Journal of Microbiology, Immunology and Infection (2013) 46, 266–270

Available online at www.sciencedirect.com

SciVerse ScienceDirect

journal homepage: www.e-jmii.com

ORIGINAL ARTICLE

Resistance profiles and *rpoB* gene mutations of *Mycobacterium tuberculosis* isolates in Taiwan

Yun-Ho Lin [a], Chun-Hsi Tai [a], Chia-Ru Li [a], Chin-Fu Lin [c], Zhi-Yuan Shi [a,b,*]

[a] *Division of Infectious Diseases, Department of Internal Medicine, Taichung Veterans General Hospital, Taichung, Taiwan*
[b] *School of Medicine, National Yang-Ming University, Taipei, Taiwan*
[c] *Microbiology Section of the Medical Laboratory, Taichung Veterans General Hospital, Taichung, Taiwan*

Received 30 April 2011; accepted 19 June 2012

KEYWORDS
Mycobacterium tuberculosis;
Resistance profiles;
rpoB

Background/Purpose: The rifampicin resistance of *Mycobacterium tuberculosis* is caused by mutations in the 81-base pair region of the *rpoB* gene encoding the β-subunit of RNA polymerase. Sequences of the *rpoB* gene of 68 isolates were analyzed to identify the mutations and to compare the mutations with their related susceptibilities.
Methods: Susceptibility tests of 68 *M. tuberculosis* isolates, collected in Taiwan during the period from 1999 to 2011, were performed by the modified agar proportion method according to Clinical and Laboratory Standards Institute recommendations. Sequences of the *rpoB* gene and the resistance profiles were analyzed and compared with the data from different geographic regions.
Results: Seven alleles were identified. Among 47 isolates of allele 1 (without mutations of *rpoB*), 46 were rifampicin-susceptible. The other 21 isolates (alleles 2 to 7, with mutations of *rpoB*) were rifampicin-resistant, including 18 isolates that were multidrug-resistant. Five mutated alleles demonstrated a single mutation. The mutations occurred in the codons 531 (68.2%), 513 (9.1%), 533 (9.1%), 516 (4.5%), and 526 (4.5%). The sensitivity and specificity of *rpoB* mutations for predicting the rifampicin-resistance of *M. tuberculosis* were 95.5% and 100%, respectively.
Conclusion: The most prevalent mutations of the *rpoB* gene were missense mutations in the critical codons, encoding Ser-531, Gln-513, Leu-533, Asp-516, and His-526. These mutations had high sensitivity and specificity for predicting the rifampicin-resistance of *M. tuberculosis* isolates. The resistance profiles and the frequencies of mutated codons of the *rpoB* gene varied in different

* Corresponding author. Division of Infectious Diseases, Department of Internal Medicine, Taichung Veterans General Hospital, 160 Section 3, Chung-Kang Road, Taichung 40705, Taiwan.
E-mail address: zyshi@vghtc.gov.tw (Z.-Y. Shi).

http://dx.doi.org/10.1016/j.jmii.2012.06.008

geographic regions, indicating that resistance evolved under the selective pressure of the therapeutic regimens and the spread of different genetic clones.

Introduction

Multidrug resistance in *Mycobacterium tuberculosis* is a global burden.[1] According to a report from World Health Organization,[1] there were an estimated 8.8 million incident cases of tuberculosis (TB) (range, 8.5 million—9.2 million) globally in 2010, 1.1 million deaths (range, 0.9 million—1.2 million) among human immunodeficiency (HIV)-negative cases of TB, and an additional 0.35 million deaths (range, 0.32 million—0.39 million) among people who were HIV-positive. There were an estimated 12.0 million prevalent cases (range, 11.0 million—14.0 million) of TB in 2010. In 2010, there were an estimated 650,000 cases of multidrug-resistant TB (MDR-TB) among the world's 12.0 million prevalent cases of TB. Among the estimated 8.8 million new and recurrent cases of TB in 2010, 5.7 million were diagnosed and reported to national TB control programs; among notified cases, there were an estimated 290,000 cases of MDR-TB, of which only 53,000 patients (18%) were reported to have been diagnosed with the condition and enrolled on appropriate treatment. According to the Taiwan Tuberculosis Control Report 2011,[2] the annual incident cases of TB were 13,237 (57.2 per 100,000 population) in 2010, and the estimated rates of MDR-TB among new cases and previously treated cases were 1.1% and 8.2% in 2009, respectively. From a study conducted by a northern Taiwanese medical center during the period from 2003 to 2004, the estimated primary resistance patterns for 486 patients were 5.4% for isoniazid, 0.4% for rifampicin, 0.4% for ethambutol, 0.8% for streptomycin, and 8.0% for pyrazinamide).[3] The resistance of the *M. tuberculosis* complex to rifampicin is caused by mutations in the 81 base pair (bp) region of the *rpoB* gene encoding the β-subunit of RNA polymerase.[4–6] The frequency of codon mutations of *rpoB* in rifampicin-resistant *M. tuberculosis* isolates varied in different geographic regions. The sequence analysis of *rpoB* of 37 isolates from Italy demonstrated mutations in codons 531 (59.4%), 526 (35.1%), and 516 (8.1%).[7] The data of 86 isolates from China showed mutations in codons 531 (41.0%), 526 (40.0%), 516 (4.0%), 513 (2.0%), and 533 (2.0%).[8] The analysis of prevalence of *rpoB* mutations was conducted by two reports in Taiwan in the past decade.[9,10] Further surveillance is clearly needed to detect the evolution of mutations and associated resistance.

In this study, *M. tuberculosis* isolates, including rifampicin-resistant and rifampicin-susceptible isolates collected in Taiwan during the period from 1990 to 2011, were tested for susceptibility to rifampicin by the modified agar proportion method according to the Clinical and Laboratory Standards Institute (CLSI) recommendations.[11] Sequences of their *rpoB* genes and resistance profiles were analyzed and compared with the mutations among different geographic regions.

Materials and methods

Collection of *M. tuberculosis* isolates

Sixty-eight *M. tuberculosis* isolates were collected for this study, including 39 isolates from Taichung Veterans General Hospital (TCVGH), a medical center in central Taiwan; 25 isolates from 8 hospitals in northern Taiwan and 4 isolates from 1 hospital in southern Taiwan during the period from 1999 to 2011. The sources of specimens included sputum (60, 88.2%), cerebrospinal fluid (5, 7.4%), pleural effusion (2, 2.9%) and abscess (1, 1.5%).

Susceptibility test

The antimycobacterial susceptibility tests were performed according to the CLSI M24-A2 modified agar proportion method.[11] The procedure is performed by inoculating equal quantities of 100× and 10,000× dilutions of a standardized inoculum (0.5—1 McFarland) onto 7H10 agar medium (Bio Star, Taichung, Taiwan) with and without the test drug and then incubating them at 35°C, 10% CO_2 for 21 days. The number of colony-forming units growing on the drug-containing medium compared with those growing on the drug-free medium are then determined and expressed as a percentage. Resistance is defined as growth on drug-containing agar is greater than 1% of the number of colonies that grow on the drug-free agar. The concentrations of antituberculosis drugs were as follows: isoniazid 0.2 μg/mL, streptomycin 2.0 μg/mL, rifampicin 1.0 μg/mL, and ethambutol 5.0 μg/mL. Multidrug resistance was defined as isolates resistant to at least isoniazid and rifampicin.[11]

DNA sequencing

The DNA was extracted by 10 mg/mL of proteinase K and 5% Tween 20 in 200 mM TRIS-HCl solution (pH 8.3) at 60°C overnight. The polymerase chain reaction (PCR) was performed in a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, CT, USA) using the primer pair 5'-TCGAATATCTGG TCCGCTTG-3' ; 3'-ACACGATCTCGTCGCTAAC-5'. The PCR began with an initial 4-minute denaturation at 94°C, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 2 minute, and extension at 72°C for 2 minute, and finally an extension step at 72°C for 5 minute. The sequence reactions were performed by adding fluorescent terminators (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) and the primers used in the initial PCR. The sequences were determined with an ABI PRISM 310 and 3100 automated DNA sequencer (Perkin-Elmer Applied Biosystems).

Table 1 Mutations of *rpoB* and resistance profiles

Alleles[a]	Codon and amino acid change	No. of isolates	Resistance profiles (*n*)
Allele 1	No mutation	47	susceptible to 4 drugs:(27); Rif+SM:(1); EMB:(2); INH:(9); INH+EMB:(3); INH+EMB+SM:(5)
Allele 2	531 TCG(Ser)→TTG(Leu)	14	INH+Rif+SM:(8); INH+Rif:(4); Rif:(2)
Allele 3	526 CAC(His)→TGC(Cys)	1	INH+Rif+SM:(1)
Allele 4	516 GAC(Asp)→GTC(Val)	1	INH+Rif+SM:(1)
Allele 5	531 TCG(Ser)→TGG(Try)	1	INH+Rif:(1)
Allele 6	533 CTG(Leu)→CCG(Pro)	2	INH+Rif+SM+EMB:(1); Rif:(1)
Allele 7	513 CAA(Gln)→AAA(Lys)	2	INH+Rif: (2)

[a] The GenBank accession numbers of alleles 1 to 7 are AY155355 to AY155361. Allele 1 is identical to the reference sequence, L27989.
EMB = ethambutol; INH = isoniazid; RIF = rifampicin; SM = streptomycin.

The sequences obtained from all 68 isolates were compared and each different sequence was assigned a unique allele number.

Results

The mutations of the *rpoB* gene and the resistance profiles are listed in Table 1. Seven alleles were identified. The GenBank sequence database accession numbers of the seven alleles were AY155355 to AY155361. Allele 1 represented the original sequence without mutation, and alleles 2 to 7 demonstrated mutated sequences. Forty-seven isolates were assigned as allele 1, including 27 isolates susceptible to isoniazid, rifampicin, ethambutol, and streptomycin; one isolate resistant to rifampicin and streptomycin; and 19 isolates with other resistance profiles. Five mutated alleles (alleles 2, 4, 5, 6, 7) possessed a single mutation in the 81-bp hot region. The mutations occurred in codons 531 (63.6%, TCG→TTG; 4.5%, TCG→TGG), 513 (9.1%, CAA→AAA), 533 (9.1%, CTG→CCG), 516 (4.5%, GAC→GTC), and 526 (4.5%, CAC→TGC). Among the 21 isolates of alleles 2 to 7, 18 isolates were multidrug-resistant; two isolates of allele 2 and one isolate of allele 6 were resistant to rifampicin only. The sensitivity and specificity of *rpoB* mutations for predicting the rifampicin-resistance of the *M. tuberculosis* complex were 95.5% and 100%, respectively.

The comparison of the frequency of codon mutations in rifampicin-resistant isolates from different geographic regions is listed in Table 2. The frequency of mutations, e.g., codons 531 (41.0%-63.6%), 513 (0%-6.0%), 533 (0%-7.5%), 526 (10.5%- 40.0 %), and 516 (4.0%-16.7%) varied in different geographic regions.[7–10,12,13]

Discussion

In this study, no novel mutation was detected beyond the 23 mutations described in previous studies.[10,14] The most common mutations were missense mutations at codons 531 (Ser→Leu; Ser→Try), 513 (Gln→Lys), 533 (Leu→Pro), 516 (Asp→Val), and 526 (His→Cys). This finding is comparable to the results of previous studies for isolates from the United States,[15] Europe,[7] Asian countries,[8,12,13,16] and Taiwan.[9,10] The frequency of mutations varied in different geographic regions, such as codons 531 (41.0 % in China,[8] to 63.6% in India[13]), 513 (0% in Italy[7] and Kaohsiung, Taiwan,[9] to 6.0% in East Asia[12]), 533 (0% in Italy[7] to 7.5% in Kaohsing, Taiwan[9]), 516 (4.0% in China[8] to 16.7% in East Asia[12]), and 526 (10.5% in East Asia[12] to 40.0% in China[8]). The sequence analysis of *rpoB* in 162 MDR-TB isolates, from Taipei (located in northern Taiwan) during the period from 1998 to 2003, showed mutation in codons 531 (49.4%), 526 (20.4%), 516 (8.6%), 513 (4.9%), and 533 (3.7%).[10] The most common mutations of 53 (84.1%) of 63 rifampicin-resistant *M. tuberculosis* isolates, from Kaoshiung (located in southern Taiwan) during the period from 1996 to 1998 were at codons 531 (41.5%), 526 (18.9%), 516 (15.1%), and 533 (7.5%).[9] In this study, codon 531 accounted for 68.2% of mutations, higher than previously reported results in the world. It could be due to the spread of a prevalent clone or sampling bias due to a small number of isolates. The limited

Table 2 Frequency of *rpoB* mutations in rifampicin-resistant *M. tuberculosis* isolates from different geographic regions

Country (reference; no. of isolates)	Frequency (%) of mutated codon								
	508	511	513	516	522	526	531	533	No mutation within hot-spot region
Italy (7; $n = 37$)	0	2.7	0	8.1	0	35.1	59.4	0	0
India (13; $n = 44$)	2.2	6.8	2.2	4.5	0	22.7	63.6	2.2	2.2
East Asia (12; $n = 66$)	0	0	6.0	16.7	0	10.5	51.5	3.0	10.5
China (8; $n = 86$)	0	2.0	2.0	4.0	3.0	40.0	41.0	2.0	10.0
Kaohsiung, Taiwan (9; $n = 63$)	0	1.9	0	15.1	1.9	18.9	41.5	7.5	15.9
Taipei, Taiwan (10; $n = 162$)	0.6	0.0	4.9	8.6	2.4	20.4	49.4	3.7	9.9
This study ($n = 22$)	0	0	9.1	4.5	0	4.5	68.2	9.1	4.5

mutations for the isolates collected over a long period of time in different geographic regions and the high sensitivity and specificity of the *rpoB* gene mutation for predicting rifampicin-resistance, indicate that molecular diagnostic techniques (such as the microarray,[17–19] and the Xpert MTB/RIF test[20–22]) are powerful and reliable methods for detection of rifampicin-resistant or multidrug-resistant isolates.

Mutations at different codons of *rpoB* could be associated with different levels of rifampicin resistance. The mutation at codon 531 or 526 was associated with high-level resistance to rifampicin (minimum inhibitory concentration [MIC] $>$ 64 μg/mL)[14,23,24] and high-level cross-resistance to all rifamycins, whereas mutation at codon 516 was associated with medium-level resistance to rifampicin (MIC = 32 μg/mL), but susceptibility to rifabutin.[14] However, some isolates with mutation at codon 516 or 533 in India were shown to have high-level resistance to rifampicin (MIC $>$ 128 μg/mL).[13] Although the MIC tests were not performed in this study, multidrug resistance appeared to be prevalent in all the mutated alleles in this study.[11,23]

In earlier studies, strains with the same *rpoB* genotype but from different geographic areas had similar patterns of drug resistance.[14,23] In contrast, the 14 isolates of allele 2 demonstrated three different resistance profiles in this study. The resistance profiles could evolve by the selective pressure of therapeutic regimens over a period of time in different geographic regions.[12]

One isolate of allele 1, without mutation in the 81-bp region, was resistant to rifampicin. Although resistance to rifampicin is mostly mediated by mutations located in the 81-bp region of *rpoB*, mutations outside this region could be found occasionally to be associated with resistance to rifampin. Examples were mutations at codon 176 (GTC→TTC),[25] codon 381 (GCG→GTG),[26] codon 572 (ATC to TTC),[27,28] and codon 490 (CAG to CAT).[16,29] Another possible mechanism of rifampicin resistance could be due to efflux pumps.[30,31]

In conclusion, the most prevalent mutations of the *rpoB* gene were missense mutations at the critical codons, encoding Ser-531, Gln-513, Leu-533, Asp-516, and His-526. These mutations had high sensitivity and specificity for predicting the rifampicin resistance of *M. tuberculosis* isolates. The resistance profiles and the frequencies of mutated codons of the *rpoB* gene, which varied in different geographic regions, indicate that resistance profiles evolved under the selective pressure of the therapeutic regimens and the spread of different genetic clones.

Acknowledgments

The authors thank the staff in the Microbiology Laboratory at TCVGH for collection of the *M. tuberculosis* isolates and technical help. This study was approved by the Institutional Review Board of Taichung Veterans General Hospital (IRB no. CE12118).

References

1. World Health Organization. Global Tuberculosis Control 2011. Available at: http://www.who.int/tb/publications/global_report/en/index.html. [accessed 01.07.12].
2. Centers for Disease Control , R.O.C.(Taiwan). *Taiwan tuberculosis control report* 2011. Available at: http://www2.cdc.gov.tw/public/data/219124871.pdf [accessed 10.07.12].
3. Su WJ, Feng JY, Huang CC, Perng RP. Increasing drug resistance of *Mycobacterium tuberculosis* isolates in a medical center in northern Taiwan. *J Formos Med Assoc* 2008;**107**:259–64.
4. Musser JM. Antimicrobial agent resistance in mycobacteria: molecular genetic insights. *Clin Microbiol Rev* 1995;**8**:496–514.
5. Telenti A, Imboden P, Marchesi F, Lowrie D, Cole S, Colston MJ, et al. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. *Lancet* 1993;**341**:647–50.
6. Miller LP, Crawford JT, Shinnick TM. The *rpoB* gene of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 1994;**38**:805–11.
7. Pozzi G, Meloni M, Iona E, Orrù G, Thoresen OF, Ricci ML. *rpoB* mutations in multidrug resistant strains of *Mycobacterium tuberculosis* isolated in Italy. *J Clin Microbiol* 1999;**37**:1197–9.
8. Yue J, Shi W, Xie J, Li Y, Zeng E, Wang H. Mutations in the *rpoB* of multidrug-resistant *Mycobacterium tuberculosis* isolates from China. *J Clin Microbiol* 2003;**41**:2209–12.
9. Hwang HY, Chang CY, Chang LL, Chang SF, Chang YH, Chen YJ. Characterization of rifampicin-resistant *Mycobacterium tuberculosis* in Taiwan. *J Med Microbiol* 2003;**52**:239–45.
10. Jou R, Chen HY, Chiang CY, Yu MC, Su IJ. Genetic diversity of multidrug-resistant *Mycobacterium tuberculosis* isolates and identification of 11 novel *rpoB* alleles in Taiwan. *J Clin Microbiol* 2005;**43**:1390–4.
11. Clinical and Laboratory Standards Institute. *Susceptibility testing of mycobacteria, nocardiae, and other aerobic actinomycetes; approved standard*. CLSI document M24-A2. 2nd ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2011.
12. Qian L, Abe C, Lin TP, Yu MC, Cho SN, Wang S, et al. *rpoB* genotypes of *Mycobacterium tuberculosis* Beijing family isolates from east Asian countries. *J Clin Microbiol* 2002;**40**:1091–4.
13. Mani C, Selvakumar N, Narayanan S, Narayanan PR. Mutations in the *rpoB* gene of multidrug-resistant *Mycobacterium tuberculosis* clinical isolates from India. *J Clin Microbiol* 2001;**39**:2987–90.
14. Williams DL, Spring L, Collins L, Miller LP, Heifets LB, Gangadharam PRJ, Gillis TP, et al. Contribution of *rpoB* mutations to development of rifamycin cross-resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 1998;**42**:1853–7.
15. Williams DL, Waguespack C, Eisenach K, Crawford JT, Portaels F, Salfinger M, et al. Characterization of rifampin-resistance in pathogenic mycobacteria. *Antimicrob Agents Chemother* 1994;**38**:2380–6.
16. Minh NN, Van Bac N, Son NT, Lien VTK, Ha CH, Cuong NH, et al. Molecular characteristics of rifampin- and isoniazid-resistant *Mycobacterium tuberculosis* strains isolated in Vietnam. *J Clin Microbiol* 2012;**50**:598–601.
17. Caoili JC, Mayorova A, Sikes D, Hickman L, Plikaytis BB, Shinnick TM. Evaluation of the TB-biochip oligonucleotide microarray system for rapid detection of rifampin resistance in *Mycobacterium tuberculosis*. *J Clin Microbiol* 2006;**44**:2378–81.
18. Li J, Xin J, Zhang L, Jiang L, Cao H, Li L. Rapid detection of *rpoB* mutations in rifampin resistant *M. tuberculosis* from sputum samples by denaturing gradient gel electrophoresis. *Int J Med Sci* 2012;**9**:148–56.
19. Yue J, Shi W, Xie J, Li Y, Zeng E, Liang L, et al. Detection of rifampin-resistant *Mycobacterium tuberculosis* strains by using a specialized oligonucleotide microarray. *Diagn Microbiol Infect Dis* 2004;**48**:47–54.
20. Scott LE, McCarthy K, Gous N, Nduna M, Van Rie A, Sanne I, et al. Comparison of Xpert MTB/RIF with other nucleic acid

technologies for diagnosing pulmonary tuberculosis in a high HIV prevalence setting: a prospective study. *PLoS Med* 2011 Jul;**8**(7):e1001061.
21. Boehme CC, Nabeta P, Hillemann D, Nicol MP, Shenai S, Krapp F, et al. Rapid molecular detection of tuberculosis and rifampin resistance. *N Engl J Med* 2010;**363**:1005—15.
22. Blakemore R, Story E, Helb D, Kop JA, Banada P, Owens MR, et al. Evaluation of the analytical performance of the Xpert MTB/RIF assay. *J Clin Microbiol* 2010;**48**:2495—501.
23. Moghazeh SL, Pan X, Arain T, Stover CK, Musser JM, Kreiswirth BN. Comparative antimycobacterial activities of rifampin, rifapentine, and KRM-1648 against a collection of rifampin-resistant *Mycobacterium tuberculosis* isolates with known *rpoB* mutations. *Antimicrob Agents Chemother* 1996; **40**:2655—7.
24. Ohno H, Koga H, Kohno S, Tashiro T, Hara K. Relationship between rifampin MICs for and *rpoB* mutations of *Mycobacterium tuberculosis* strains isolated in Japan. *Antimicrob Agents Chemother* 1996;**40**:1053—6.
25. Heep M, Rieger U, Beck D, Lehn N. Mutations in the beginning of the *rpoB* gene can induce resistance to rifamycins in both *Helicobacter pylori* and *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2000;**44**:1075—7.
26. Taniguchi H, Aramaki H, Nikaido Y, Mizuguchi Y, Nakamura M, Koga T, et al. Rifampicin resistance and mutation of the *rpoB* gene in *Mycobacterium tuberculosis*. *FEMS Microbiol Lett* 1996;**144**:103—8.
27. Aktas E, Durmaz R, Yang D, Yang Z. Molecular characterization of isoniazid and rifampin resistance of *Mycobacterium tuberculosis* clinical isolates from Malatya, Turkey. *Microb Drug Resist* 2005;**11**:94—9.
28. Yuen LKW, Leslie D, Coloe PJ. Bacteriological and molecular analysis of rifampin-resistant *Mycobacterium tuberculosis* strains isolated in Australia. *J Clin Microbiol* 1999;**37**: 3844—50.
29. Cavusoglu C, Hilmioglu S, Guneri S, Bilgic A. Characterization of *rpoB* mutations in rifampin-resistant clinical isolates of *Mycobacterium tuberculosis* from Turkey by DNA sequencing and line probe assay. *J Clin Microbiol* 2002;**40**:4435—8.
30. Siddiqi N, Das R, Pathak N, Banerjee S, Ahmed N, Katoch VM, et al. *Mycobacterium tuberculosis* isolate with a distinct genomic identity overexpresses a tap-like efflux pump. *Infection* 2004;**32**:109—11.
31. Louw GE, Warren RM, Van Pittius NC, McEvoy CRE, Van Helden PD, Victor TC. A balancing act: efflux/influx in mycobacterial drug resistance. *Antimicrob Agents Chemother* 2009;**53**:3181—9.