC → ACC | WT | WT |
| HS913 | R, I | D516G, GAC → GGC | S315T, AGC → ACC | WT | M306V, ATG → GTG |
| HS937 | R, I, E | S531L, TCG → TTG | S315T, AGC → ACC | WT | WT |

[a]Drug susceptibility testing done by proportion method. R, rifampin; I, isoniazid; E, ethambutol.
[b]*rpoB* codon numbering is based on the *Escherichia coli rpoB* numbering system.
[c]WT, wild type.
[d]Double mutation: L511P, CTG → CCG; H526Q, CAC → CAG.
[e]Double mutation: L511R, CTG → CGG; H516Y, GAC → TAC.

H526Q (CAC → CAG); the other carried L511R (CTG → CGG) and H516Y (GAC → TAC). Mutations in *katG* codon 315 were detected in 28 of the 36 MDR-TB strains (78%). There were 25 strains with the S315T (AGC → ACC) mutation, the most common base substitution mutation; two carried the S315N (AGC → AAC) mutation, and one carried the S315T (AGC → ACA) mutation. Sequencing analysis of the *inhA* regulatory region revealed base substitutions (C → T) at nucleotide position −15 in three resistant isolates (8%). Of these, two had mutations only in the *inhA* regulatory region, and one had an accompanying *katG* mutation at codon 315. Of the MDR-TB isolates, 83% had at least one mutation in the *katG* and *inhA* regulatory region. Mutations in *embB* were detected in 13 of 22 EMB-resistant isolates (59%), and three of the *embB306* mutants were EMB susceptible. The observed mutations included M306V, M306I, and M306L (Table II).

## DICUSSION

Mutations leading to RIF resistance were first elucidated by studies on the DNA-dependent RNA polymerase of *Escherichia coli,* and mutations conferring resistance to RIF were found to be located exclusively in the core region of *rpoB* (Jin and Gross, 1988). RIF acts by binding the $\beta$-subunit of the RNA polymerase, thus interfering with transcription and RNA elongation. Subsequent studies revealed that ca. 95% of RIF-resistant MTB strains carried mutations within an 81-bp region determining rifampin resistance (RRDR) of *rpoB* (Chaves *et al*., 2000). Later studies showed targeted molecular analysis of *rpoB* to be effective for detecting RIF resistance in over 90% of RIF-resistant strains from diverse geographic regions (Kapur *et al*., 1994; Caugant *et al*., 1995; Qian *et al*., 2002). Moreover, disequilibrium has been reported in the distribution of resistance mutations among different regions or different genotype strains isolated from TB patients (Caugant *et al*., 1995; Hillemann *et al*., 2005). In this study, we report the presence of mutations in the core region of *rpoB* in 92% (33/36) of *M. tuberculosis* strains that exhibited RIF resistance. The most common mutation was S531L (TCG → TTG), identified in 19 of the 33 mutated isolates (58%). One multidrug-resistant isolate presented another kind of codon 531 mutation, S531W (TCG → TGG). The mutations at codon position 531 amounted to 61% (20/33). Other mutations were detected at *rpoB* codons 526, 516, 533, and 513. Two double mutations, both affecting codon 511, were found. The double mutation of L511R (CTG → CGG) and H516Y (GAC → TAC) has not been reported previously. None of the 30 susceptible control strains carried any mutation in *rpoB*.

The molecular mechanisms of INH resistance are highly complex and affect several genes that are involved in mycolic acid biosynthesis or are overexpressed as a response to the buildup of cellular toxicity of INH (Ramaswamy and Musser, 1998; Herrera *et al*., 2004). Frequencies of the *katG* 315 mutations from different

geographic regions show obvious discrepancies (Herrera *et al*., 2004; Coll *et al*., 2005). Our study detected mutations of *katG* codon 315 in 28 of the 36 MDR-TB strains (78%), with the most common nucleotide change being from AGC to ACC (S315T). Additionally, three (8%) had mutations in the putative *inh*A regulatory region. It is thought that the mutation in the putative *inh*A regulatory sequences may cause overexpression of the InhA protein, which could elevate levels of the drug target for INH, resulting in a more resistant phenotype (Banerjee *et al*., 1994). As a whole, 83% (30/36) of MDR-TB isolates had at least one mutation in the *katG* and *inh*A regulatory region.

Several lines of evidence suggest that EMB exerts its toxic effect on mycobacteria by inhibiting the embABC-encoded proteins and finally inhibits the growth of *M. tuberculosis* by blocking the synthesis of arabinogalactan (Khoo *et al*., 1996, 2001). Associations between EMB resistance and mutations in *embB* have been reported in clinical strains of *M. tuberculosis* (Telenti *et al*., 1997; Rinder *et al*., 2001). Mokrousov *et al*. (2002) and Hazbon *et al*. (2005) recently reported, however, that some clinical *M. tuberculosis* isolates from different regions were susceptible to EMB yet had mutations in *embB*306. These results strongly suggest that *embB*306 mutations do not cause EMB resistance in *M. tuberculosis*. But *embB*306 mutations may predispose *M. tuberculosis* to become resistant to any antibiotic and to become multidrug resistant. Our results revealed 13 (59%) *embB*306 mutants in the 22 EMB-resistant MDR isolates and 3 *embB*306 mutants in EMB-susceptible MDR isolates. No mutations in *embB* were detected in the 30 strains susceptible to INH, RIF, and EMB. Although recent studies demonstrated that EMB-resistant isolates with *embB*306 mutations were also resistant to isoniazid (Lee *et al*., 2004; Parsons *et al*., 2005), our results primarily revealed that *embB*306 mutants were more frequently accompanied by *rpoB* mutations (100%, 16/16) than by *katG*315 mutations (75%, 12/16). Alterations at *embB*306 may not confer resistance to EMB and may be only polymorphisms, which increase the likelihood that any type of drug resistance will develop (Hazbon *et al*., 2005). Obviously, further investigations are imperative in order to understand the involvement of these drug-resistant genes in the molecular genetic mechanism of EMB resistance.

This is the first report on simultaneous genotypic analysis of the *rpoB, katG*, *inh*A regulatory region, and *embB* in multidrug-resistant *M. tuberculosis* isolates recovered from the WHO drug resistance surveillance program in Central China. Our results show geographic variations in the molecular mechanism responsible for RIF and INH resistance. The relatively high prevalence of mutations within the *rpoB* core region, *katG* codon 315, and *inh*A regulatory region is favorable for developing a rapid and reliable molecular drug susceptibility test for multidrug-resistant *M. tuberculosis*. Our results primarily reveal the association between *embB*306 mutants and *rpoB* mutations and *katG*315 mutations in multidrug-resistant *M. tuberculosis* isolates. Further genotypic analysis of multidrug-resistant

*M. tuberculosis* isolates from diverse geographic origins would facilitate research on molecular mechanisms of drug resistance in *M. tuberculosis* and the development of a rapid approach to molecular screening for drug resistant *M. tuberculosis*, which is necessary for the effective control and prevention of tuberculosis and MDR-TB.

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