



Frequency and Type of Disputed *rpoB* Mutations in *Mycobacterium tuberculosis* Isolates from South Korea

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Background: A disputed *rpoB* mutation is a specific type of *rpoB* mutation that can cause low-level resistances to rifampin (RIF). Here, we aimed to assess the frequency and types of disputed *rpoB* mutations in *Mycobacterium tuberculosis* isolates from South Korea.

Methods: Between August 2009 and December 2015, 130 patients exhibited RIF resistance on the MTBDR_{plus} assay at Asan Medical Center. Among these cases, we identified the strains with disputed *rpoB* mutation by *rpoB* sequencing analysis, as well as among the *M. tuberculosis* strains from the International Tuberculosis Research Center (ITRC).

Results: Among our cases, disputed *rpoB* mutations led to RIF resistance in at least 6.9% (9/130) of the strains that also exhibited RIF resistance on the MTBDR_{plus} assay. Moreover, at the ITRC, sequencing of the *rpoB* gene of 170 strains with the *rpoB* mutation indicated that 23 strains (13.5%) had the disputed mutations. By combining the findings from the 32 strains from our center and the ITRC, we identified the type of disputed *rpoB* mutation as follows: CTG511CCG (L511P, n=8), GAC516TAC (D516Y, n=8), CTG533CCG (L533P, n=8), CAC526CTC (H526L, n=4), CAC526AAC (H526N, n=3), and ATG515GTG (M515V, n=1).

Conclusion: Disputed *rpoB* mutations do not seem to be rare among the strains exhibiting RIF resistance in South Korea.

Keywords: Rifampin; Biological Assay; *Mycobacterium tuberculosis*; *rpoB* protein, *Mycobacterium tuberculosis*; Mutation

Introduction

Isoniazid (INH) and rifampin (RIF) are the most important drugs administered for the treatment of tuberculosis (TB). Resistance to both of these drugs results in multidrug-resistant (MDR) TB, which is a major threat to TB control in South Korea¹. RIF resistance is rare among INH-susceptible strains, as the mutation to develop spontaneous INH resistance occurs in approximately one in every 10⁶ *Mycobacterium tuberculosis* bacilli present; in contrast, the mutation rate is one in every 10⁸ *M. tuberculosis* bacilli for RIF². Mutations in the well-defined 81-bp region of the *rpoB* gene, known as the RIF-resistance-determining region (RRDR), have been detected in approximately 97% of all RIF-resistant *M. tuberculosis* strains³.

The degree of resistance among the mutations in the RRDR region is not equal. In particular, some RRDR mutations—

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called disputed *rpoB* mutations—can cause low-level resistance to RIF. A previous report defined low-level resistance to RIF as a minimal inhibitory concentration (MIC) of 0.063–0.5 µg/mL for RIF using the 7H9 Middlebrook medium⁴. *M. tuberculosis* isolates with the disputed *rpoB* mutation exhibit discrepant susceptibility results of RIF resistance between genotypic and phenotypic tests⁵. That is, the phenotypic drug susceptibility test (DST) may indicate susceptibility to RIF, despite genotypic resistance to RIF. The commonly observed disputed *rpoB* mutations in previous studies include CTG511CCG (L511P)⁴, GAC516TAC (D516Y)⁶, CAC526CTC (H526L)⁷, and CTG533CCG (L533P)⁵. In contrast, undisputed *rpoB* mutations, including GAC516GTC (D516V), CAC526TAC (H526Y), CAC526GAC (H526D), and TCG531TTG (S531L), are known to cause high-level RIF resistance^{8,9}.

Disputed *rpoB* mutations have previously been considered to be very rare¹⁰. However, a recent report suggested that they frequently contribute to RIF resistance⁷. To our knowledge, no studies on disputed *rpoB* mutations have been conducted in South Korea thus far. Therefore, in the present study, we aimed to investigate the frequency and types of disputed *rpoB* mutations, as well as the treatment outcomes for patients with *M. tuberculosis* isolates with disputed *rpoB* mutations.

Materials and Methods

1. Study subjects and data collection

The study was conducted at the Asan Medical Center, which is a 2,700-bed referral hospital in Seoul, South Korea (an intermediate TB burden country). A total of 1,551 Genotype MTBDR*plus* assays (Hain Lifescience, Nehren, Germany), one of the World Health Organization (WHO) endorsed line probe assays¹¹, were performed between August 2009 and December 2015. Of the 1,389 MTBDR*plus* assays from which corresponding phenotypic DST results were available, 130 (9.4%) exhibited RIF resistance. Among these 130 cases, those with disputed *rpoB* mutation on *rpoB* sequencing analysis were enrolled in the present study. The medical records of these patients with *M. tuberculosis* isolates with disputed *rpoB* mutations were reviewed retrospectively in August 2016.

In addition, we identified the *M. tuberculosis* strains with available *rpoB* sequencing results from the International Tuberculosis Research Center (ITRC) in Masan, South Korea. Over a 5-year period from 2005 and 2010, 170 strains with *rpoB* mutation identified via *rpoB* sequencing were collected at the ITRC, through the South Korean MDR-TB cohort study¹². Of these 170 strains, we identified those with disputed mutations on *rpoB* sequencing analysis.

The study protocol was approved by the Institutional Review Board of Asan Medical Center, which waived the requirement for informed consent due to the retrospective nature of the analysis.

2. MTBDR*plus* assay and DNA sequencing

The MTBDR*plus* assay was performed at our center according to the manufacturer's instructions¹³. The attending physician made the decision regarding whether to perform an MTBDR*plus* assay.

DNA sequencing was performed using a polymerase chain reaction (PCR)–direct sequencing system (Cosmo Co., Seoul, Korea). A 501-bp fragment of the *rpoB* gene was sequenced. The following forward and reverse primers (5'–3') were used: TCAAGGAGAAGCGCTACGACCTGGC and ACGGGTG-CACGTCGCGGACCTCCA. The primers were synthesized by JieLi Bio Co. (Shanghai, China). The PCR conditions included denaturation at 94°C for 10 minutes, followed by 35 cycles of amplification at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. The PCR mixtures were prepared using 2× GoldStar Best MasterMix (CWBio Co., Beijing, China). The PCR products were used as templates for targeted DNA sequencing. To confirm the sequence-amplified PCR product, direct sequencing was performed using an ABI 373 DNA analyzer (Applied Biosystems, Foster City, CA, USA) according to the protocol supplied by the manufacturer, along with the BigDye Terminator v3.1 Cycling Sequencing Kit (Applied Biosystems). Sequencing data were assembled and analyzed using BioEdit software (Isis Pharmaceuticals, Inc., Carlsbad, CA, USA). The mutations were determined via comparison with the H37Rv sequence of the *rpoB* genes from the GenBank database (<http://www.ncbi.nih.gov/gene>). The decision regarding whether to perform a DNA sequencing analysis was made by the attending physician.

3. Bacteriological study and DSTs

Acid-fast bacillus (AFB) smears were examined following Ziehl-Neelsen staining. The AFB culture was carried out using both solid (Ogawa medium; Korean Institute of Tuberculosis, Korea) and liquid (BACTEC 960 Mycobacterial Growth Indicator Tube; Becton Dickinson, Sparks, MD, USA) media. Positive liquid media cultures or colonies on the solid medium were subjected to Ziehl-Neelsen staining and a PCR assay using Seeplex TB detection (Seegen, Seoul, Korea) to differentiate between the *M. tuberculosis* complex and nontuberculous mycobacteria.

When the growth of *M. tuberculosis* was detected in the cultures, DST was requested according to the Korean national TB guidelines. DSTs were performed using the absolute concentration method on Löwenstein-Jensen medium at the Korean Institute of Tuberculosis, which is the reference laboratory for TB in South Korea. Wells contained the following critical concentrations of anti-TB drugs: 0.2 µg/mL of INH and 40 µg/mL of RIF. Growth exceeding that of the control wells by >1% was considered to indicate drug resistance.

4. Treatment outcome

Patient treatment was not standardized. Treatment was individualized according to the previous treatment history and DST results for other drugs, such as INH. The treatment outcome categories were assessed in accordance with the revised 2013 WHO definitions¹⁴. In addition, we assessed the incidence of recurrence within 1 year following the completion of treatment.

5. Statistical analyses

Data are presented as the means for continuous variables

and percentages for discrete variables. Variables were analyzed using simple descriptive statistics.

Results

1. Frequency of disputed *rpoB* mutations

Among the 130 cases with RIF resistance on the MTBDR_{plus} assay, 15 were found to be RIF-susceptible on DSTs. Of these 15 cases, 11 underwent *rpoB* sequencing analysis. With the exception of two cases with wild-type strains on *rpoB* sequencing, the remaining nine exhibited disputed *rpoB* mu-

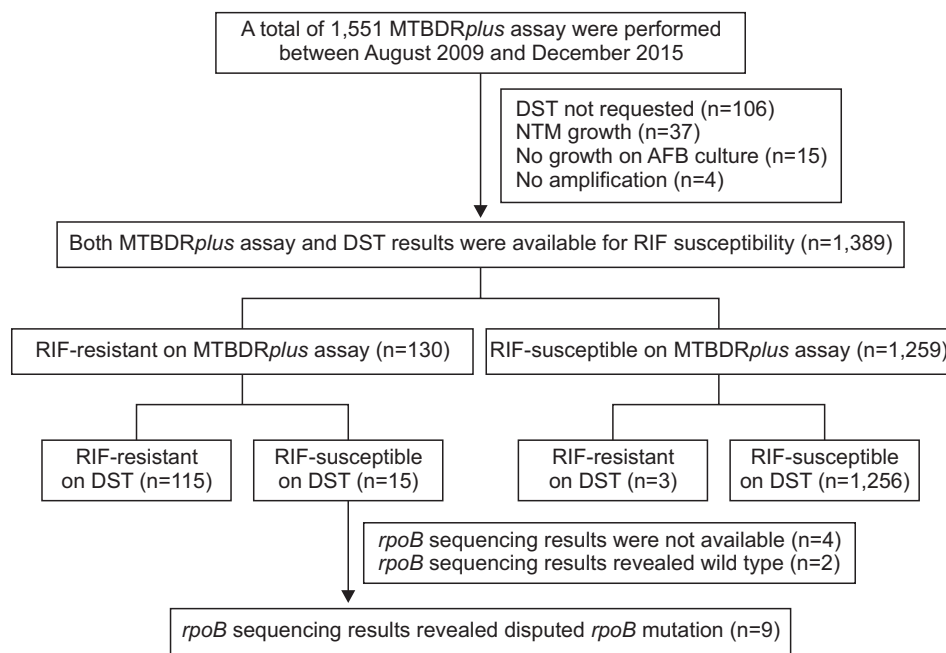


Figure 1. Study flow chart. DST: drug susceptibility test; NTM: non-tuberculous mycobacteria; AFB: acid-fast bacillus; RIF: rifampin.

Table 1. Results of the MTBDR_{plus} assay and *rpoB* sequencing in the nine study patients with disputed *rpoB* mutations

Patient No.	MTBDR _{plus} assay results		Sequencing results
	Wild-type loss	Mutation detected	<i>rpoB</i> mutation
1	<i>rpoB</i> WT2	Not detected	CTG511CCG (L511P)
2	<i>rpoB</i> WT2	Not detected	CTG511CCG (L511P)
3	<i>rpoB</i> WT2	Not detected	CTG511CCG (L511P)
4	<i>rpoB</i> WT2	Not detected	CTG511CCG (L511P)
5	<i>rpoB</i> WT2	Not detected	CTG511CCG (L511P)
6	<i>rpoB</i> WT4	Not detected	GAC516TAC (D516Y)
7	<i>rpoB</i> WT4	Not detected	GAC516TAC (D516Y)
8	<i>rpoB</i> WT7	Not detected	CAC526AAC (H526N)
9	<i>rpoB</i> WT7	Not detected	CAC526AAC (H526N)

WT: wild type.

tations (Figure 1). Thus, disputed *rpoB* mutations were identified in at least 6.9% of the cases (9/130) with RIF resistance, as determined via the MTBDR*plus* assay, at our center. In addition, the sequencing of the *rpoB* gene of 170 strains with *rpoB* mutations at the ITRC showed that 23 (13.5%) had disputed *rpoB* mutations¹².

2. Types of disputed *rpoB* mutations

Table 1 presents the results of the MTBDR*plus* assay and *rpoB* sequencing for the nine patients with *rpoB* mutations in their *M. tuberculosis* isolates. Among these nine patients, the most common disputed mutation was CTG511CCG (n=5), followed by GAC516TAC (n=2) and CAC526AAC (n=2). These sequencing patterns were precisely consistent with the location of the wild-type loss indicated in the MTBDR*plus* assay. In addition, the order of frequency of the disputed *rpoB* mutations in the 23 strains stored in the ITRC was as follows: CTG533CCG (n=8), GAC516TAC (n=6), CAC526CTC (n=4), CTG511CCG (n=3), CAC526AAC (n=1), and ATG515GTG (n=1).

After combining the results of the 32 strains from both our center and the ITRC, the order of frequency of the disputed *rpoB* mutations was as follows: CTG511CCG (n=8), GAC516TAC (n=8), CTG533CCG (n=8), CAC526CTC (n=4), CAC526AAC (n=3), and ATG515GTG (n=1).

3. Treatment regimen and outcomes in the nine patients with disputed *rpoB* mutations

Table 2 summarizes the treatment regimen, duration, and treatment outcome of the nine patients with disputed *rpoB* mutations at our center. All of these patients were diagnosed with pulmonary TB and more than 50% were treated with an MDR-TB regimen (patients 1, 3, 6, 7, 8, and 9). The remaining three patients received a treatment regimen comprising first-line drugs (patients 2, 4, and 5). In particular, two patients (patients 4 and 5) were prescribed a regimen including high-dose RIF (20 mg/kg). Most of the patients (except patient 7) exhibited favorable outcomes. We could not assess the treatment outcomes of 23 cases with disputed mutations in the ITRC.

Discussion

Among *rpoB* mutations, the most commonly detected have been GAC516GTC, CAC526TAC, CAC526GAC, and TCG531TTG, all of which are included as mutation probes in the MTBDR*plus* assay. In contrast to these four undisputed mutations that cause high-level RIF resistance, some *rpoB* mutations can cause low-level RIF resistance and are referred to as disputed mutations⁷. The most important finding of this study was that disputed *rpoB* mutations do not appear to be

Table 2. Isoniazid susceptibility, treatment regimen and duration, and treatment outcome in the nine study patients with disputed *rpoB* mutations

Patient No.	Age (yr)	Sex	INH-susceptibility: MTBDR <i>plus</i> assay	INH-susceptibility: DST	Initial regimen	Duration of first regimen (mo)	Subsequent regimen	Duration of subsequent regimen (mo)	Treatment outcome	1 Year recurrence
1	43	Male	Resistant	Resistant	RIF, EMB, PZA	4	MXF, KM, PTH, CS, PAS	17	Cured	No recurrence
2	50	Male	Susceptible	Susceptible	INH, RIF, PZA	6	-	-	Cured	No recurrence
3	52	Male	Susceptible	Resistant	INH, RIF, EMB, PZA	5	MXF, KM, PTH, CS, PAS	13	Cured	No recurrence
4	56	Male	Susceptible	Resistant	INH, HD-RIF, EMB	1	MXF, HD-RIF, EMB	11	Cured	No recurrence
5	59	Male	Susceptible	Susceptible	INH, RIF, EMB, PZA	2	INH, HD-RIF	8	Treatment completed	No recurrence
6	24	Female	Resistant	Resistant	LVFX, KM, PZA, PTH, CS	14	-	-	Cured	No recurrence
7	39	Female	Resistant	Resistant	MXF, KM, PZA, PTH, CS	8	-	-	Treatment failed	N/A
8	69	Female	Resistant	Resistant	LVFX, KM, PZA, PTH, PAS	3	MXF, RIF, CS, AMOX	9	Cured	No recurrence
9	55	Male	Resistant	Resistant	INH, RIF, PZA, CS	0.5	LZD, PZA, CS	-	On treatment	N/A

INH: isoniazid; DST: drug susceptibility test; RIF: rifampicin; EMB: ethambutol; PZA: pyrazinamide; MXF: moxifloxacin; KM: kanamycin; PTH: prothionamide; CS: cycloserine; PAS: p-aminosalicylic acid; HD-RIF: high dose rifampin; LVFX: levofloxacin; N/A: not available; AMOX: amoxicillin/davulanate; LZD: linezolid.

rare among those exhibiting RIF resistance in South Korea.

Van Deun et al.¹⁵ reported that disputed mutations comprise 13.1% and 10.6% of all *rpoB* mutations in strains derived from patients with first failure or relapse in Bangladesh and Kinshasa, respectively. In a recent study, most of the non-silent RRDR mutations detected directly from diagnostic sputum specimens of previously untreated cases in Bangladesh belonged to the disputed group⁷. Another study conducted in Australia reported that *M. tuberculosis* isolates that are genotypically resistant but phenotypically susceptible to RIF accounted for 9% of all cases⁵. A recent study performed in Germany reported that 2.8% of strains (4/143) submitted to the German National Reference Laboratory had disputed *rpoB* mutations in INH-resistant and RIF-susceptible *M. tuberculosis* isolates¹⁶. In the present study, a disputed *rpoB* mutation was identified as the cause of phenotypic or genotypic RIF resistance from at least 6.9% (our center) and 13.5% (ITRC) cases. It should be noted that we could not assess the exact prevalence of the disputed *rpoB* mutations at our center, because *rpoB* sequencing was not performed for all cases, as it was dependent on the attending physician.

Not all disputed mutations exhibit low-level resistance. One previous study reported that most strains with disputed mutations were RIF-resistant in a routine Löwenstein-Jensen medium DST (78.7%)¹⁵. Given that the strains with CTG511CCG or CTG533CCG were found to be RIF-susceptible by a phenotypic DST in approximately half of the tested cases^{15,17-19}, the level of resistance appears to be dependent on the type of the disputed mutation. As *rpoB* sequencing was conducted for only a portion of the cases that were RIF-resistant by the MTBDR*plus* assay at our center, it is highly likely that we overlooked the disputed *rpoB* mutations with high-level resistance, which would have been reported as RIF-resistant in the DST. In fact, among the 115 strains that were RIF-resistant both by the MTBDR*plus* assay and DST, 17 exhibited a pattern suspected as being a disputed mutation on the MTBDR*plus* assay (the loss of the wild-type probe without any hybridization of the mutation probe). Therefore, the prevalence of disputed *rpoB* mutations may have been at lot higher if some of these 17 cases actually had disputed *rpoB* mutations; as high as 20.0% (26 out of 130 stains with RIF resistance on the MTBDR*plus* assay).

Although the WHO guidelines recommend that any patient with RIF-resistant TB should be treated with the recommended MDR-TB regimen irrespective of the INH susceptibility²⁰, patients infected with *M. tuberculosis* strains exhibiting low-level RIF resistance caused by disputed mutations present a therapeutic challenge in terms of high-dose RIF based treatment. Van Ingen et al.⁶ reported the feasibility of high-dose RIF therapy for the GAC516TAC mutation, with an MIC of 0.5–2 µg/mL, determined using 7H10 agar dilution methods. They found that a 20 mg/kg dose of RIF is likely to overcome an MIC of 1 µg/mL, but not 2 µg/mL, thus suggesting that high-

dose RIF treatment (20 mg/kg) might be feasible for isolates with disputed mutations for an MIC up to 1 µg/mL⁶. In fact, evidence in the literature for the current standard dose of RIF (600 mg) is scarce, and a high-dose RIF has been shown to increase bactericidal activity without significantly increasing the toxicity²¹. Therefore, the use of a higher dose of RIF (e.g., 900 mg or 1,200 mg) should be re-explored in the context of low-level RIF resistance⁵. In our present study, two patients (patients 4 and 5) were treated with a high dose of a RIF-containing regimen, and showed favorable outcomes. Although there may be concerns regarding the potential hazardous adverse effects of higher RIF doses, there is little evidence that higher daily RIF doses lead to increased toxicity in the literature²¹. In fact, one randomized phase II trial recently found that there was no significant increase in adverse events when the RIF dose was increased from 10 to 15 or 20 mg/kg²². Nevertheless, further studies are needed to determine the efficacy and optimal duration of a high-dose RIF-based regimen for disputed *rpoB* mutations with low-level resistance.

Previous studies have shown that *M. tuberculosis* isolates with disputed *rpoB* mutations had poorer outcomes if they were treated with standard dosing RIF-based regimens; in fact, the rate of treatment failure ranged from 25% to 70%, even though the cases were found to be RIF-susceptible via DST^{5,23,24}. Therefore, in cases found to be RIF-resistant via an MTBDR*plus* assay and RIF-susceptible via the DST, *rpoB* sequencing should be performed to identify the cause of the discrepancy, particularly regarding the presence of disputed mutations. If *rpoB* sequencing cannot be performed, treatment options other than the standard first-line anti-TB regimens (e.g., regimens containing a higher dose of RIF or second-line drugs) should be considered. In addition, considering that this resistance pattern is not detected by the current phenotypic DST methods, it may be necessary to perform genotypic DST for all *M. tuberculosis* isolates, if the burden of disputed *rpoB* mutations cannot be ignored. In this context, we have developed a rapid molecular test kit, which can rapidly detect disputed mutations without the need for sequencing analysis (Supplementary Methods, Supplementary Figures S1, S2).

Our study had several notable limitations. Most significantly, it was conducted with strains from only two centers, and thus included only a few cases with disputed *rpoB* mutations. The number of strains from these centers appears to be insufficient to draw highly reliable conclusions regarding the frequency and common types of mutations in South Korea. In addition, as the decision for performing sequencing analysis was dependent on the attending physician, gene sequencing was not performed in some cases with discrepant results at our center. Moreover, we did not measure the MIC of each strain with disputed mutations. Finally, the isolates yielding discrepant results were not re-tested by all the methods to ascertain the reproducibility of the initial testing result.

In conclusion, disputed *rpoB* mutations do not appear

to be rare among the *M. tuberculosis* strains exhibiting RIF resistance in South Korea. We have identified the types of disputed *rpoB* mutations from 32 strains with this mutation at our center and the ITRC. All the patients with strains showing disputed mutations received individualized treatment regimens at our center, with favorable outcomes observed in most cases, including two that were treated with a high-dose RIF-containing regimen.

Supplementary Material

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

Supplementary Figure S1. (A) A schematic illustration of each probe from the upgraded version of the MolecuTech REBA MTB-MDR Kit. (B) Detection of the CTG511CCG (L511P) disputed *rpoB* mutation using the upgraded version of the MolecuTech REBA MTB-MDR Kit.

Supplementary Figure S2. The detection of undisputed (A, B) and disputed (C–F) mutations using the upgraded version of the MolecuTech REBA MTB-MDR Kit.

Conflicts of Interest

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

Acknowledgments

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