Drug-resistance mechanism, pathogenesis and genomics of tuberculosis: Molecular characteristics and associations of rifampicin resistance to other antituberculosis drugs in *Mycobacterium tuberculosis*

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Summary

Rifampin (RIF) is bactericidal, and acts on both intracellular and extracellular organisms. RIF is a potent drug for tuberculosis (TB) treatment and is a marker for multidrug-resistant (MDR) TB. However, RIF has limitation in treating MDR-TB and cases with side-effects and co-infected with HIV. To understand genetic alterations of the *rpo*B gene conferring RIF resistance, genetic differences to rifabutin (RBT), and the association with other anti-tuberculosis drugs, we conducted a population-based analysis of 800 MDR *Mycobacterium tuberculosis* isolates. Of the 800 isolates, predominant *rpoB* mutations conferred RIF resistance were S531L (61.8%), H526Y (6.7%) and H526D (4.4%). We found that the cross-resistant rate between RIF and RBT was 87.0%. Among isolates with single mutation in the *rpoB* gene, mutations at codon 146, 513 and 531 were only observed in RBT-resistant isolates, whereas, mutations at codons 143, 511, 516, 522 and 529 were only in RBT-susceptible ones. Interestingly, isolates with amino acid substitutions at codon 526 (H to C, L, T, N) and codon 529 (R to L) were susceptible to RBT. We further analyzed 568 isolates with results of the first and second-line drug susceptibility testing (DST), and found codon 531 mutation of the *rpoB* gene was significantly associated with ofloxacin and ethionamide resistance, and negatively associated with kanamycin resistance ($p<0.05$). Whereas, codons 513 and 533 mutations were not found in isolates resistant to

amikacin or capreomycin. Consequently, specific mutations of the *rpoB* gene can be used to determine RBT susceptibility and to predict drug resistance to other anti-TB drugs.

Purpose

The objective of this study is to understand genetic alterations of the *rpo*B gene conferring RIF resistance, genetic differences to RBT, and the association with other anti-TB drugs, we conducted a population-based analysis of 800 MDR *Mycobacterium tuberculosis* isolates.

Materials and Methods

Study populations. *M. tuberculosis* isolates identified as MDR based on bacteriological (culture in Löwenstein-Jensen or MGIT® medium), biochemical and molecular identification were collected from each of the 803 cases in certified clinical Mycobacteriology laboratories throughout Taiwan from January 2006 to December 2010. We excluded one confirmed mixed culture and two isolates without RBT DST results. The spare 800 isolates were used to evaluate the cross-resistant rate between RIF and RBT. In addition, we also evaluated the relationships between specific *rpo*B mutations and the percentage of resistance to the first and second-line anti-TB drugs from 568 isolates with complete DST data.

Drug susceptibility testing. The agar proportion method on either Middlebrook 7H10 or 7H11 (Creative Microbiologicals or Sancordon, Taiwan), and BACTEC™ MGIT™ 960 SIRE Kits (Becton Dickinson Diagnostic Systems, Sparks, MD) with a

liquid culture system were used. The critical first-line drug concentrations for the agar proportion method on 7H10 were 0.2 μg/ml and 1.0 μg/ml for INH, 1.0 μg/ml for RIF, 5.0 μg/ml and 10μg/ml for ethambutol (EMB), 2.0 μg/ml and 10μg/ml for streptomycin (STR). Isolates resistant to at least INH and RIF were considered MDR and were subjected to the second-line drug DST. The critical concentrations of second-line drugs for the agar proportion method on 7H11 were 2 μg/ml for ofloxacin (OFX), 6 μg/ml for AMK, 6 μg/ml for KAN, 10 μg/ml for CM, 8μg/ml for p-aminosalicylic acid (PAS), 10μg/ml for ethionamide (ETH), and 0.5μg/ml for RFB. Growth on the control medium was compared to growth on the drug-containing medium to determine susceptibility. The DST results were categorized as resistant or susceptible. The tests were validated by comparison to the susceptibility of *M. tuberculosis* H37Rv included in the same DST. MDR was defined as *M. tuberculosis* isolates resistant to at least INH and RIF.

DNA sequencing of the *rpo*B gene. Two primer sets were used to analyze the variation at the *rpo*B gene. A 541-bp fragment which contains the 81-bp hotspot region was amplified and sequenced with the oligonucleotide primers *rpoB*-F (5′-TCGGCGAGCCCATCACGTCG-3′) and *rpoB*-R (5′-GCGTACACCGACAGCGAGCC-3′) [1]. A 365-bp fragment targeting the V146F (V176F according to the *M. tuberculosis* numbering system) mutation was amplified and sequenced with the oligonucleotide primers TB-176-F (5′-CTTCTCCGGGTCGATGTCGTTG-3′) and TB-176-R (5′-CGCGCTTGTCGACGTCAAACTC-3′) [2]. The PCR reactions were performed as follows: 35 cycles at 96°C for 1 min; annealing at 64°C for 1 min; and elongation at 72°C for 1 min. Thereafter, the PCR products were analyzed with an ABI 3730 automated sequencer (Applied Biosystems, USA), and the sequence data were

assembled and edited using the Sequencing Analysis 5.2.0 software (Applied Biosystems, USA). In this study, codons were numbered according to the *E. coli* numbering system.

Statistical analysis. The percentage of resistant to various anti-TB drugs between wild-type and mutated groups at specific location of the *rpo*B gene was compared using binomial test. *P*-value < 0.05 was considered as statistically significant.

Results

***rpoB* gene sequence conferring RIF resistance.** Predominant *rpoB* mutations conferring RIF resistance of the 800 MDR *M. tuberculosis* isolates were at codons S531L (61.8%), H526Y (6.7%) and H526D (4.4%). The cross-resistant rate between RBT and RIF was 87.0% (696/800). Of the 800 isolates, 740 (92.5%) had single mutation, 40 (5%) had double mutations, 1 had triple mutations, 6 had deletion, and 13 (1.6%) showed wild-type. We revealed 13 novel *rpoB* gene variations conferring RIF resistance including 11 isolates with single mutation (R143C, V144A, Q148R, S164P, D444V, E458K, T480I, A501T, R529Q, R529L, M558K) and two deletions (509-511 or 510-512, and 9 bp discontinuous deletion at 513-516).

***rpoB* gene sequence conferring RIF but not RBT resistance.** Correlations between specific *rpoB* mutations confer RBT resistance among 740 MDR *M. tuberculosis* isolates with single mutation in the *rpoB* gene, and DST results using minimum inhibitory concentration (MIC) tests were summarized in Table 1. Of the 740 isolates, 68.5% harbored mutations in codon 531, 16.8% in codon 526, 5.9% in codon 516, 3.6% in codon 533 and 3.0% in codon 513. Nevertheless, 12.3% (91/740) of the

studied isolates with single mutation in the *rpoB* gene were RBT-susceptible. Interestingly, 11 single mutations (R143C, L511P, D516Y, D516V, D516F, S522L, H526C, H526L, H526T, H526N, R529L) were identified only in RBT-susceptible isolates (Table 1). In addition, for MDR isolates with more than one mutated codon, changes in their resistance to RBT could be found in isolates with multiple mutations at codon 511 (71.4%, 5/7) and 516 (84.6%, 11/13).

Drug susceptibility of MDR *Mycobacterium tuberculosis* isolates. Since PZA, PAS and ETH resistance were not tested in the beginning of our laboratory program, only 568 MDR *M. tuberculosis* isolates had complete results of the first and second-line DST were included in the analysis. The drug susceptibility patterns of the 568 clinical MDR isolates were summarized in Table 2. Of the 568 isolates, 52.8% were resistant to EMB, 43.3% to STR, 34.0% to PZA, 23.4% to OFX, 11.4% to PAS, 29.0% to ETH, 5.5% to AMK, 9.9% to KAN, and 4.9% to CM (Table 2). Overall 27.8% (158/568) were resistant to all four first-line drugs, 23.4% (133/568) were pre-XDR and 5.3% (30/568) were XDR. Moreover, DST profiles were highly diversified with 120 patterns identified. Of the 568 MDR isolates, 15 (2.6%) isolates were susceptible to all other tested drugs and 88 (15.5%) isolates had concurrent resistance to RBT. Major patterns were MDR isolates with concordant mono-resistant to RFB (88, 15.5%), concordant double-resistant to RBT and EMB (55, 9.7%), followed by concordant double-resistant to RBT and STR (37, 6.5%) (Table 2).

Correlations between *rpoB* mutations and drug resistance. Interestingly, we found associations between specific *rpoB* mutations and drug-resistant rates of various drugs. Isolates with a mutation at codon 516 had higher resistant rate to PZA ($p<0.05$), PAS, and KAN. Isolates with a mutation at codon 526 had higher resistant rate to STR

($p<0.05$). As compared to RBT-resistant MDR isolates, RBT-susceptible ones harbored mutation at codon 526 tended to have higher resistant rates to EMB, ETH and three injectable second-line drugs; however, lower resistant rate to OFX was found (Table 2). In addition, isolates with a mutation at codon 531 had higher resistant rate to OFX ($p<0.05$) and ETH ($p<0.05$), and lower resistant rate to KAN ($p<0.05$). Furthermore, isolates with a mutation at codon 533 had lower resistant rate to PZA ($p<0.05$), STR ($p<0.05$), and ETH ($p<0.05$). In contract to MDR isolates harbored mutations at codons 513, 516, 526 and 531 had high resistant rates (16.3% to 46.2%) to ETB, none of MDR isolates with mutated codon 533 was resistant to ETH ($p<0.01$) (Table 2). Moreover, MDR isolates with codons 513 and 533 mutations were not found in isolates resistant to AM or CAP.

Discussion

RBT is as effective as RIF for TB treatment and is an alternative for treating MDR-TB patients, TB cases with serious side-effects, and HIV co-infected TB cases. However, RBT is not included in routine DST of first-line drugs for *M. tuberculosis* isolates, and subsequent testing of isolates is time-consuming. In addition, the cross-resistant rate is usually high between RIF and RBT. In this study, the cross-resistant rate between RIF and RBT is 87.0%. It was comparable to that of Australia et al. (88%, 18/22) [3] and Turkey et al. (85.4%, 35/41) [4] studies, however, higher than another research later from study of Turkey et al.(73.1%) [5]. We revealed 13 novel mutations and deletions in RIF-resistant *M. tuberculosis* clinical isolates. Moreover, we found RIF-resistant isolates with specific single mutation at codon 143, 511, 516, 522, 529 and ones with amino acid changes at codon 526 (H to C, L, T, N) of the *rpoB* gene were susceptible to RBT, and can be used as robust markers for RBT-susceptible isolates. Furthermore, we observed association of predominant mutations in the *rpo*B genes and other anti-tuberculosis drugs, and that might be used as predictors for probable resistance to various drugs during treatment and for selection of treatment options.

Previous studies revealed that variations of MICs of RBT in RIF-resistant strains carrying *rpo*B mutations depend on specific mutations in the *rpo*B gene. It has been reported that V176F (*M. tuberculosis* numbering), Q513K, Q513L, Q513P, S522W, H526R, H526Y, H526D, H526Q, H526P, S531L, S531W (*E. coli* numbering) coherently confer resistance to both RIF and RFB; whereas, D516Y, D516V, S522L, H526C confer resistance, and L511P confers low level resistance only to RIF [2, 6, 7, 8, 9, 10]. However, resistance to RIF or RBT remains controversial in isolates with

H526L and L533P [9, 10]. Our data were consistent with pervious findings, and we also confirmed 11 isolates with H526L were RBT-susceptible without ambiguous. It was postulated that simple amino acid substitution could interfere or enhance protein polar/hydrophobic enzyme and RIF interactions; while, induction of hydrogen bonding and changes in van der Waals interaction between protein and drug also had profound influence on drug resistance [11, 12, 13].

RIF had drug-drug interactions with several drugs, including antiretrovirals. Some mechanisms that RIF influences the susceptibilities of different structural drugs were postulated. RIF exposure induces multidrug-resistant gene (MDR1) expression which encodes an efflux pump contributing to fluconazole resistance in *Candida albicans*, compared to non-exposed control was up to 122-fold dose-dependent induction. However, RBT and rifamycin are not active [14]. RIF induces enzymes that transport and metabolize moxifloxacin [15]. RBT is mostly used in HIV co-infected patients because it has fewer drug interactions with antiretroviral agents than RIF. Furthermore, Srivastava et al. revealed that resistance to INH, EMB, RIF, ciprofloxacin (CIP), STR, tetracyclines (TETs), OFX and KAN were found to be related to mechanisms of efflux pump systems [16]. However, one antibiotic may induce a pump that also extrude other antibiotics or induce a particular single pathway which then leads to inductions of many different efflux pumps. Srivastava et al. proposed an evolution of simultaneous resistance to anti-tuberculosis drugs [16]. This may able to explain that some resistant strains do not harbor any mutation at resistance-related genes and partial cross-resistance phenomenon.

The frequency of major mutations was shown in Table 1. The most common mutation was at codon 531 (64%), codon 526 (17.1%) and codon 526 (7.1%).

However, only 1% of isolates with codon 531 mutation were multiple mutation cases while isolates with codon 511 or 146 were 70% and 45.5% respectively. In addition, 71.4% and 84.6% of multiple mutation cases with one common mutation at codon 511 and 516 changed their susceptibilities to RFB and became resistant.

In conclusion, RIF had high cross-resistant rate with RBT. However, RBT remains potent to isolates with certain genetic alterations. Besides, genetic mutation of certain condon of the *rpoB* gene might be used to predict drug-drug interaction to other anti-TB drugs.

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Table 1. MDR *Mycobacterium tuberculosis* isolates with multiple mutations in the *rpoB* gene

Mutated position	No. of isolates with any mutation (%)	No. of isolates with multiple mutations (%)
146	11 (1.4)	5 (45.5)
511	10 (1.3)	7 (70.0)
513	25 (3.1)	3 (12.0)
516	57 (7.1)	13 (22.8)
526	137 (17.1)	13 (9.5)
531	512 (64.0)	5 (1.0)
533	39 (4.9)	12 (30.8)
Total	800	58 (7.5)

Table 2. Associations of various mutation codons at the *rpo*B gene and the first and the second-line drugs of 568 multidrug-resistant *Mycobacterium tuberculosis* isolates

Mutation[f]		No. of MDR isolates	EMB	PZA	STR	PAS	OFX	ETH	KAN	CM	AMK
			No. of resistant isolates (%)								
513[a]	wt	547	289 (52.8)	186 (34.0)	241(44.1)	61 (11.2)	131 (23.9)	159 (29.1)	54 (9.9)	28 (5.1)	31 (5.7)
	mut	21	11 (52.4)	7 (33.3)	5 (23.8)	4 (19.0)	2 (9.5)	6 (28.6)	2 (9.5)	0 (0.0)	0 (0.0)
516[b]	wt	537	283 (52.7)	177 (33.0)	229 (42.6)	56 (10.4)	123 (22.9)	154 (28.7)	49 (9.1)	26 (4.8)	28 (5.2)
	mut	31	17 (54.8)	**16 (51.6)***	17 (54.8)	**9 (29.0)**[g]**	10 (32.3)	11 (35.5)	**7 (22.6)**[g]**	2 (6.5)	3 (9.1)
526[c]	wt	482	263 (54.6)	170 (35.3)	200 (41.5)	57 (11.8)	120 (24.9)	151 (31.3)	45 (9.3)	25 (5.2)	25 (5.2)
	mut	86	**37 (43.0)***	23 (26.7)	**46 (53.5)***	8 (9.3)	**13 (15.1)***	**14 (16.3)****	11 (12.8)	3 (3.5)	6 (7.0)
526[d]	wt	555	291 (52.4)	189 (34.1)	237 (42.7)	64 (11.5)	133 (24.0)	159 (28.6)	53 (9.5)	26 (4.7)	28 (5.0)
	mut	13	9 (69.2)	4 (30.8)	**9 (69.2)*[g]**	1 (7.7)	**0 (0.0)*[g]**	6 (46.2)	3 (23.1)	2 (15.4)	**3 (23.1)**[g]**
531[e]	wt	183	93 (50.8)	58 (31.7)	84 (45.9)	25 (13.7)	33 (18.0)	41 (22.4)	25 (13.7)	8 (4.4)	12 (6.6)
	mut	385	207 (53.8)	135 (35.1)	162 (42.1)	40 (10.4)	**100 (26.0)***	**124 (32.2)***	**31 (8.1)***	20 (5.2)	19 (4.9)

533	wt	549	290 (52.8)	191 (34.8)	242(44.1)	65 (11.8)	129 (23.5)	165 (30.1)	55 (10.0)	28 (5.1)	31 (5.6)
	mut	19	10 (52.6)	**2 (10.5)***	**4(21.1)***	0 (0.0)	4 (21.1)	**0 (0.0)****	1 (5.3)	0 (0.0)	0 (0.0)
	Total	568	300 (52.8)	193 (34.0)	246 (43.3)	65 (11.4)	133 (23.4)	165 (29.0)	56 (9.9)	28 (4.9)	31 (5.5)

* P value <0.05

** P value <0.01

[a] Include codon 513 CAA to AAA, CTA, GAA, and CCA

[b] Include codon 516 GAC to TAC, GGC, GTC, and TTC

[c] Include codon 526 CAC to CGC, TAC, GAC, CAA, and CCC, which were associated with RFB resistance

[d] Include codon 526 CAC to TGC, CTC, AAC, and ACC, which were not associated with RFB resistance

[e] Include codon 531 TCG to TTG and TGG

[f] wt, wild-type; mut, mutant

[g] Significance may not be valid, because the sample size is too small.