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Patterns of *rpoC* Mutations in Drug-Resistant *Mycobacterium tuberculosis* Isolated from Patients in South Korea

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Background: Rifampicin (RFP) is one of the principal first-line drugs used in combination chemotherapies against *Mycobacterium tuberculosis*, and its use has greatly shortened the duration of chemotherapy for the successful treatment of drug-susceptible tuberculosis. Compensatory mutations have been identified in *rpoC* that restore the fitness of RFP-resistant *M. tuberculosis* strains with mutations in *rpoB*. To investigate *rpoC* mutation patterns, we analyzed 93 clinical *M. tuberculosis* isolates from patients in South Korea.
Methods: Drug-resistant mycobacterial isolates were cultured to determine their susceptibility to anti-tubercular agents. Mutations in *rpoC* were identified by sequencing and compared with the relevant wild-type DNA sequence.
Results: In total, 93 *M. tuberculosis* clinical isolates were successfully cultured and tested for drug susceptibilities. They included 75 drug-resistant tuberculosis species, of which 66 were RFP-resistant strains. *rpoC* mutations were found in 24 of the 66 RFP-resistant isolates (36.4%). Fifteen different types of mutations, including single mutations (22/24, 91.7%) and multiple mutations (2/24, 8.3%), were identified, and 12 of these mutations are reported for the first time in this study. The most frequent mutation involved a substitution at codon 452 (nt 1356) resulting in amino acid change F452L.
Conclusion: Fifteen different types of mutations were identified and were predominantly single-nucleotide substitutions (91.7%). Mutations were found only in dual isoniazid- and RFP-resistant isolates of *M. tuberculosis*. No mutations were identified in any of the drug-susceptible strains.

Keywords: *Mycobacterium tuberculosis*; Drug Resistance, Multiple; Beta' Subunit of RNA Polymerase; Mutation

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Introduction

The worldwide emergence of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) threatens global efforts to control tuberculosis[1,2]. Isoniazid (INH) and rifampicin (RFP) are the main components of first-line anti-tuberculosis treatment and are effective drugs for the treatment of tuberculosis[3-5]. MDR-TB is resistant to INH and RFP, and the emergence of MDR-TB is placing an increasing burden on South Korea[4-6].

Mycobacterium tuberculosis can acquire resistance to RFP through mutations in *rpoB*, which encodes the β subunit of RNA polymerase[7-9]. Mutations in *rpoC*, which encodes the β′ subunit of RNA polymerase, have also been associated with

increased *in vitro* fitness and were overrepresented in MDR-TB isolates from countries with high MDR-TB burdens[10,11]. One study showed that *M. tuberculosis* isolates harbouring an *rpoB* mutation also carried nonsynonymous mutations in *rpoC*[10].

In this study, we investigated *rpoC* mutation patterns in drug-resistant and susceptible *M. tuberculosis* isolates from patients in South Korea to determine the epidemiological relevance of *rpoC* nonsynonymous mutations in a high-incidence setting of MDR- and XDR-TB.

Materials and Methods

1. *Mycobacterial* isolates and susceptibility testing

Ninety-three *M. tuberculosis* isolates with clinically observed drug resistance were collected at National Masan Hospital and Pusan National University College of Medicine in South Korea and were cultured to determine their susceptibility to anti-tubercular agents (Table 1). Each isolate was cultured on Löwenstein-Jensen medium at 37°C for 3–4 weeks and tested for resistance to critical concentrations of capreomycin (40 μg/mL), ethambutol (2.0 μg/mL), INH (0.2 μg/mL), kanamycin (40 μg/mL), ofloxacin (2 μg/mL), streptomycin (4 μg/mL), pyrazinamide (100 μg/mL, Wayne's pyrazinamidase assay[12]), and RFP (40 μg/mL). *M. tuberculosis* H37Rv (American Type Culture Collection [ATCC] 27294) was used as a positive control in all of the experiments. The following drug-resistant profiles were defined: MDR, resistance to both RFP and INH; MDR-plus, resistance to any of the second-line injectable drugs (INH+RFP+Inj.D) or to any fluoroquinolone drugs (INH+RFP+FQ); XDR, extensively drug-resistant; DR, drug resistance other than MDR (including MDR-plus and

Table 1. Drug resistance profiles of 93 *Mycobacterium tuberculosis* isolates

No.	Drug resistance	Drug resistance profile	No.	Drug resistance	Drug resistance profile
1	SM, INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	48	None detected	S
2	SM, INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	49	CPM	DR
3	SM, INH, RFP, EMB, CPM, KM, OFX, MFX, PZA	XDR	50	INH, RFP, OFX, MFX, PZA	MDR
4	INH, RFP, CPM, KM, PZA	MDR	51	INH, RFP, KM, OFX, MFX, PZA	XDR
5	SM, INH, RFP, EMB, OFX, MFX, PZA	MDR	52	INH, RFP	MDR
6	INH, RFP, OFX, MFX	MDR	53	SM, INH, RFP, EMB, CPM, KM, OFX, MFX, PZA	XDR
7	SM, INH, RFP, EMB, CPM, KM, MFX, PZA	XDR	54	SM, INH, RFP, EMB, OFX, PZA	XDR
8	SM, INH, RFP, MFX, PZA	MDR	55	SM, INH, RFP, OFX, MFX, PZA	XDR
9	INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	56	SM, INH, RFP, CPM, OFX, MFX, PZA	XDR
10	SM, INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	57	SM, INH, RFP, EMB, CPM, KM, OFX, MFX, PZA	XDR
11	SM, INH, RFP, EMB, CPM, KM, OFX, MFX, PZA	XDR	58	INH, RFP, CPM, OFX, MFX, PZA	XDR
12	SM, INH, RFP, KM, OFX, MFX, PZA	XDR	59	SM, INH, RFP, CPM, KM, OFX, MFX, PZA	XDR
13	INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	60	RFP, CPM	DR
14	SM, INH, RFP, EMB, CPM, KM, OFX, MFX	XDR	61	INH, RFP	MDR
15	INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	62	INH	DR
16	SM, INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	63	INH, RFP, LEV, OFX, MFX,	MDR-plus
17	SM, INH, RFP, CPM, KM, PZA	MDR	64	INH, RFP, LEV, OFX, MFX, KM, AMK, CPM	XDR
18	INH, RFP, OFX, MFX, PZA	MDR	65	INH, RFP, LEV, OFX, MFX, KM, AMK, CPM	XDR
19	INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	66	INH, RFP, OFX, KM	XDR
20	INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	67	RFP	DR
21	INH, RFP, CPM, KM, MFX, PZA	XDR	68	INH, RFP,	MDR
22	None detected	S	69	INH, RFP, CPM	MDR-plus
23	None detected	S	70	None detected	S
24	None detected	S	71	INH, RFP, LEV, OFX	MDR-plus
25	None detected	S	72	INH, RFP	MDR

Table 1. Continued

No.	Drug resistance	Drug resistance profile	No.	Drug resistance	Drug resistance profile
26	None detected	S	73	INH, RFP	MDR
27	SM, INH, CPM, PZA	DR	74	INH, RFP	MDR
28	INH	DR	75	INH, RFP	MDR
29	None detected	S	76	RFP, CPM	DR
30	None detected	S	77	None detected	DR
31	None detected	S	78	INH, RFP, LEV, OFX, MFX, KM, AMK, CPM	XDR
32	SM, INH, RFP, EMB, KM, OFX, MFX, PZA	XDR	79	INH, RFP, LEV, OFX	MDR-plus
33	None detected	S	80	INH, RFP, LEV, OFX	MDR-plus
34	SM, INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	81	CPM	DR
35	None detected	S	82	INH, RFP, LEV, OFX	MDR-plus
36	None detected	S	83	None detected	S
37	INH	DR	84	INH, RFP	MDR
38	SM, INH, RFP, EMB, CPM, KM, MFX, PZA	XDR	85	None detected	S
39	INH, RFP, EMB, OFX, MXF, PZA	MDR	86	CPM	DR
40	INH, RFP, CPM, KM, PZA	MDR	87	CPM	DR
41	SM, INH, RFP, EMB, CPM, KM, OFX, MFX, PZA	XDR	88	None detected	S
42	SM, INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	89	INH, RFP	MDR
43	SM, INH, RFP, EMB, CPM, KM, PZA	MDR	90	INH, RFP, MFX, CPM	XDR
44	SM, INH, RFP, CPM, KM, MFX, PZA	XDR	91	INH, RFP, CPM	MDR-plus
45	SM, INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	92	None detected	S
46	SM, INH, RFP, CPM, KM, OFX, MFX	XDR	93	None detected	S
47	SM, INH, RFP, OFX, MFX, PZA	MDR			

SM: streptomycin; INH: isoniazid; RFP: rifampicin; CPM: capreomycin; KM: kanamycin; OFX: ofloxacin; MFX: moxifloxacin; PZA: pyrazinamide; EMB: ethambutol; XDR: extensively drug-resistant; MDR: multidrug-resistant; S: susceptibility to all of the drugs; DR: drug resistance other than MDR (including MDR-plus and XDR); MDR-plus: INH+RFP+fluoroquinolone or INH+RFP+injectable drugs.

XDR); and S, susceptibility to all of the drugs. Sixty-six *M. tuberculosis* isolates were RFP resistant (Table 1).

This study was approved by the institutional review board (IRB) at the International Tuberculosis Research Centre, and all subjects signed an informed consent form.

2. Polymerase chain reaction and sequencing of *rpoC*

The *rpoC* region (1,730 bp) was amplified by polymerase chain reaction (PCR) using the GeneAmp PCR System 9600 (PerkinElmer, Foster City, CA, USA) with primers 5′-CGAAAACCTCTACCGCGAAC-3′ and 5′-CACGGAAGGAGGACTTGACC-3′[10].

Briefly, the PCR parameters were 5 minutes at 95°C, followed by 40 cycles of 45 seconds at 94°C, 45 seconds at 60°C, and 60 seconds at 72°C, with a final extension step at 72°C for 10 minutes. The PCR products were purified using a QIAEX II Gel Extraction Kit (Qiagen Inc., Mainz, Germany) according to the manufacturer's instructions and sequenced using a BigDye Terminator cycle sequencing kit with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA) using primers 5′-CGAAAACCTCTACCGCGAAC-3′ and 5′-CACGGAAGGAGGACTTGACC-3′[10]. The nucleotide sequences were analyzed using BioEdit software version 5.0.9.1 (Ibis Biosciences, Carlsbad, CA, USA), Chromas version 2.33 (Technelysium, Brisbane, QLD, Australia, http://www.technelysium.com.au/chromas.html), and the Basic Local Alignment Search Tool (BLAST, National Center for Biotechnology Information, Bethesda, MD, USA, http://blast.ncbi.nlm.nih.gov/Blast.cgi). Mutations in the *rpoC*-encoding regions were defined as any nucleotide changes that led to translational changes in RpoC compared with the RFP-susceptible strain, H37Rv (ATCC 27294).

Results

Ninety-three clinical isolates were included in this study, and all were from South Korean patients. Drug susceptibility testing identified 75 multidrug-resistant isolates: 20 were categorized as MDR-TB, seven were categorized as MDR-plus, 36 were categorized as XDR-TB, and 12 were categorized as DR-TB. Moreover, 66 of the cultured *M. tuberculosis* isolates were found to be RFP-resistant. Eighteen isolates were categorized as S (Table 1).

rpoC PCR products were amplified from 93 isolates and sequenced. Fifteen different types of mutations were identified in 24 isolates (24/93, 25.8%), all of which were resistant to both INH and RFP. The *rpoC* mutation rate of the MDR- and XDR-TB isolates was 37.0% (10/27) and 38.9% (14/36), respectively (Table 2).

Single mutations (22/24, 91.7%) and multiple mutations (2/24, 8.3%) in the *rpoC* region were identified, but no deletion nor insertion mutations were detected in any of the isolates. No mutations were identified in the *rpoC* region of any drug-susceptible strains. A mutation at codon 452 (nt 1356) was the most common mutation (7/24, 29.2%) and a mutation at codon 531 (nt 1594), which is the most frequently mutated nucleotide in *rpoB*, was also detected in these isolates (Supplementary Table S1)[7-9]. Twelve different mutation sites (at codon 281 [nt 843], 416 [nt 1249], 434 [nt 1302], 446 [nt 1338], 561 [nt 1683], 575 [nt 1726], 581 [nt 1745], 728 [nt 2186], 747 [nt 2242], 801 [nt 2403], 812 [nt 2437], and 813 [nt 2441]) are reported for the first time in this study[10,11]; these new mutations are shown in Table 3.

Discussion

RFP is one of the principal first-line drugs used in combination chemotherapy for tuberculosis, and RFP resistance is a valuable surrogate marker of MDR-TB. Over 90% of RFP-resistant clinical *M. tuberculosis* isolates possess genetic alterations in *rpoB*[2,8].

rpoC encodes the β′ subunit of RNA polymerase, and Comas et al.[11] suggested that the acquisition of particular mutations in *rpoC* by RFP-resistant *M. tuberculosis* strains leads to the emergence of MDR strains with high fitness over time. Additionally, de Vos et al.[10] showed that nonsynonymous mutations in the *rpoC* region were prevalent among RFP-resistant isolates in a South African high-burden setting; these mutations were strongly associated with the transmission of RFP-resistant strains.

rpoC mutations have not been studied in South Korea; thus, we investigated the *rpoC* mutation patterns in drug-resistant and susceptible *M. tuberculosis* isolates from patients in South Korea. Fifteen different types of mutations were identified, 12 of which were reported for the first time in this study (Table 3)[10,11]. A mutation at codon 452 was the most common mutation (7/24, 29.2%), and a mutation at codon 531, which is the most frequently mutated nucleotide in *rpoB*, was also detected in these isolates (Supplementary Table S1)[7-9].

Mutations were found only in the MDR-TB isolates and no

Table 2. Isolates with *rpoC* mutations (n=24)

No.	Drug resistance	Drug resistance profile	No.	Drug resistance	Drug resistance profile
2	SM, INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	50	INH, RFP, OFX, MFX, PZA	MDR
8	SM, INH, RFP, MFX, PZA	MDR	53	SM, INH, RFP, EMB, CPM, KM, OFX, MFX, PZA	XDR
9	INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	54	SM, INH, RFP, EMB, OFX, PZA	XDR
11	SM, INH, RFP, EMB, CPM, KM, OFX, MFX, PZA	XDR	57	SM, INH, RFP, EMB, CPM, KM, OFX, MFX, PZA	XDR
13	INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	63	INH, RFP, LEV, OFX, MFX,	MDR-plus
16	SM, INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	65	INH, RFP, LEV, OFX, MFX, KM, AMK, CPM	XDR
17	SM, INH, RFP, CPM, KM, PZA	MDR	71	INH, RFP, LEV, OFX	MDR-plus
20	INH, RFP, CPM, KM, OFX, MFX, PZA	XDR	73	INH, RFP	MDR
32	SM, INH, RFP, EMB, KM, OFX, MFX, PZA	XDR	74	INH, RFP	MDR
38	SM, INH, RFP, EMB, CPM, KM, MFX, PZA	XDR	79	INH, RFP, LEV, OFX	MDR-plus
44	SM, INH, RFP, CPM, KM, MFX, PZA	XDR	80	INH, RFP, LEV, OFX	MDR-plus
46	SM, INH, RFP, CPM, KM, OFX, MFX	XDR	89	INH, RFP	MDR

Isolates with mutations in *rpoC* are resistant to both INH and RFP.
SM: streptomycin; INH: isoniazid; RFP: rifampicin; CPM: capreomycin; KM: kanamycin; OFX: ofloxacin; MFX: moxifloxacin; PZA: pyrazinamide; EMB: ethambutol; XDR: extensively drug-resistant; MDR-plus: INH+RFP+fluoroquinolone or INH+RFP+injectable drugs.

Table 3. Mutations detected in the *rpoC* gene of 93 *Mycobacterium tuberculosis* isolates

Nucleotide change (nucleotide No.)	Translational change (codon No.)	Cultured isolates (n=93)	MDR-TB isolates (n=20)	XDR-TB isolates (n=36)	MDR-plus isolates (n=7)	DR isolates (n=12)	S isolates (n=18)	No.
Substitution								
843 A>G	I281V*	3 (3.23)	2 (2.15)	-	1 (1.08)	-	-	71, 73, 89
1249 A>G	N416S*	1 (1.08)	-	1 (1.08)	-	-	-	20
1302 C>A	P434T*	1 (1.08)	-	-	1 (1.08)	-	-	79
1338 C>A	L446M*	1 (1.08)	-	-	1 (1.08)	-	-	63
1356 T>C	F452L	7 (7.53)	1 (1.08)	6 (6.45)	-	-	-	2, 11, 13, 17, 44, 53, 57
1450 T>C	V483A	1 (1.08)	-	1 (1.08)	-	-	-	38
1450 T>G	V483G	1 (1.08)	-	1 (1.08)	-	-	-	46
1683 T>C	S561P*	1 (1.08)	-	1 (1.08)	-	-	-	54
1726 C>T	A575V*	1 (1.08)	-	1 (1.08)	-	-	-	16
2186 C>T	G728G*	1 (1.08)	1 (1.08)	-	-	-	-	74
2242 A>G	D747G*	2 (2.15)	1 (1.08)	1 (1.08)	-	-	-	8, 65
2437 C>T	T812I*	1 (1.08)	1 (1.08)	-	-	-	-	50
2441 G>C	Q813H*	1 (1.08)	-	1 (1.08)	-	-	-	32
Multi-site mutations				-				
1683 T>C, 1745 G>A	S561P*, M581I*	1 (1.08)	-	1 (1.08)	-	-	-	9
1302, 1303 CC>GT 2403A>T	P434V, T801S*	1 (1.08)	-	-	1 (1.08)	-	-	80

Values are presented as number (%).
*New mutation not reported in previous studies. Isolates with mutations in *rpoC* are resistant to both INH and RFP.
MDR-TB: multidrug-resistant tuberculosis; XDR-TB: extensively drug-resistant tuberculosis; MDR-plus: INH+RFP+fluoroquinolone or INH+RFP+injectable drugs; DR: drug resistance other than MDR (including MDR-plus and XDR); S: susceptibility to all of the drugs; INH: isoniazid; RFP: rifampicin.

rpoC mutations were identified in any of the drug-susceptible strains (Tables 2, 3).

Therefore, we suggest that *rpoC* mutations could be used DNA-based diagnosis for detection of INH and RFP drug resistance; however, more extensive studies on larger collections of isolates are needed.

In summary, 15 different types of mutations were identified. Substitutions in a single nucleotide were the most common mutation found (22/24, 91.7%), and mutations were found only in dual INH- and RFP-resistant isolates in this study.

Authors' Contributions

Conceptualization: Lee KH. Methodology: Yun YJ, Lee JS, Yoo JC, Cho E, Park D. Formal analysis: Lee KH, Yun YJ, Lee JS, Yoo JC, Park D. Data curation: Yun YJ, Lee JS, Yoo JC, Lee KH, Cho E, Park D. Validation: Lee KH, Yun YJ, Lee JS, Yoo JC, Kook YH. Investigation: Lee KH. Writing-original draft preparation: Yun YJ, Lee KH. Writing-review and editing: Yun YJ, Lee JS, Yoo JC, Lee KH. Approval of final manuscript: all authors.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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Supplementary Material

Supplementary material can be found in the journal homepage (http://www.e-trd.org).

Supplementary Table S1. Mutations detected in the *rpoB* gene of 80 isolates.

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