The Beginning of the *rpoB* Gene in Addition to the Rifampin Resistance Determination Region Might Be Needed for Identifying Rifampin/Rifabutin Cross-Resistance in Multidrug-Resistant *Mycobacterium tuberculosis* Isolates from Southern China

Yaoju Tan,^a Zuqiong Hu,^a Yanlin Zhao,^b Xingshan Cai,^a Chunming Luo,^a Cairong Zou,^a and Xin Liu^a

Department of Clinical Laboratory, Guangzhou Chest Hospital, Guangdong Province, Guangzhou, China,^a and National Tuberculosis Reference Laboratory, China Center for Disease Control and Prevention, Beijing, China^b

We aimed to study the distribution and contribution of mutations in the *rpoB* whole gene in rifampin-resistant/rifabutin-resistant (RIF^r/Rfb^r) (or RIF/Rfb cross-resistant) clinical *Mycobacterium tuberculosis* isolates. One standard *M. tuberculosis* strain (H37Rv) and 392 other clinical *M. tuberculosis* isolates mainly from Guangdong Province of China whose susceptibilities to rifampin (RIF), rifabutin (Rfb), streptomycin (SM), ethambutol (EMB), and isoniazid (INH) were previously determined were subjected to DNA sequencing of their *rpoB* whole genes. H37Rv and the 30 drug-susceptible clinical isolates had no mutations in *rpoB* whole genes. In 43 rifampin-resistant/rifabutin-susceptible (RIF^r/Rfb^s) isolates, the most frequent mutation codons were 516 (62.80%), 526 (14.0%), and 533 (6.98%), but codon 531 had no mutation. Twenty-one of the 43 isolates (48.84%) had single mutations of H526L, H526S, D516V, D516Y, and D516F. In 319 RIF^r/Rfb^r isolates, the most frequent mutation codons were 531 (73.7%) and 526 (18.8%); the mutation frequency for codon 516 was 2.5%, and that for codon 533 was only 0.31%. A total of 82.8% (264/319) of them had single mutations of S531L, S531W, H526D, H526Y, H526R, Q513K, Q513P, Q510H, V176F, P206^TR, Y314^TC, and H323^TY (the superscript T indicates *M. tuberculosis* numbering; the remaining codons use the *E. coli* numbering), among which V176F, P206^TR, Y314^TC, and H323^TY were located in the beginning of *rpoB*, and all of them were present in 1.9% (6/319) of RIF^r/Rfb^r isolates. The multiple mutations in RIF^r/Rfb^r isolates and in RIF^r/Rfb^s isolates were also different from each other either in mutation positions or in types of mutation combinations. In conclusion, the mutations of *rpoB* in RIF-R/Rfb^s and in RIF-R/Rfb-R isolates differ significantly from each other not only in the most frequent mutation codons (516, 531, and 533) but also in the most frequent single mutations (S531L, H526L, D516V, D516Y, and D516F), and the beginning of *rpoB* may confer a RIF/Rfb cross-resistance phenotype in *M. tuberculosis*. Molecular assays for identifying RIF/Rfb cross-resistance in *M. tuberculosis* might be improved in terms of accuracy by including this region, in addition to the rifampin resistance determination region.

According to the data reported by the World Health Organization (WHO) in 2010, an estimated 440,000 cases of multidrug-resistant tuberculosis (MDR-TB) (primary and acquired) arose in 2008. Among all incident TB cases globally, 3.6% are estimated to have MDR-TB (19), so a series of new drugs or alternatives for treatment of MDR-TB has been recommended by WHO since 2008 (20). Rifabutin (Rfb) has been one of the most efficacious drugs and the first-line oral agent recommended by WHO for MDR-TB, but Rfb sometimes has cross-resistance to rifampin (RIF) (2, 4, 15), which implies that the characterization of *rpoB* mutations in Rfb-resistant (Rfb^r) isolates is not completely the same as that of *rpoB* mutations in RIF-resistant (RIF^r) isolates. There have been few data supporting this conjecture to this day because of a lack of characterization of *rpoB* mutations in Rfb^r clinical *Mycobacterium tuberculosis* isolates, so it is very hard to identify the Rfb^r isolates from RIF^r isolates, even though so many molecular assays for prediction of rifampin susceptibility have been established (8, 1, 13, 3, 5). Furthermore, phenotypic drug susceptibility testing is too time-consuming, which is a tremendous obstacle for MDR-TB therapy with rifabutin.

According to the results of research presented previously, the mechanism of resistance to rifampin and rifabutin is commonly amino acid exchange in the β subunit (*rpoB*) of the DNA-directed RNA polymerase. So, the *rpoB* mutations in *M. tuberculosis* iso-

lates, whose susceptibility to rifampin, rifabutin, and isoniazid had been previously determined, were characterized after their *rpoB* whole genes were sequenced.

MATERIALS AND METHODS

Clinical *M. tuberculosis* isolates. *M. tuberculosis* clinical strains ($n = 392$) were originally isolated from August 2008 to February 2011 from 392 different pulmonary tuberculosis patients (age range, 17 to 84 years) of the Guangzhou Chest Hospital in southern China. Of these patients, 347 came from Guangdong Province, 13 from Guangxi Province, 16 from Hunan Province, 9 from Jiangxi Province, and 7 from Hainan Province. Of the 392 drug-resistant isolates, 362 were from patients after retreatment for RIF^r pulmonary tuberculosis. Thirty drug-susceptible isolates were from patients with new cases of pulmonary tuberculosis. Among the 362

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Address correspondence to Yaoju Tan, gzchtan@163.com, or Yanlin Zhao, zhaoyanlin@tb123.org.

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

No. of strains	Susceptibility to ^a :				
	RIF	Rfb	INH	SM	EMB
37	R	R	R	S	R
13	R	R	R	S	S
154	R	R	R	R	R
86	R	R	R	R	S
12	R	R	S	S	S
17	R	R	S	R	S
12	R	S	R	S	S
14	R	S	R	S	R
2	R	S	R	R	S
12	R	S	R	R	R
3	R	S	S	S	R
30	S	S	S	S	S

^a R, resistant; S, susceptible.

drug-resistant isolates, 319 isolates were rifampin resistant/rifabutin resistant (RIF^r/Rfb^r), or RIF/Rfb cross resistant, including 290 isoniazid (INH)-resistant isolates and 29 INH-susceptible isolates, 257 streptomycin (SM)-resistant and 62 SM-susceptible isolates, and 191 ethambutol (EMB)-resistant and 128 EMB-susceptible isolates. Forty-three isolates were rifampin resistant/rifabutin susceptible (RIF^r/Rfb^s), including 40 INH-resistant isolates and 3 INH-susceptible isolates, 14 SM-resistant and 29 SM-susceptible isolates, and 29 EMB-resistant and 14 EMB-susceptible isolates. One standard *M. tuberculosis* H37Rv strain conserved by the National Tuberculosis Reference Laboratory, Beijing, China, was used as a control in this study.

Primer design. According to the *rpoB* entire gene sequence of H37Rv recorded by GenBank (GenBank accession no. [BX842574.1](#)), primers F1 and F4 were designed by the software Primer Premier, version 5.0 for Windows and Power Macintosh (Premier, Canada), and were synthesized (Sangon, Shanghai, China) (primer F1, 5'-ACAAAATTATCGCGGCGA AC-3'; primer F4, 5'-CCGTCCTTCTCCGGCTTAAGCG-3').

DNA extraction. The GenoLyse reagent (version 1.0; Hain Lifescience GmbH, Nehren, Germany) was used for DNA extraction according to the instructions of the manufacturer.

PCR amplification. PCR was carried out in 50.25-μl volumes of reaction mixture containing 0.25 μl PrimerSTAR HS polymerase (TaKaRa), 5 μl 10× PrimerSTAR buffer (TaKaRa), 8 μl 2.5 mM deoxynucleoside triphosphate mixture (TaKaRa), 0.5 μl 20 μM each primer, 31 μl water, and 5 μl of DNA extract. Thermal cycling was performed in a thermocycler (Eppendorf) by the following program: 94°C for 3 min, 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min, and a final step at 72°C for 5 min. The full length of the PCR product was 3,782 bp, which covered the −85 bp upstream of the start codon to +178 bp downstream of the terminator codon of the *rpoB* gene.

DNA sequencing and sequence analysis. The PCR products were confirmed by agarose gel electrophoresis, and the sequences were then determined by standard Sanger DNA sequencing on a 3730XL genetic analyzer (Applied Biosystems), after they were purified. The sequences of the *rpoB* genes from DNA sequencing were analyzed by ClustalX and DNASTar software.

RESULTS

Drug susceptibility patterns of clinical isolates. The drug susceptibility patterns of the clinical isolates are summarized in Table 1.

Main mutations inside and outside the RRDR of *rpoB* whole gene in 362 drug-resistant isolates. Among the 319 RIF^r/Rfb^r clinical *M. tuberculosis* isolates, 313 (98.1%) isolates had mutations inside the rifampin resistance determination region (RRDR)

TABLE 2 Comparison of the most frequent point mutations between the RIF^r/Rfb^r isolates and the RIF^r/Rfb^s isolates

Mutation position	Frequency (%) of mutation		
	RIF ^r /Rfb ^r isolates (n = 319)	RIF ^r /Rfb ^s isolates (n = 43)	P ^a
533	0.31	6.98	0.006
531	73.70	0.00	0
526	18.80	14.0	0.439
516	2.50	62.80	0

^a The P values were obtained from comparison of the frequencies of the most frequent mutation points between the RIF^r/Rfb^r isolates and the RIF^r/Rfb^s isolates.

and 6 (1.9%) isolates had single mutations outside the RRDR. There were 55 kinds of mutations observed in RIF^r/Rfb^r isolates, of which 37 were located outside the RRDR and 18 were located inside the RRDR, but the most frequently observed mutation codons were 531 (73.7%) and 526 (18.8%), while codon 516 had a mutation frequency of 2.50% and codon 533 had one of only 0.31%. It might be of interest that there were no combined mutations among codons 516, 526, and 531. All of the 43 RIF^r/Rfb^s isolates had mutations inside the RRDR in the *rpoB* gene. There were 23 types of mutations present in the 43 isolates, of which 6 were located outside the RRDR and 17 were inside the RRDR, but the codons that most frequently had mutations were 516 (62.80%), 526 (14.0%), and 533 (6.98%). There were also no combined mutations among the 3 codons and no codon 531 mutation in RIF^r/Rfb^s isolates. The mutation frequency of codon 531 was significantly higher in RIF^r/Rfb^r isolates than in RIF^r/Rfb^s isolates (73.7% versus 0.0%; P = 0), and the mutation frequencies of codons 516 and 533 were separately significantly lower in RIF^r/Rfb^r isolates than in RIF^r/Rfb^s isolates (2.5% versus 62.8% [P = 0] and 0.31% versus 6.98% [P = 0.006], respectively). The mutations for all codons are summarized in Table 2. The reference strain H37Rv and the 30 drug-susceptible isolates had no mutations in the *rpoB* whole gene.

Novel mutations found in 362 drug-resistant isolates. Thirty-three novel mutations, mainly located outside the RRDR, were found in RIF^r/Rfb^r isolates. These included 3 single mutations (P206^TR, Y314^T, 323^TY [the superscript T indicates *M. tuberculosis* numbering; the remaining codons use the *E. coli* numbering]) outside the RRDR, and the remaining 30 novel mutations were in combination with other mutations (marked with italics in Table 3 and Table 4). Seven novel mutations were present in RIF^r/Rfb^s isolates, but no single novel mutations occurred (Table 3).

Single mutations and multiple mutations in RIF^r/Rfb^r isolates. All of the 319 RIF^r/Rfb^r isolates were found to have mutations in the *rpoB* gene, of which 264 isolates had single mutations: S531L (201 isolates), S531W (5 isolates), H526D (20 isolates), H526Y (15 isolates), H526R (10 isolates), Q513K (3 isolates), Q513P (2 isolates), Q510H (2 isolates), V176F (2 isolates), P206^TR (1 isolate), Y314^TC (2 isolates), and H323^TY (1 isolate). Their mutation frequencies (Fig. 1) were compared to those in RIF^r/Rfb^s isolates and are described in Table 3. The remaining 55 isolates had multiple mutations, of which 3 isolates possessed triple mutations: S531W/S345P/S955Y in 1 isolate, 516G/L511P/S509R in 1 isolate, and Q513K/G920C/K1167R in 1 isolate. Fifty-two isolates had double mutations. These are listed in Table 4 and described in Fig. 1.

Single mutations and multiple mutations in RIF^r/Rfb^s isolates. All of the 43 RIF^r/Rfb^s isolates had mutations inside the

TABLE 3 Single point mutations in RIF^r/Rfb^r and RIF^r/Rfb^s isolates

Single amino acid change ^a	No. of isolates with single point mutation		<i>P</i> ^b
	RIF ^r /Rfb ^r isolates (<i>n</i> = 319)	RIF ^r /Rfb ^s isolates (<i>n</i> = 43)	
S531L	201	0	0
S531W	5	0	1
H526L	0	4	0
H526S	0	1	0.119
H526D	20	0	0.182
H526Y	15	0	0.296
H526R	10	0	0.495
D516V	0	10	0
D516Y	0	4	0
D516F	0	2	0.014
Q513K	3	0	1
Q513P	2	0	1
Q510H	2	0	1
V176F	2	0	1
P206 ^T R	1	0	1
Y314 ^T C	2	0	1
H323 ^T Y	1	0	1

^a In this study, codons with superscript T designations were derived from the *M. tuberculosis* numbering. The remaining codon designations were derived from the *E. coli* numbering. Italics indicate the novel mutations found in our results.

^b The *P* values were obtained from comparison of the mutation frequencies of the single amino acid changes between the RIF^r/Rfb^r isolates and the RIF^r/Rfb^s isolates.

RRDR in the *rpoB* gene, of which 21 isolates had separate single mutations: H526L (4 isolates), H526S (1 isolate), D516V (10 isolates), D516Y (4 isolates), and D516F (2 isolates). These are described in Table 3 and in Fig. 2. The other 22 isolates had multiple mutations, including 3 isolates with triple mutations: D516G/Q513H/M515V in 2 isolates and H526Q/L511P/L420V in 1 isolate. Nineteen isolates had double mutations, and these are listed in Table 4 and described in Fig. 2.

DISCUSSION

In order to provide more information than those studies that analyzed only the RRDR of the *rpoB* gene, we sequenced the *rpoB* whole gene of *M. tuberculosis*. The DNA sequences of the *rpoB* whole gene demonstrated that all of the 362 rifamycin-resistant isolates had missense mutations in the *rpoB* entire gene, while 30 susceptible isolates and 1 reference strain (strain H37Rv) of *M. tuberculosis* had no mutations in the *rpoB* whole gene, which was in line with the previous findings that the susceptibility to rifampin and that to rifabutin in *M. tuberculosis* are closely correlated with mutation of the *rpoB* gene (9, 12, 18). Furthermore, more than 95% of RIF^r isolates in our study possessed mutations inside the RRDR of the *rpoB* gene, which was consistent with the data reported by previous researchers (10, 17, 11). However, there were still 33 kinds of novel mutations outside the RRDR in RIF^r/Rfb^r isolates, including in 6 (1.9%) isolates with single mutations (2 isolates with V176F, 1 with P206^TR, 2 with Y314^TC, 1 with H323^TY) in the beginning of the *rpoB* gene, and also 4 kinds of novel mutations outside the RRDR in RIF^r/Rfb^s isolates. These mutations may be responsible for the detection of rifampin susceptibility when only the RRDR in the *rpoB* gene is targeted, a result that fails to match the resistance phenotype (14). This implies that use of only the RRDR as the target region for detection of RIF/Rfb susceptibility is not enough for *M. tuberculosis*. Perhaps

TABLE 4 Multiple mutations in 362 drug-resistant isolates

Positions and type of mutation ^a	No. of isolates with multiple mutations	
	RIF ^r /Rfb ^r isolates	RIF ^r /Rfb ^s isolates
L533P/D346G		2
L533P/E562A		1
S531L/S103L	1	
S531L/P111S	1	
S531L/P126A	1	
S531L/P126S	3	
S531L/K272E	1	
S531L/S335P	1	
S531W/S345P/S955Y	1	
S531L/A367V	1	
S531L/V386L	2	
S531L/T480I	1	
S531L/T481A	2	
S531W/H526N	1	
S531L/I561T	1	
S531L/I572V	4	
S531L/V577G	1	
S531L/F584S	2	
S531L/S621A	1	
S531L/D626E	1	
S531L/V643 M	1	
S531L/D652G	1	
S531L/D655E	1	
S531L/L1060R	1	
S531L/V1112A	1	
H526R/Q510H	3	
H526R/R952H	1	
H526Y/L459R	1	
H526Y/S509R	1	
H526Y/E541G	2	
H526N/L511P	1	
H526D/Q513K	1	
H526D/D1251V	1	
H526L/S509G		1
H526Q/L511P/L420V		1
D516G/L511P	2	
D516G/L511P/S509R	1	
D516G/L511R		3
D516G/R529Q	1	
D516G/L533P	1	
D516G/Q513H/M515V		2
D516G/S522L		1
D516V/P520D	1	
D516V/N518D		6
D516Y/Q253R	1	
D516Y/L511P	1	
D516N/R529Q		1
D516E/I572T		1
Q513L/E247G	1	
Q513K/Q880K	1	
Q513K/H916R	1	
Q513K/G920C/K1167R	1	
L511P/I572 M	1	
L511P/S574L		3
Total	55	22

^a In this study, codons with superscript T designations were derived from the *M. tuberculosis* numbering. The remaining codon designations were derived from the *E. coli* numbering. Italics indicate the novel mutations found in our results.

FIG 1 Single point mutations and combined mutations in RIF^r/Rfb^r isolates of *M. tuberculosis*. Mutation means that there is a point mutation in the *rpoB* whole gene of *M. tuberculosis*. Single mutation means only one mutation point in the *rpoB* whole gene of *M. tuberculosis*. Multiple mutation means more than one mutation point in the *rpoB* whole gene of *M. tuberculosis*. Double mutation means two mutations in the *rpoB* whole gene of *M. tuberculosis*. Triple mutation means three mutations in the *rpoB* whole gene of *M. tuberculosis*.

the beginning of the *rpoB* gene should also be added as another target for detecting RIF/Rfb cross-resistance in *M. tuberculosis*.

The distribution and frequency of main mutations of *rpoB* in RIF^r/Rfb^r isolates and in RIF^r/Rfb^s isolates were different from each other and had no correlation with their susceptibility to INH, SM, and EMB. In the RIF^r/Rfb^r isolates, the most frequently observed mutation codons were 531 (73.7%) and 526 (18.8%); codon 516 had a mutation frequency of 2.50% and codon 533 had one of only 0.31%, both of which were significantly lower than that in RIF^r/Rfb^s isolates, while the mutation frequency of codon 531 was significantly higher than that in RIF^r/Rfb^s isolates. Though the mutation frequency of codon 526 in RIF^r/Rfb^r isolates was not significantly higher than that in RIF^r/Rfb^s isolates (18.8% versus 14.0%; $P = 0.439$), the types of amino acid substitutions of their single mutations and the combination types of their multiple mutations were completely different from each other.

Some 82.8% (264/319) of RIF^r/Rfb^r *M. tuberculosis* isolates separately possessed single mutations S531L, S531W, H526D, H526Y, H526R, Q513K, Q513P, Q510H, V176F, P206^TR, Y314^TC, and H323^TY, while 48.8% (21/43) of RIF^r/Rfb^s *M. tuberculosis* isolates contained single mutations of H526L, H526S, D516V, D516Y, and D516F, all of which were completely distinct from those in RIF^r/Rfb^r isolates either in mutation positions or in the types of amino acid substitutions. The frequency of the S531L mutation in RIF^r/Rfb^r isolates was especially significantly higher than that in RIF^r/Rfb^s isolates, while the frequencies of the H526L, D516V, D516Y, and D516F mutations were significantly lower than those in RIF^r/Rfb^s isolates. This finding implied that the S531L mutation is mainly associated with cross-resistance to RIF/Rfb, and the single mutation H526L, D516V, D516Y, or D516F is more likely to have a correlation with resistance to rifampin but

FIG 2 The single point mutations and multiple mutations in RIF^r/Rfb^s isolates of *M. tuberculosis*. Mutation means that there is a point mutation in the *rpoB* whole gene of *M. tuberculosis*. Single mutation means only one mutation point in the *rpoB* whole gene of *M. tuberculosis*. Multiple mutation means more than one mutation point in the *rpoB* whole gene of *M. tuberculosis*. Double mutation means two mutations in the *rpoB* whole gene of *M. tuberculosis*. Triple mutation means three mutations in the *rpoB* whole gene of *M. tuberculosis*.

susceptibility to rifabutin. The insufficient number of isolates investigated may be responsible for the lack of a significant difference between the two groups of isolates for the rest of the single mutations.

Another valuable finding was the 3 novel single mutations Y314^TC, P206^TR, and H323^TY and the rare single mutation V176F in association with a high level of rifampin resistance and with resistance to rifabutin, confirmed by other researchers (6, 7), in RIF^r/Rfb^r *M. tuberculosis* isolates. All of them were located within a narrow region in the beginning of the *ropB* gene, together with the mutation V146F, which has been found to be associated with rifampin resistance, in addition to the RRDR in *M. tuberculosis*, as recently proven in Hong Kong (16). We have reason to believe that the isolates might harbor a novel hot-spot region for RIF/Rfb cross-resistance in *M. tuberculosis* outside the RRDR near the beginning of the *rpoB* gene.

Although all multiple mutations in RIF^r/Rfb^r isolates differed from those in RIF^r/Rfb^s isolates either in mutation positions, in the types of amino acid substitutions, or in the types of combination mutations in this study, the frequencies were too low and the positions were too disperse for a significant difference between the two groups of isolates to be calculated. Maybe a larger number of samples and multicenter research can disclose the correlations between the mutations and RIF/Rfb cross-resistance in *M. tuberculosis*.

In conclusion, our results demonstrated that the specific mutations of the *rpoB* gene in RIF^r/Rfb^r isolates differ sharply from those in RIF^r/Rfb^s isolates in terms of the most frequent mutations, single mutations, and multiple mutations. All of the disparities have implications for improving the accuracy of molecular tests for identifying RIF/Rfb cross-resistance in *M. tuberculosis* if at least the beginning region of the *rpoB*, in addition to the RRDR, is

included. This improved accuracy will facilitate the treatment of patients infected with MDR *M. tuberculosis* isolates and will be helpful in controlling the transmission of TB.

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