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rpoB gene mutations in rifampin-resistant *Mycobacterium tuberculosis* isolates from rural areas of Zhejiang, China

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Abstract

Objective: The aim was to analyze genetic mutations in the *rpoB* gene of rifampin-resistant *Mycobacterium tuberculosis* isolates (RIF^R-MTB) from Zhejiang, China.

Methods: We prospectively analyzed RIF^R-associated mutations in 13 rural areas of Zhejiang. Isolates were subjected to species identification, phenotype drug susceptibility testing (DST), DNA extraction, and *rpoB* gene sequencing.

Results: A total of 103 RIF^R isolates were identified by DST (22 RIF^R only, 14 poly-drug resistant, 49 multidrug resistant, 13 pre-extensively drug resistant [pre-XDR], and 5 extensively drug resistant [XDR]) from 2152 culture-positive sputum specimens. Gene sequencing of *rpoB* showed that the most frequent mutation was S450L (37.86%, 39/103); mutations P280L, E521K, and D595Y were outside the rifampicin resistance-determining region (RRDR) but may be associated with RIF^R. Mutations associated with poly-drug resistant, pre-XDR, and XDR TB were mainly located at codon 445 or 450 in the RRDR.

Conclusions: The frequency of *rpoB* RRDR mutation in Zhejiang is high. Further studies are needed to clarify the relationships between RIF^R and the TTC insertion at codon 433 in the RRDR and the P280L and D595Y mutations outside the RRDR.

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Introduction

Tuberculosis (TB), which is usually caused by *Mycobacterium tuberculosis* complex (MTB) infection, has existed for millennia and remains a major global health problem. According to the World Health Organization (WHO) TB report in 2017, more than 160,000 patients with TB have multidrug resistance (MDR) or rifampin (RIF) resistance (MDR-TB/RIF^R).[1] Rapid identification and effective treatment are critical for TB control. Antibiotics have revolutionized the treatment of TB; however, the appearance of drug-resistant TB has complicated the traditional use of antibiotics to treat TB. Since 1994, WHO has regularly reported drug resistance to TB. MDR, pre-extensive drug resistance (pre-XDR), and extensive drug resistance (XDR) of TB cases results in high mortality (>90%), especially in patients co-infected with human immunodeficiency virus (HIV).[2,3] Globally, an estimated 3.5% of new TB patients and 20.5% of retreated TB patients have MDR-TB.[4] These expanding pools of individuals with unidentified MDR-TB are a crucial and underestimated source of infection, exposing healthy people in the community. Epidemiological control of MDR-TB is one of the most challenging issues globally for WHO's End TB Strategy.

Rifampin has been used for about 50 years. Unlike other antibiotics, which require active growth and metabolism of the target bacteria to exert their antibacterial effects,[5] RIF is a specially selected agent that acts against slow-growing and even non-replicating MTB.[6] It is especially vital for persistent MTB infection, in which non-replication and low metabolic activity are factors.[7] By binding to the β subunit of RNA polymerase, which is encoded by *rpoB* gene, RIF prevents elongation of the RNA transcript beyond two or three nucleotides and thus inhibits transcription of the RNA polymerase. As a result, MTB is killed by RIF.[8] RIF^R is usually accompanied by mutations located in an 81-bp region of the *rpoB* gene,[9] known as the RIF resistance-determining region (RRDR). Previous research evaluated an automated molecular test, the Xpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA), which includes all reagents required for bacterial lysis, DNA extraction, amplification, and amplicon detection.[10] It can identify MTB and RIF^R in less than 2 hours and has greater than 95% sensitivity on smear-positive respiratory and non-respiratory specimens.[11] Although RIF^R mutation sites outside the RRDR have been reported in the past decade, mutations within the 81-bp RRDR have also been reported.[12]

This prospective study complies with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement: guidelines for reporting observational studies (https://www.equator-network.org/reporting-guidelines/strobe/). We aimed to determine RIF^R isolates with *rpoB* mutations from 13 rural areas of Zhejiang, China. Isolates were

identified by biochemical culture, and RIF^R isolates were screened out by drug susceptibility testing (DST) with four first-line drugs: isoniazid (INH), RIF, streptomycin (SM), and ethambutol (EMB), and two second-line drugs: the fluoroquinolone ofloxacin (OFX), and an injectable drug, kanamycin (KM). RIF^R isolates were subjected to *rpoB* gene amplification and sequencing.

Materials and methods

Ethics statement

The institutional review board of Hangzhou Center for Disease Control and Prevention (HZCDC, Hangzhou, China) approved the study protocol and waived the need for informed consent because no patients were at risk (HZCDC/LSY no. 2020-41).

Isolates, species identification, and phenotype DST

For this prospective study, sputum culture-positive specimens were collected from 1 January 2016 to 31 December 2017. Using Löwenstein–Jensen (LJ) medium, species identification using *p*-nitrobenzoic acid and 2-thiophenecarboxylic acid hydrazide and DST were conducted for 3 to 4 weeks at 37°C, with cultures checked weekly for visible colony growth. Species identification and the proportional method of DST followed standard procedures.[13] For DST, the concentrations of drugs were as follows: 0.2 mg/L INH, 40 mg/L RIF, 4.0 mg/L SM, 2.0 mg/L EMB, 4.0 mg/L OFX, and 30 mg/L KM. Sterile deionized water (ddH_2O) and a standard isolate H37Rv (*M. tuberculosis* ssp. *tuberculosis* ATCC 27294) were used as negative and positive controls, respectively, in all experiments in this study.

DNA extraction, primers, and rpoB gene amplification

A loopful from one colony was ground, added to 500 μL of ddH_2O, and incubated for 20 minutes at 95°C in a heating block. The supernatant was transferred into a new tube after centrifugation at $10{,}000 \times g$ for 15 minutes. Genomic DNA of inactivated MTB was extracted using the MTB DNA extraction kit (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA), and 5 μL of DNA was used as a template for *rpoB* amplification. The PCR reaction conditions included a denaturation step of 12 minutes at 94°C, followed by 29 cycles of 15 s at 94°C, 15 s at 56°C, and 30 s at 72°C, followed by a final extension step of 7 minutes at 72°C.

Sanger sequencing

For the *rpoB* fragment (3519 bp), five pairs of primers (Rpo-1 to -5) were used, and Sanger sequencing and assembly were conducted according to previous studies.[14] Primer synthesis and sequencing was conducted by Sangon Biotech (Shanghai, China). All regions containing variants were amplified by PCR and sequenced using a 3730xl DNA Analyzer (Thermo Fisher Scientific) according to standard procedures.

Data analysis

Test results were based on comparison of RIF^R derived from phenotypic DST and *rpoB* gene sequencing. Data were expressed as numbers or percentages. Data collection was performed using Excel, version 2017 (Microsoft Corp., Redmond, WA, USA).

Results

Drug resistance profiles of 103 RIF^R-TB isolates and rpoB gene fragments

A total of 4.79% (103/2152) RIF^R isolates were identified by DST from 2152 sputum culture-positive specimens from patients suspected of having TB in two specialized TB hospitals (The Affiliated Hospital of Hangzhou Normal University and Hangzhou Chest Hospital, College of Medicine, Zhejiang University). In total, 21.36% (22/103) of the isolates were resistant to RIF only, 13.59% (14/103) isolates were poly-drug resistant (resistant to two or three first-line anti-TB drugs). Overall, 47.57% (49/103) were MDR, of which 63.27% (31/49) isolates were resistant to more than three drugs; 12.63% (13/103) isolates were pre-XDR and 4.85% (5/103) were XDR. In total, 65.05% (67/103) isolates were resistant to both INH and RIF (Table 1). The *rpoB* gene fragments were successfully amplified sequenced and assembled.

Mutations of rpoB gene and changes in corresponding amino acids

Nine mutated gene sites were found, assigned to three regions to facilitate analysis: pre-RRDR, RRDR, and post-RRDR. In the pre-RRDR region, the P280L mutation was observed in isolate M318 (GenBank accession no. MN221385). Six mutations at positions from 431 to 450 were found within the RRDR, and two mutations were found in the post-RRDR region: E521K in isolate M318 and D595Y in isolate M643 (GenBank accession no. MN221386). Additionally, a TTC insertion at codon 433 was observed in isolate M471 (GenBank accession no. MN221387). The frequency of mutations outside the RRDR was 1.94% (2/103) and

Table 1. Drug resistance profiles of 103 patients in Zhejiang, China, with drug-resistant tuberculosis

Profile	Resistant to:	Drug	Frequency (n)	Total (N = 103)
RIF resistance only	1 drug	RIF	22	22
Poly-drug resistance	2 drugs	RIF, SM	6	14
		RIF, EMB	2	
		RIF, OFX	3	
	3 drugs	RIF, SM, OFX	3	
MDR	2 drugs	INH, RIF	18	49
	3 drugs	INH, RIF, SM	19	
		INH, RIF, EMB	2	
	4 drugs	INH, RIF, SM, EMB	10	
Pre-XDR	4 drugs	INH, RIF, SM, OFX	3	13
		INH, RIF, EMB, OFX	5	
	5 drugs	INH, RIF, SM, EMB, OFX	5	
XDR	4 drugs	INH, RIF, OFX, KM	2	5
	5 drugs	INH, RIF, SM, OFX, KM	1	
	6 drugs	INH, RIF, SM, EMB, OFX, KM	2	

INH, isoniazid; RIF, rifampin; SM, streptomycin; EMB, ethambutol; OFX, ofloxacin; KM, kanamycin; Poly-drug resistance, resistant to more than one first-line anti-TB drug, in addition to INH and RIF simultaneously; MDR, multi-drug resistance, resistant to at least INH and RIF simultaneously; Pre-XDR, extensively drug resistant (MDR plus resistant to either OFX or KM); XDR, extensively drug resistant (MDR plus resistant to OFX and KM).

Table 2. Mutations of *rpoB* and changes in corresponding amino acids

Location	Gene site	Codon variation	Amino acid change	Frequency (N = 103)	
				n	%
RRDR (426–452)	431 (AGC)	AGC→ACC	S431T	1	0.97
	433 (TTC)	TTC insertion	—	1	0.97
	435 (GAC)	GAC→GTC	D435V	3	2.91
	441 (TCG)	TCG→CAG	S441Q	2	1.94
		TCG→TTG	S441L		
	445 (CAC)	CAC→GAC	H445D	31	30.10
		CAC→TAC	H445Y		
		CAC→CGC	H445R		
		CAC→AAC	H445N		
		CAC→TGC	H445C		
	450 (TCG)	TCG→TTG	S450L	63	61.17
		TCG→TGG	S450W		
		TCG→TTT	S450F		
Outside RRDR	**280 (CCC)**	**CCC→CTC**	**P280L**	1	0.97
	521(GAG)	GAG→AAG	E521K		
	595 (GAC)	**GAC→TAC**	**D595Y**	1	0.97

New mutations that have never been reported are in bold.
RRDR, rifampicin resistance-determining region.

that inside the RRDR was 98.06% (101/103). Overall, 91.26% (94/103) isolates were mutated at codon 445 or 450 (Table 2).

Distribution of mutations by types of drug resistance

The most frequent mutation was at codon 450, accounting for 61.17% (63/103) of all mutations (Table 3); these included 39 S450L, 23 S450W, and 1 S450F mutations, respectively. The second most frequent mutation was at codon 445, accounting for 30.10% (31/103): 15 H445D, 7 H445Y, 6 H445R, 2 H445N, and 1 H445C mutations. Two rare mutations, P280L and the TTC insertion at codon 433, were found in two RIF^R-only isolates. Of the 94 isolates associated with mutations at codon 445 or 450, 19.15% (18/94) were RIF^R only, 14.89% (14/94) were poly-drug resistant, and 46.81% (44/94) were MDR. Of the 67 MDR (49), pre-XDR (13), and XDR (5) isolates in Table 1, 92.54% (62/67) were mutated at codon 445 or 450.

Discussion

In combination chemotherapy for TB, RIF is one of the main first-line drugs, and RIF^R is a valuable surrogate marker of MDR-TB.[15] More than 90% of clinical RIF^R-TB cases have genetic mutations in *rpoB*[16] and more than 80% are MDR-TB.[17] In this study, 98.06% (101/103) of RIF^R-TB cases had mutations in the *rpoB* gene, and 47.57% (49/103) were simultaneously resistant to INH and RIF. In addition, 1.94% (2/103) RIF^R isolates had mutations outside the RRDR; these cases cannot be detected by rapid diagnostic methods such as the Xpert MTB/RIF assay; however, they can be detected by time-consuming culture methods. It is uncertain whether similar isolates with undetected mutations

Table 3. Mutations in *rpoB* and drug resistance profiles of patients with tuberculosis

		Number of isolates (N = 103)				
Location	Amino acid variations	RIF^R only	Poly-drug resistance	MDR	Pre-XDR	XDR
RRDR	S431T			1		
	— (433)	1				
	D435V			3		
	S441Q	1				
	S441L			1		
	H445D	2		9	3	1
	H445Y	1	1	5		
	H445R	3	3			
	H445N			2		
	H445C			1		
	S450L	8	6	17	5	3
	S450W	3	4	10	5	1
	S450F	1				
Outside RRDR	P280L	1				
	E521K					
	D595Y	1				
Total		22	14	49	13	5

RIF^R, rifampin resistance; MDR, multidrug resistant; pre-XDR, pre-extensive drug resistance; XDR, extensive drug resistance; RRDR, rifampicin resistance-determining region.

(outside the RRDR) exist in rural areas in Zhejiang, China, where MDR-TB detection by Xpert MTRB/RIF has been widely implemented. We identified three isolates with synonymous mutations; however, these three isolates had non-synonymous mutations simultaneously. Studies have reported some false-resistant Xpert MTB/RIF results associated with silent *rpoB* mutations.[18] All isolates in our study had phenotypic RIF^R, and thus the synonymous mutations may not be related to RIF^R. In our study, we identified mutations of both P280L and E521K outside the RRDR in isolate M318; however, no mutations were found in the RRDR in M318. Significantly, P280L has not been reported elsewhere. In addition, we detected an insertion of TTC at codon 433 in M471 (Table 3). This type of insertion was previously reported by Suresh et al. (GenBank accession no. AY793005) and Ahmad et al. (GenBank accession no AJ870394) in 2004. Ours is the first report of the TTC insertion mutation in Zhejiang, China. Some RIF^R MTB isolates mutants selected *in vitro* have reduced fitness and carry multiple mutations in the RNA polymerase genes. These multiple mutations support a role for compensatory evolution in global epidemics of drug-resistant *M. tuberculosis*. The TTC insertion may be a type of compensatory mutation,[19,20] but whether it is related to fitness cost needs further research.

In this study, 10 RIF-susceptible (RIF^S) isolates were included as negative controls for the *rpoB* sequencing; however, we did not find any mutations in these RIF^S isolates by sequencing. Genetic mutations within the RRDR were present at high frequency (98.06%; 101/103) in our RIF^R isolates from rural areas in Zhejiang, China. The frequency was higher than that

reported by Luo et al.[21] in Jiangxi, China (93.6%). Similar to other studies worldwide,[22,23] the most frequent mutation site was codon 450 (61.17%, 63/103), followed by codon 445 (30.10%, 31/103). Mutations at codon 450 or 445 were found in 91.26% (94/103) of our RIF^R isolates, a higher frequency than was reported in Jiangxi (57.3% and 16.56%, respectively)[21] or Hebei Province (58.5% and 15.46%, respectively).[24] Therefore, the high rate of mutations within the RRDR of *rpoB* demonstrates that targeting the RRDR is still feasible for determination of RIF^R-TB in Zhejiang, China.

Our study has notable limitations. First, and most importantly, although both forward and reverse primers were used for confirmation when double peaks or low peaks occurred, Sanger sequencing has inherent limitations.[25] Therefore, next-generation sequencing approaches should be used to confirm the less-common *rpoB* mutations (e.g., P280L and D595Y in Table 2) reported in this study. Furthermore, some isolates with low-level resistance might have been missed because they could be susceptible to the concentrations used in the DST but still lead to poor treatment outcomes. Mutations in these isolates with low-level RIF^R may induce a delay in growth on solid medium and reduce binding affinity but not completely prevent RIF from binding to *rpoB*.[26] Therefore, identification of RIF^R-TB patients based on critical concentrations in DST may result in some patients being missed and thus experiencing delay in diagnosis, treatment, and subsequent management. Universal clinical whole-genome sequencing could overcome the limitations of critical concentration phenotyping and partial gene sequencing and provide a more complete understanding of the prevalence and type of *rpoB* mutations and their association with RIF^R.[27] Second, we focused only on MTB isolates of RIF^R using LJ medium and the DST proportional method ; therefore, we were unable to associate treatment outcomes with various mutation types. Finally, all isolates were isolated from culture-positive sputum specimens from patients with suspected TB, and we did not include patients without isolates available for drug resistance profiles and *rpoB* mutations in our analyses.

Through our analysis of drug resistance profiles and *rpoB* gene sequencing, we gained further understanding of RIF^R and genetic mutations in MTB isolates from rural areas in Zhejiang, China. Given the focus of genotypic assays on the RRDR for detecting RIF^R,[12] it is inevitable that any mutations outside of the RRDR will be underrepresented in molecular epidemiological surveys of resistance. Using the Xpert MTB/RIF assay, mutations outside the RRDR cannot be detected. Thus, patients with RIF^R-TB and mutations outside the RRDR experience delays in adjustment of their chemotherapy and suffer side effects associated with intolerance to RIF. Furthermore, more attention should be paid to patients with poly-drug resistant TB, and optimal patient management should include checking for mutations outside the RRDR because the failure or relapse rates are similar in isolates with a canonical or disputed *rpoB* mutation.[28] Mutations outside the RRDR are rarely reported, accounting for $<5\%$ of RIF^R isolates[29]; however, given the large number of RIF^R-TB patients worldwide, patients with mutations outside the RRDR represent hidden sources of RIF^R-TB infection and pose a major threat to public health. Therefore, appropriate molecular tests should be developed to detect such mutations for early and reliable prediction of RIF^R in clinical MTB isolates.

In conclusion, we discovered three mutations outside the RRDR that may be associated with RIF^R; two of these, P280L and D595Y, were the first to be reported in

Zhejiang Province. The frequency of mutations outside the RRDR was 1.94% (2/103). Furthermore, we revealed a TTC insertion at codon 433, and this is also the first report of this mutation in Zhejiang, China.

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Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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Author contributions

Mei-chun Zeng provided writing assistance and proof reading; Qing-jun Jia contributed to study design and writing; and Lei-ming Tang provided data collection and language help.

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