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Mutations in the *rpoB* Gene of Multidrug-Resistant *Mycobacterium tuberculosis* Isolates from China

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Mutations in the 81-bp rifampin resistance determining region (RRDR) and mutation V176F locating at the beginning of the *ropB* gene were analyzed by DNA sequencing of 86 *Mycobacterium tuberculosis* clinical isolates (72 resistant and 14 sensitive) from different parts of China. Sixty-five mutations of 22 distinct kinds, 21 point mutations, and 1 insertion were found in 65 of 72 resistant isolates. The most common mutations were in codons 531 (41%), 526 (40%), and 516 (4%). Mutations were not found in seven (10%) of the resistant isolates. Six new alleles within the RRDR, along with five novel mutations outside the RRDR, are reported. None of isolates contained the V176 mutation.

Tuberculosis (TB) remains one of the main threats to humans, causing 8 million new cases and 2 million deaths each year. The problem is becoming more critical with the emergence and spread of multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis*, defined as resistant to at least isoniazid and rifampin (RIF). About 2 to 3% of all new TB cases worldwide are due to MDR strain, and the highest MDR populations among new cases have been found in China (11%) and eastern Europe (7 to 14%) (1, 6, 7). China is not only one of the 22 high-burden countries that collectively account for ca. 80% of the world's TB cases but it is also the hotspot area of very high prevalence of MDR TB identified by the World Health Organization (4, 5). Because of the very large financial implications of the treatment and spread of MDR strains due to globalization, MDR TB has been classified as a global pandemic more deadly than AIDS, with the potential to destabilize society.

RIF is one of the principal first-line drugs used in combination chemotherapy and RIF resistance (Rif^r) is a valuable surrogate marker of MDR TB (3). RIF interferes with transcription and elongation of RNA by binding to the β-subunit of RNA polymerase. It has been observed that >90% of Rif^r strains of *M. tuberculosis* possess genetic alterations within an 81-bp fragment, the so-called Rif^r-determining region (RRDR), of the *rpoB* gene, which codes for the beta subunit of the RNA polymerase (17). The types of mutations include single-nucleotide changes and deletions and insertions.

Compilation of data available from many studies indicated that Rif^r clinical isolates of *M. tuberculosis* from diverse geographic regions had 87 distinct point mutations or short insertions and deletions located in an 81-bp core RRDR of *rpoB* codons 507 to 533 encoding 27 amino acids (2, 11, 12, 14–16, 18, 19, 21). Few of these findings are associated with Chinese isolates. Therefore, the aim of the present study was to characterize mutations in the RRDR of the *rpoB* gene by DNA sequencing, the prevalence of the recently described mutation V176F (10), located at the beginning of the *rpoB* gene and associated with Rif^r, was also determined. A total of 72 MDR *M. tuberculosis* isolates from five provinces of China (Guangxi Zhuang autonomous region [GX], Jilin [JL], Zhejiang [ZJ], Jiangsu [JS], and Anhui [AH]). GX is located in the southern region. ZJ, JS, and AH are located in the eastern region. JL is located in the northern region.

***M. tuberculosis* isolates.** A total of 72 Rif^r and 14 RIF-sensitive (Rif^s) strains were recovered from 86 patients from five province in China. Of the 72 Rif^r strains, the numbers isolated from each province were as follows: GX, 26; JL, 16; ZJ, 15; JS, 8; and AH, 7. Of the 14 Rif^s strains, 5 were collected from GX, 4 were collected from JL, and 5 were collected from ZJ.

Drug susceptibility testing. Drug susceptibility was determined on Lowenstein-Jensen medium by absolute concentration method and/or the modified proportion method in a Bactec 460TB apparatus in accordance with the manufacturer's instructions. RIF sensitivity was determined again by the MIC method with serial dilutions of RIF. Resistance was defined by an MIC of ≥128 mg/liter. The drug susceptibility profiles of these isolates are shown in Table 1.

Sequencing of *rpoB*. Two regions of *rpoB* associated with Rif^r were sequenced after amplication by PCR. Template DNA for PCR was prepared by chloroform-isoamyl alcohol extraction as previously described (8). Two oligonucleotide primers designed by Primer 5 software, rpoB101F (5′-TACG GTCGGCGAGCTGATCC-3′) and rpoB101R (5′-TACGGC GTTTCGATGAACC-3′), were used to amplify a 411-bp fragment of the *rpoB* gene, from nucleotides 2201 to 2611 (GenBank accession no. L27989), containing the 81-bp hypervariable region. For analysis of the presence of the V176F mutation, a 365-bp fragment of the *rpoB* gene was amplified by using primers TB-176-F (5′-CTTCTCCGGGTCGATGTCGT TG-3′) and TB-176-R (5′-CGCGCTTGTCGACGTCAAACT C-3′) as recently described by Heep et al. (10). The PCR product was sequenced by using a Prism 377 automated DNA sequencer (Applied Biosystems, Inc., Foster City, Calif.). For

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TABLE 1. Drug susceptibility patterns of MDR *M. tuberculosis* strains isolated in China

No. of strains	Susceptibility to[a]:			
	INH	RIF	EMB	STR
42	R	R	R	R
13	R	R	R	
15	R	R		
2	R	R		R

[a] INH, isoniazid; EMB, ethambutol; STR, streptomycin; R, resistant.

each sample, the sequence was examined twice in one direction by using as a template the products of two independent amplification reactions. The isolates that showed new mutations were sequenced again by cloning PCR products into the M13 plasmid. Sequence data were assembled and analyzed by CLUSTAL W.

IS*6110*-based restriction fragment-length polymorphism (RFLP). *Pvu*I-digested DNA of *M. tuberculosis* was probed with the insertion element IS*6110* according to the standardized protocol of van Embden et al. (22).

Mutations in the *ropB* gene of Rif^r *M. tuberculosis* isolates from GX. In this group, DNA sequence analysis of 26 resistant isolates showed that 24 had 9 different kinds of missense mutations within a 411-bp region of the *rpoB* gene containing 81-bp RRDR. All isolates had a single point mutation, and the highest frequency of mutation was observed in the codon 526 (50%). Point mutations in codons 531 (35%), 516 (8%), and 511 (4%) were also observed. No mutations were found in the 411-bp *rpoB* segment from two Rif^r isolates and five Rif^s isolates.

Mutations in the *rpoB* gene of Rif^r *M. tuberculosis* isolates from JL. Twenty *M. tuberculosis* isolates (16 Rif^r and 4 Rif^s) were analyzed. Single-point mutations were found in 14 of 16 Rif^r isolates. Six different kinds of nucleotide substitution were detected in three codons of RRDR in the *rpoB* gene. Mutations in codon 531 (50%), 526 (31%), and 522 (6%) were also detected. Two *M. tuberculosis* Rif^r isolates contained no mutations within the region of *rpoB* gene examined. No mutations were observed in the Rif^s isolates.

Mutations in the *rpoB* gene of Rif^r *M. tuberculosis* isolates from the eastern region (ZJ, AH, and JS). DNA sequence analysis of the 30 Rif^r isolates from eastern region of China showed that 23 had a single mutation, two had double mutations, and two had quadruple mutations in the 411-bp fragment of the *ropB* gene. Three isolates did not contain any mutation. Eighteen kinds of mutation, seventeen point muatations, and one insertion were observed in the 81-bp RRDR of the *ropB* gene. Most mutations occurred in codons 531 (40%) and 526 (30%). Two isolates from ZJ that contained four mutations had four novel mutations. One of the two isolates had mutations at codon 505 (TTC to TCC), codon 522 (TCG to CCG), codon 526 (CAC to CGC), and codon 531 (TCG to TTG), whereas the other shows changes in codon 500 (GCC to GTC), codon 507 (GGC to GGT), codon 518 (AAC to TAC), and codon 538 (CTG to CCG). Two Rif^r isolates from JS contained point mutations in two separate codons, resulting in two amino acid substitutions for each isolate (506 and 507, 502 and 526). None of the five sensitive strains contained any mutation.

TABLE 2. Distribution of mutations found in the RRDR of the *rpoB* gene in Rif *M. tuberculosis* isolates from China

Mutated codon	Specific mutation	Distribution (province, no.)[a]
507	GGC→GAC	ZJ, 1
	GGC→GGT[b]	ZJ, 1
511	CTG→CCG	AH, 1
513	CAA→AAA	JS, 1
514	TTC→Ins[d]	ZJ, 1
516	GAC→GGC	GX, 1
	GAC→TAC	GX, 1
	GAC→GTC	ZJ, 1
518	AAC→TAC[b]	AH, 1
522	TCG→CCG[b]	JL, 1; ZJ, 1
526	CAC→GAC	GX, 9; JL, 1; JS, 1; ZJ, 1
	CAC→CTC	GX, 2; JL, 1; ZJ, 3
	CAC→CCC	GX, 1
	CAC→CGC	ZJ, 1; AH, 1
	CAC→TAC	JL, 3
	CAC→AAC	AH, 1
	CAC→GCC[b,c]	JS, 1
531	TCG→TTG	GX, 1; JL, 7; JS, 3; AH, 2; ZJ, 5
	TCG→TGG	GX, 6
	TCG→CAG	JL, 1
	TCG→TAC[b,c]	ZJ, 1; GX, 1
533	CTG→CCG[b]	AH, 1

[a] Provinces are abbreviated as in the text.
[b] New allele.
[c] Double mutations in the same codon.
[d] Ins, insertion.

General analysis. The MICs of RIF for all 72 Rif^r isolates were >128 mg/liter, whereas the MICs of RIF for all 14 Rif^s isolates were <32 mg/liter. The analysis of 72 Rif^r isolates revealed that 65 had mutations within a 411-bp fragment of the *rpoB* gene (Fig. 1). Twenty-two different types of mutations were identified in the 81-bp RRDR of the *rpoB* gene among 72 Rif^r *M. tuberculosis* clinical isolates, and six new alleles were identified (Table 2). Most of them were single-nucleotide mutations (90%) involving nine codons, whereas only one isolate had an insertion. The codons most frequently affected by point mutations were 531, 526, and 516, with frequencies of 41, 40, and 5%, repectively. No mutations were revealed in the *rpoB* segment sequenced from 14 Rif^r isolates. Seven Rif^r isolates (10%) contained no mutations in this sequenced region, although these isolates were resistant to RIF as determined by the MIC method. The V176F mutation could not be found in the beginning of the *ropB* gene in all organisms.

In our study, we observed that 90% of the *M. tuberculosis* isolates with the Rif^r phenotype contained missense mutations that led to amino acid substitutions at the Ser-531 (41%), His-526 (40%), and Asp-516 (5%) residues. This finding is similar to results reported by Ramaswamy and Musser, who determined frequencies of 41 and 36% for various mutations occurring at codons 531 and 526, respectively, in 478 isolates obtained from various parts of the world (17). Although Qian et al. (16) reported a low frequency of the mutation at codon 526 (4%) in China isolates, we found a high frequency of this mutation (40%). Similarly high frequencies of this mutation at codon 526 have found in isolates from Korea (38%) (13), Japan (33%) (16), Italy (30%) (15), and Greece (19%) (14). The likelihood of a mutation at codon 526 is higher than at codon 531 in isolates from GX. There was no specific geo-

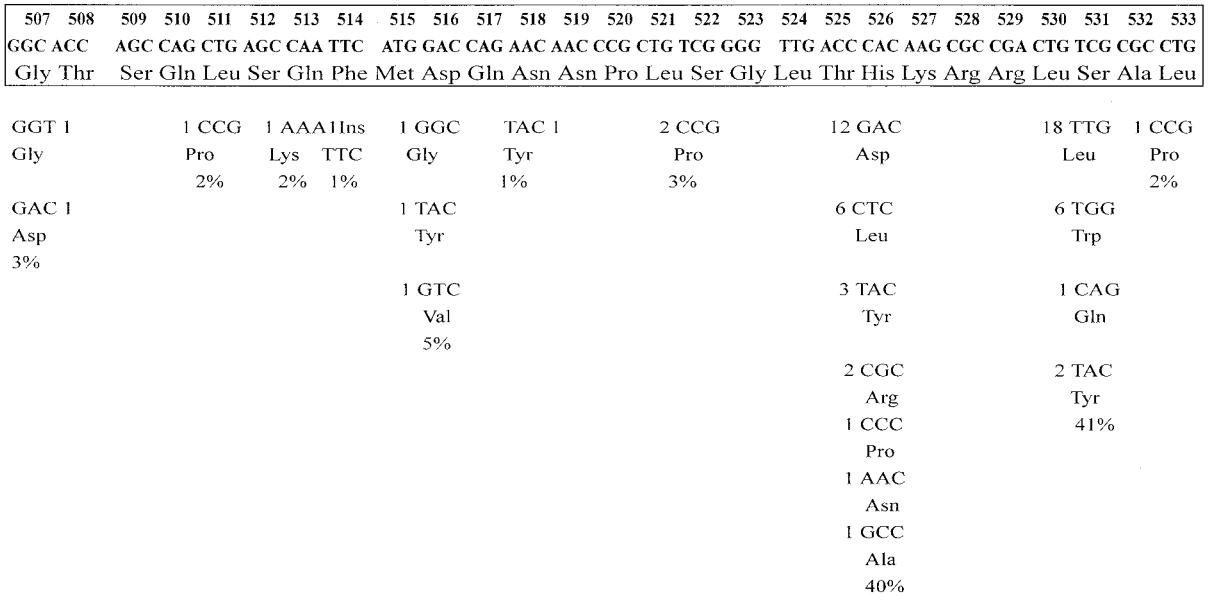

FIG. 1. Mutations located in the RRDR of the *M. tuberculosis rpoB* gene in 65 Rif^r isolates from China. The bottom panel shows the mutated codons with corresponding amino acids. The original sequence is boxed. Numbers to the left or below amino acid designations indicate numbers of isolates showing the mutation, while the percentages denote the frequencies of occurrence of mutations at the particular codon.

graphical clustering of isolates in terms of mutations. Analysis with a large number of samples is the only way to reach definitive conclusions.

Six new alleles within the RRDR, along with five novel mutations outside the RRDR, were recognized in this investigation. New alleles include mutations from GGC (Gly) to GGT (Gly) at codon 507, AAC (Asn) to TAC (Tyr) at codon 518, TCG (Ser) to CCG (Pro) at codon 522, CAC (His) to GCC (Ala) at codon 526, TCG (Ser) to TAC (Tyr) at codon 531, and CTG (Leu) to CCG (Pro) at codon 533. New mutations outside the RRDR were seen in four isolates with double or multiple mutations (GCC to GTC at codon 500, ATC to GTC at codon 502, TTC to TCC at codon 505, TTC to TTT at codon 506, and CTG to CCG at codon 538). Similar findings were reported worldwide. (2, 14, 18, 23).

In contrast to results revealed by Taniguchi et al. (20), who suggest a strong correlation of specific amion acid substitutions and MIC, our results showed high MICs for two isolates with a mutation at codon 514 (MIC = 256 mg/liter) or codon 533 (MIC = 512 mg/liter). Similar findings were reported by Cheruvu et al. (2).

No mutations were detected in V176 residue of the *rpoB* gene from these isolates. Mutation V176 appears to confer high-level resistance in clinical *M. tuberculosis* isolates and may account for >1% of all Rif^r strains (9). No mutation V176 was detected in the present study; an inadequate number of samples may be the reason. Another possibility is that these strains lack the V176 mutation.

Ten percent of Rif^r *M. tuberculosis* isolates in the present study did not show any mutation in the RRDR and the V176 codon of *rpoB* gene. These results differ from the data showing that only ~4% of the Rif^r isolates lack RRDR changes. We retested the resistance of these isolates to RIF, and the results that demonstrated our drug sensitivity test is reliable and accurate. The presence of a mutation outside the regions investigated in this gene or mutation of these genes whose products participate in antibiotic permeation or metabolism or some other resistant mechanism, such as unexplained resistance or heteroresistance, may account for the Rif^r phenotype in these isolates. The alarmingly high percentage of isolates lacking mutations seems to represent an important impediment to molecular drug resistance testing. The currently available molecular methods are designed to determine the expected mutations within the RRDR of the *rpoB* gene. Therefore, although the molecular methods may aid in the rapid detection of mutations associated with drug resistance, the tested results must always be confirmed by phenotypic methods.

Knowing the strain genotype would distinguish whether this rare mutation occurred twice in two populations or happened once and then spread to another region. We analyzed 40 isolates of *M. tuberculoisis* in which the mutation of the *rpoB* gene was observed more than once by IS*6110* RFLP. Most of isolates shared 8 to 21 copies. There were 37 unique fingerprints. A total of 27 (68%) of 40 isolates had an IS*6110*-based banding pattern characteristic of the Beijing genotype of *M. tuberculosis*. This Beijing family of isolates was highly related to MDR. Some isolates with the same point mutation in the *rpoB* gene from two provinces had different IS*6110* patterns, suggesting that there was no direct transmission within regions. Two isolates with similar IS*6110* fingerprints have different mutations in *rpoB*. *rpoB* genotyping can also be used to discriminate between Rif^r *M. tuberculosis* isolates with identical IS*6110* fingerprints.

In conclusion, the analysis of the 81-bp RRDR and V176 of the *rpoB* gene of 86 *M. tuberculosis* clinical isolates from various parts of China was performed and mutations were recognized. Six new alleles and five novel mutations were recognized. The profile of mutations in the 81-bp RRDR is similar

to that of the majority of isolates worldwide. More information about these mutations would be helpful in the development of novel molecular diagnostic methods such as the DNA line probe and DNA microarray, which will be implemented in China. The high percentage of Rif^r isolates with no mutations suggests that phenotypic methods remain an important complement to genotypic methods for drug susceptibility testing.

Nucleotide sequence accession numbers. The sequences with mutations in the new alleles are deposited in GenBank under accession numbers AY147213, AY147214, AY147215, AY147216, AY147217, and AY147218. Those with mutations outside RRDR are deposited under accession numbers AY147208, AY147209, AY147210, AY147211, and AY147212.

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