Rifampin resistance-associated mutations in the RIF resistance-determining region (RRDR) of the rpoB gene of Mycobacterium tuberculosis clinical isolates in Shanghai, China

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Abstract

Background: Resistance to rifampin (RIF) in Mycobacterium tuberculosis infection is associated with mutations in the rpoB gene coding for the beta-subunit of RNA polymerase. The contribution of many individual rpoB mutations to the development and level of RIF resistance remains elusive. Our objective for this study was to investigate the relationship between specific rpoB mutations and the minimum inhibitory concentrations (MICs) of RIF and rifabutin (RFB) against M.tuberculosis. Methods: We collected 195 clinical isolates of M. tuberculosis including 105 RIF-resistant and 90 RIF-susceptible isolates from Shanghai Pulmonary Hospital in China. The MICs of antituberculosis drugs in 7H10 Middlebrook medium for clinical isolates of M. tuberculosis were determined. Strains were screened for rpoB mutations by DNA extraction, rpoB gene amplification, and DNA sequencing analysis. Results: Twenty different types of mutations were identified in the rpoB gene. One hundred isolates (95.24%) were found to have mutations in the RIF resistance-determining region (RRDR) of the rpoB gene. Three rpoB mutations were identified in 90 RIF-susceptible isolates. Out of 105 isolates, 86 (81.90%) were cross-resistant to both RIF and RFB. The most frequent mutation occurred at codon 531 (65.71%), followed by 526 (8.57%). We also found a novel nine-nucleotide (ATCATGCAT) deletion (between positions 1543 and 1551) in the rpoB gene among two strains (1.90%) with resistance to RIF, but susceptibility to RFB. In addition, the mutation frequency at codon 531 was significantly higher in RIF-resistant/RFB-resistant (RIFR/RFBR) strains than in RIFR/RFBS strains (75.58% versus 21.05%), whereas the mutation frequency at codon 516 was significantly lower in RIFR/RFBR strains than in RIFR/RFBS strains (1.16% versus 26.32%). The MICs of RIF against 87.62% (92/105) of the M.tuberculosis isolates were ≥ 16 μg/mL. Conclusions: Our data supported previous findings that various rpoB mutations are associated with differential levels of resistance to RIF. The specific mutations of the rpoB gene in RIFR/RFBR isolates differed from those in RIFR/RFBS isolates. A novel deletion mutation in the RRDR might be associated with resistance to RIF, but not to RFB. Further clinical studies are required to investigate the efficacy of RFB in the treatment of M. tuberculosis infections, which harbor the mutations.

Background

Tuberculosis (TB), a disease affecting 10 million persons in 2017, remains a major global health problem [1]. Resistance of *Mycobacterium tuberculosis* to a single drug has increased in different areas of the world. Recently, there has also been an increase in the number of reports of multiple drug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB), both of which are associated with considerable mortality and have resulted in serious outbreaks. In addition, more than half of all MDR-TB cases are estimated to occur in India, China, and the Russian Federation [2]. Thus, the control of MDR-TB has become an urgent public health problem in different parts of the world, particularly in developing countries.

Rifampin (RIF) has long been used in combination first-line therapy for TB. The widespread use of RIF has led to the emergence of RIF-resistant strains. Resistance to RIF is high among patients with MDR-TB, and it represents an important surrogate marker for MDR-TB isolates. Therefore, it is important to

understand the molecular basis for this resistance. The cellular target of RIF is the beta-subunit of bacterial RNA polymerase encoded by the *rpoB* gene [3]. Approximately 95% of RIF-resistant *M. tuberculosis* is due to a single mutation in an 81-bp region corresponding to codons from 507 to 533 of *rpoB,* termed the RIF resistance-determining region (RRDR) of the *rpoB* gene [4]. Among different mutations types, non-synonymous mutations are more common than deletion, insertion, and frameshift mutations. However, not all mutations within the RRDR display the same loss of RIF susceptibility [5, 6]. Mutations in *rpoB* can render the organism resistant to RIF, owing to decreased binding affinity, and can result in high-level resistance [7]. At the same time, parts of *rpoB* mutations have been associated with RIF-susceptible phenotypes [8–10].

Rifabutin (RFB) is another member of the RIF family [11, 12] that is recommended as an alternative treatment for *M. tuberculosis* in HIV-infected patients, because it tends to have fewer interactions with protease inhibitor drugs [13]. The importance of exploring the use of RFB in RIF-resistant, RFB-susceptible *M. tuberculosis* treatment is highlighted by the threat RIF-resistance poses to TB control worldwide [1].

In this study, we describe the distribution of *rpoB* mutations found by direct DNA sequencing and analyze drug susceptibility. Our primary aim was to reliably investigate specific *rpoB* mutations and correlations between *rpoB* mutations and the minimum inhibitory concentrations (MICs) of RIF and RFB.

Methods

M. tuberculosis clinical isolates

A total of 2017 *M. tuberculosis* strains were isolated from suspected TB patients between March and August 2018 from Shanghai Pulmonary Hospital, China. In this study, 105 RIF-resistant isolates and 90 RIF-susceptible isolates were retrospectively and randomly selected, respectively. Each strain corresponded to a single patient. All strains were identified as *M. tuberculosis* using the BACTEC MGIT (Mycobacteria growth indicator tube) 960 culture (BD Biosciences, New Jersey, USA) and conventional biochemical methods, and were confirmed by 16S rRNA sequencing. All strains were cultured on Lowenstein–Jensen (LJ) and MGIT liquid media.

Phenotypic drug susceptibility testing

Susceptibility testing of all isolates tested was performed by the broth microdilution method with Roch broth containing OADC (oleic albumin dextrose catalase) supplement (Thermo, USA). The bacterial concentration was adjusted to a McFarland no. 0.5 standard by dilution with Middlebrook 7H9 broth. All isolates were subjected to standardized drug susceptibility testing against RIF, isoniazid (INH), RFB, streptomycin (SM), ethambutol (EMB), ofloxacin (OFX), amikacin (AK), kanamycin (KAN), moxifloxacin (MXF), pyrazinamide (PAS), ethionamide (ETH), and cycloserine (CYC). The critical concentrations of these drugs were 1 mL/L for RIF, 0.2 mL/L for INH, 0.5 mL/L for RFB, 2.0 mL/L for SM, 5.0 mL/L for EMB, 2.0 mL/L for OFX, 4.0 mL/L for AK, 5.0 mL/L for KAN, 0.5 mL/L for MXF, 2.0 mL/L for PAS, 5.0 mL/L for ETH, and 25.0 mL/L for CYC. If MICs of RIF were ≤ 1 μg/mL, the strain was considered susceptible,

according to the World Health Organization recommendation. The pan-susceptible *M. tuberculosis* strain, H37Rv (ATCC27294), was employed as a reference.

Genomic DNA extraction

Frozen isolates were sub-cultured on L-J medium for 4 weeks. The DNA was extracted using the rapid boiling method. This was carried out by suspending a loopful of *M. tuberculosis* colonies in a screw-cap tube containing 200 µL 1× TE (Tris-EDTA)-buffer, and incubating at 100 ºC for 10 min, followed by centrifugation at 12000 ×*g* for 10 min, after which the supernatant was collected. The DNA was stored at −20 ºC until further use. The *M. tuberculosis* reference strain H37Rv was used as the control.

PCR amplification and sequencing of rpoB

Amplification and sequencing primers of the *rpoB* gene were described in a previous study [14]. The PCR mixture was prepared in a volume of 50 µL as follows: 25 µL 2× PCR Mixture (Sangon, Shanghai, China); 2 µL of DNA template; 17 µL ddH_2O; and 1 µM of each primer set. Thermo-cycling conditions were as follows: 94°C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and final elongation at 72 °C for 10 min. The PCR product was confirmed on electrophoresis gel. The 688-bp fragments containing the RRDR of the *rpoB* gene were sent to TSINGKE Company (Hangzhou, China) for DNA sequencing. Alignment of the *rpoB* gene was performed by comparison with the standard *M. tuberculosis* reference strain, H37Rv. The Basic Local Alignment Search Tool (BLAST) sequencing program (NCBI) was used for DNA sequence comparisons (https://blast.ncbi.nlm.nih.gov).

Statistical analysis

The SPSS statistical software package (v20.0; SPSS Inc.) was used to perform chi-squared analysis, and differences were considered to be statistically significant when P was < 0.05.

Results

RIF and RFB resistance

Of the 105 RIF-resistant isolates tested, resistance of *M. tuberculosis* to RIF alone (RIF monoresistance) was rare, as it was observed in only one isolate. However, 90 (85.71%) isolates were MDR. In addition, 86 (81.90%) isolates were also resistant to RFB, and 19 isolates (16.81%) were RFB-susceptible. Among the 19 RFB-susceptible strains, the RFB MICs of 0.25 µg/mL and 0.5 µg/mL accounted for 8 and 11 isolates, respectively. We further analyzed the proportion of RIF-resistant strains among different susceptibility profile groups. As shown in Table 1, the resistance rates of 105 RIF-resistant isolates to the other three first-line drugs, including INH, EMB, and PAS were 85.71%, 38.10%, and 12.38%, respectively. Approximately 45% of the isolates were resistant to OFX and MXF. The resistance rates of 105 RIF-

resistant isolates to ETH, KAN, CYC, and AK were 17.14% (18/105), 20.95% (20/105), 10.48% (11/105), and 20.00% (21/105), respectively.

Mutationsof rpoB

Fifteen types of codons involving 20 types (95.24%) of mutations in theRRDR of the *rpoB* gene were identified among all of the 105 RIF-resistant isolates. Single mutation rates in the RRDR were 85.71% (90/105). In addition, five (4.76%) strains demonstrated no mutations in the RRDR of the *rpoB* gene. Double mutations were found in eight (7.62%) isolates.

We also identified a novel nine-nucleotide (ATCATGCAT) deletion (between positions 1543 and 1551) in the *rpoB* gene, among two strains (1.90%) with resistance to RIF, but susceptibility to RFB.

As shown in Table 2, the most frequent mutation of the RIF-resistant clinical strains was at codon 531 (58.10%, 69/105); followed by codons 526 (8.57%, 9/105); 522 (4.76%, 5/105); and 516 (3.81%, 4/105), respectively. Four types of mutations were found at codon 531: Ser-Leu (61, 58.10%); Ser-Trp (5, 4.76%); Ser-Tyr (2, 1.90%); and Ser-Phe (1, 0.95%). Three types of mutations were found at codon 526: His-Tyr (5, 4.76%); His-Leu (2, 1.90%); and His-Asp (2, 1.90%). Two types of mutations were observed at codon 522: Ser-Leu (4, 3.81%) and Ser-Gly (1, 0.95%). The remaining mutations were only observed in single isolates (mutations at Leu533Arg, Ala532Val, and Leu511Pro), with a distribution of 0.95% each (Table 2). Of the 90 RIF-susceptible strains, 95.56% (86/90) showed no mutations. Three types of mutations were associated with four isolates containing Leu511Pro (one isolate) His526Tyr (one isolate) and Leu533Pro (two isolates).

As shown in Table 3, we compared the most frequent mutation between the RIF-resistant/RFB-resistant (RIF^R/RFB^R) strains and RIF-resistant/RFB-susceptible (RIF^R/RFB^S) strains. All of the 19 RIF^R/RFB^S isolates had mutations in the RRDR of the *rpoB* gene. The mutation frequency of codon 531 was significantly higher in RIF^R/RFB^R strains than in RIF^R/RFB^S strains (75.58% versus 21.05%). Furthermore, the mutation frequency of codons 516 and 522 were significantly lower in RIF^R/RFB^R strains than in RIF^R/RFB^S strains (1.16% versus 26.32%, and 3.49% versus 10.53%, respectively). However, two isolates with a mutation at His526Leu were both susceptible to RFB. In addition, the two deletion mutations found in MDR-TB isolates were associated with resistance to RIF, but susceptibility to RFB.

Association between RIF MICs for M. tuberculosis clinical isolates and mutations in the RRDR of the rpoB gene

To better understand the relationship between RIF-resistance and the different *rpoB* mutations, we analyzed the association between RIF MICs for the *M. tuberculosis* clinical isolates and mutations in the RRDR of the*rpoB* gene. Among 105 RIF-resistant isolates, 92 (87.62%) were resistant to RIF with MICs of ≥ 16 μg/mL, whereas 12.38% (13/105) were < 16 μg/mL. Among 69 isolates with a mutation in codon 531, 66 isolates (95.62%) bearing a single mutation had high levels of resistance (MICs ≥ 16 μg/mL). All five isolates requiring MICs of ≥ 16 μg/mL contained a point mutation in codon 522. Isolates with the

C1576T (His526Tyr) mutation displayed a higher-level resistance to RIF than isolates with the A1578T (His526Leu) mutation. Four strains with the A1547T (Asp516Val) mutation had MICs of RIF that were lower than those of *rpoB* mutants without this alteration. Three isolates with single mutations in Leu533Arg (n = 1), Ala532Val (n = 1), and Leu511Pro (n = 1) were MDR and resistant to RFB (MIC < 16 μg/mL) and RIF (MIC ≥ 4 μg/mL). In addition, 75% (6/8) of the double-mutation isolates were resistant to RIF, with MICs of ≥ 16 μg/mL (Table 2). The MICs of RIF-resistant isolates without mutations were 4 μg/mL (one isolate) and ≥ 16 μg/mL (four isolates).

All *rpoB* mutants that were RIF-susceptible were also susceptible to RFB (MIC ≤ 0.25 μg/mL). The RIF-susceptible isolates with mutations in Leu533Pro (n = 2) and Leu511Pro (n = 1) were resistant to INH, OFX, and MXF, whereas isolates with mutations in His526Tyr (n = 1) were susceptible to these drugs.

Discussion

More than 90% of RIF-resistant isolates in the present study possessed mutations in the RRDR of the *rpoB* gene. These findings are consistent with those of previous studies, which have reported that the prevalence of *rpoB* mutations ranges from 76% [15] to 99% [16]. It is critical to understand the correlation among mutations of *M. tuberculosis* and high-level resistance to RIF with clinical states of the patients.

In the present study, the frequency of mutations at codon 531 was 65.71%, which is similar to that recorded in Turkey, Iran, India, and Singapore, and lower than that recorded in Brazil (79.3%) [17–19]. The frequency of mutations observed at codon 526 was 8.57%, which is lower than that recorded in Zhejiang (75.8%), Anhui (32.26%), and Shandong (26.7%), Provinces, China, and other countries (ranging from 12.5% to 29.7%) [19, 20]. However, the frequency of mutations at codon 522 (4.46%) was higher than that recorded in Vietnam, India, Spain, and some parts of China (Beijing, Shandong, and Zhejiang) (0.0%), but lower than that recorded in Turkey [19].

Not all mutations in the RRDR were associated with resistance to RIF [14]. In addition, the frequencies of single nucleotide polymorphisms (SNPs) in these codons were variable. These variations reflect the complex and crucial interactions between RIF and the positions of the affected nucleotide changes, which seemed to have been variable.

The distribution and frequency of the main mutations of the *rpoB* gene in RIF^R/RFB^R and RIF^R/RFB^S isolates differed. We found that *rpoB* mutations at codons 531, 526, and 516 were associated with phenotypic resistance to RIF and susceptibility to RFB in MDR-TB isolates; whereas other studies have reported diversity among mutations at codons 511, 515, 522, 516, 529, and 533 [13, 14].

It is worth noting that mutations at Ser531Leu and His526Pro in the RRDR confer cross-resistance to RFB and RIF [21]. Mutations in Leu533Pro were only found in RIF^S/RFB^S strains. Interestingly, the mutation at Asp516Val was found predominantly in RIF^R/RFB^S isolates. In a previous study, two isolates with mutations at Leu511Pro were susceptible to RFB; whereas in the present study, one isolate was resistant to RFB [14]. Four isolates with mutations at Asp516Val were RIF^R/RFB^S strains, indicating that this

mutation may be associated with susceptibility to RFB. This finding reflects the probabilistic nature of, and complexity associated with the use of mutations to predict resistant phenotypes.

Another interesting finding was a novel nine-nucleotide (ATCATGCAT) deletion (between positions 1543 and 1551) in the *rpoB* gene of two strains (1.90%) with resistance to RIF, but susceptibility to RFB. In previous studies, mutations of other nine-nucleotide deletions (codons 510 to 513 and 513 to 515) have been observed in *M.tuberculosis* clinical isolates [10, 22]. In addition, other studies have reported three-nucleotide mutations in codons 510 and 517, and nucleotides 1550 to 1552 [23–25]. Our findings imply that the deletion mutation in nucleotides 1543 to 1551 might be associated with resistance to RIF. However, the clinical significance of these findings requires further exploration. Although cross-resistance to RIF and RFB is common, RIF^R/RFB^S isolates have been reported, and RFB has been suggested as a good alternative to treat MDR-TB and XDR-TB associated with some RRDR mutations.

Many studies have reported that specific mutations of *rpoB* are associated with different levels of resistance to RIF [14, 26]. For example, the strains with Ser531Leu and His526Asp RpoB mutants showed high resistance to RIF, and they comprised 90% or more of those found among phenotypically RIF-resistant isolates [27]. It should be noted that not all variations in well described resistance genes were related to the development of high-level resistance. Specific mutations in codons 511, 516, 518, and 522 are reportedly associated with low-level resistance to RIF [28]. Conversely, in the present study, mutations at codon 522 may have been associated with high-level resistance to RIF. Two strains with mutations at His526Leu may have been associated with low levels (MIC = 4 μg/mL) of resistance; whereas mutations at His526Asp and His526Tyr were associated with high levels of resistance to RIF (MICs > 16 μg/mL). Mutations at Asp516Val were found in both high-level and low-level resistance isolates. This suggests that various substitutions in the same codon can lead to different levels of resistance [29]. In addition, double mutations in Leu511Pro-Asp516Gly were associated with high-level resistance to both drugs. Five isolates (MICs > 4 μg/mL) showed no mutations in the *rpoB* gene, suggesting that the mutations might have occurred elsewhere, or that there were other unknown mechanisms of drug resistance. It is important to note that the isolates with double mutations were all resistant to RFB (MIC ≤ 8 μg/mL), which may reflect moderate synergistic effects between the two codon mutations.

In conclusion, our findings demonstrate that various *rpoB* mutations are associated with differential resistance to RIF, and support previous studies highlighting certain mutations in the RRDR that are more likely to confer high levels of resistance to RIF. The specific mutations of the *rpoB* gene in RIF^R/RFB^R isolates differed from those in RIF^R/RFB^S isolates. A novel deletion mutation in the RRDR may be associated with resistance to RIF, but not to RFB.

Abbreviations

RIF: rifampin

RFB: rifabutin

MICs: minimum inhibitory concentrations

MDR-TB: multiple drug-resistant tuberculosis

XDR-TB: extensively drug-resistant tuberculosis

RRDR: RIF resistance-determining region

INH: isoniazid

SM: streptomycin

EMB: ethambutol

OFX: ofloxacin

AK: amikacin

KAN: kanamycin

MXF: moxifloxacin

PAS: pyrazinamide

ETH: ethionamide

CYC: cycloserine

CLSI: Clinical and Laboratory Standards Institute

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Declarations

Ethics considerations

The Shanghai Pulmonary Hospital Affiliated to Tongji University School of Medicine Ethics Committee approved the research protocols. The informed consent that was both written and informed was obtained from each patient who was treated in accordance with the Helsinki Declaration on the participation of human subjects in medical research.

Consent to publish

Not applicable.

Availability of data and materials

The datasets used during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare they have no competing interests.

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Authors' contributions

YJ, XW,JH, XC, YL and BS designed of the work and analyzed and interpreted of data for the work; YJ and FY Drafted the work and revised it critically for important intellectual content. JH, XC, YL and BS participated in the experimental design and data analysis. FY agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

Tables

Table 1. Drug susceptibility patterns of RIF-resistant *M. tuberculosis* strains (n = 105) isolated in China

Drug	Number	%
ETH	18	17.14
EMB	40	38.10
RFB	86	81.90
KAN	22	20.95
PAS	13	12.38
INH	90	85.71
OFX	48	45.71
CYC	11	10.48
MXF	47	44.76
SM	69	65.71
AK	21	20.00

Table 1. Drug susceptibility patterns of RIF-resistant *M. tuberculosis* strains (n = 105) isolated in China

RIF: rifampin; ETH: ethionamide; EMB: ethambutol; RFB: rifabutin; KAN: kanamycin; PAS: pyrazinamide; INH: isoniazid; OFX: ofloxacin; CYC: cycloserine; MXF: moxifloxacin; SM: streptomycin; AK: amikacin.

Table 2. RIF susceptibility profiles of *rpoB* mutant strains obtained in this study

ons	Nucleotide mutation	Amino acid mutation	Number (%)	MICs (μg/mL) (NO)	Reference
le ation					
	C1592T	Ser-Leu	61 (58.10)	4 (1), 8 (1), 16 (7), > 16 (50)	[30]
	C1592G	Ser-Trp	5 (4.76)	4 (1), > 16 (4)	[31]
	C1592A	Ser-Tyr	2 (1.90)	> 16	[32]
	C1592T G1593C	Ser-Phe	1 (0.95)	> 16	[33]
	A1577T	His-Leu	2 (1.90)	4	[34]
	C1577G	His-Asp	2 (1.90)	> 16	[34]
	C1577T	His-Tyr	6	0.5 (1), > 16 (5)	[34]
	A1547T	Asp-Val	4 (3.81)	2 (1), 4 (1), 16 (2)	[34]
	T1532C	Leu-Pro	2	0.25 (1), > 16 (1)	[34]
	C1575T	Ser-Leu	4 (3.81)	16 (3), > 16 (1)	[35]
	C1575G	Ser-Gly	1 (0.95)	> 16	This study
	C1595T	Ala-Val	1 (0.95)	16	[32]
	T1598C	Leu-Arg	3	0.5 (2), 4 (1)	This study
			5 (4.76)	4 (1), 16 (1), > 16 (3)	
tion	ATCATGGAT		2 (1.90)	16 (1), > 16 (1)	This study
ole ations					
515	A1547G A1543G	Asp-Gly Met-Val	1 (0.95)	> 16	This study
515	G1546T G1545T	Asp-Pro Met-Ile	1 (0.95)	2	This study
511	A1547G T1532C	Asp-Gly Leu-Pro	2 (1.90)	> 16	[36]
511	C1303T T1532C	Asp-Tyr Leu-Pro	1 (0.95)	4	[37]
511	G1283A C1531A	His-Asn Leu-Met	1 (0.95)	16	This study
511	C1578G T1532C	Leu-Pro His-Glu	2 (1.90)	16	This study

Table 3. Frequency of mutations in the RRDR of the *rpoB* gene among RIF^R/RFB^R and RIF^R/RFB^S *M. tuberculosis* isolates

	Frequency of mutation	
Mutation position	RFB/R (n = 86)	RFB/S (n = 19)
531	65 (75.58)	4 (21.05)
511	4 (4.65)	3 (15.79)
516	1 (1.16)	5 (26.32)
526	7 (8.14)	2 (10.53)
522	3 (3.49)	2 (10.53)
532	1 (1.16)	0 (0.00)
533	1 (1.16)	0 (0.00)
N[a]	4 (4.65)	1 (5.26)
Deletion	0 (0.00)	2 (10.53)

a: No mutation

RIF: rifampin; RFB: rifabutin;

RRDR: RIF resistance-determining region;

RIF^R/RFB^R: RIF-resistant/RFB-resistant;

RIF^R/RFB^S: RIF-resistant/RFB-susceptible.