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rpoB Genotypes of *Mycobacterium tuberculosis* Beijing Family Isolates from East Asian Countries

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The 81-bp region of the *rpoB* gene in 66 Rif^r *Mycobacterium tuberculosis* isolates from China, Japan, Korea, and Taiwan was analyzed. Twelve single-nucleotide substitutions in the *rpoB* gene were detected. The most prevalent mutations were at Ser-531 (52%), Asp-516 (17%), and His-526 (11%). Mutations were not found in seven (11%) of the isolates. Higher mutation rates in 50 Beijing family isolates were found than in other isolates for mutations at Asp-516 (18 and 12.5%, respectively) and His-526 (12 and 6.3%, respectively). The different rates of mutation may reflect the choice of rifamycin analogs.

Antibiotic resistance is a growing impediment to the control of infectious diseases worldwide, and multidrug-resistant (MDR) tuberculosis (TB) continues to be a serious part of this problem, particularly in Asia and developing countries (6, 8). In 1999, the World Health Organization reported that 80% of all incident TB cases were found in 22 countries, with more than half the cases occurring in five Southeast Asian countries, followed by the Western Pacific region, which accounted for 1.96 million cases (7). These findings indicated that the constant creation and increased circulation of MDR TB threatens not only the countries of origin, but also other countries through immigration.

Resistance to rifampin is one of the key components in antituberculosis therapeutic regimens and is a primary marker of MDR TB. The resistance to rifampin in most cases is due to the genetic alterations in the *rpoB* gene, encoding the β-subunit of DNA-dependent RNA polymerase (10, 13, 18, 20, 28, 32). It was demonstrated that more than 90% of *Mycobacterium tuberculosis* isolates with the rifampin-resistant (Rif^r) phenotype possess single-nucleotide substitutions within an 81-bp fragment, the so-called hot spot region, of the *rpoB* gene (24, 33). Similar information on the frequency of occurrence of *rpoB* mutations among isolates from different geographic origins has accumulated in recent years (9, 11, 17, 22, 25–27, 29).

Our finding of *M. tuberculosis* Beijing family, a dominant clone in China and neighboring countries in Asia, led to our interest in tracking this distinct family and other isolates from Asia (23, 30). Our recent data demonstrated that the Beijing family dominates in Taiwan, Japan, Korea and China, where it comprises 50 to 80% of all isolates (L. Qian, S. Yamamoto, X. Zheng, and J. T. Douglas, Abstr. 101st Gen. Meet. Am. Soc. Microbiol. 2001, p. 699, 2001). The Beijing family, as characterized by spoligotyping, has nine spacers from lanes 35 to 43 and typically has 15 to 20 bands by IS*6110* restriction fragment length polymorphism. This family has similar DNA typing patterns to the W family and W-like family, which also includes fully susceptible and MDR isolates (2, 30).

Since acquisition of drug resistance could play a role in contributing to the survival and spread of the Beijing family and other *M. tuberculosis* isolates, we decided to investigate the mutations in the *rpoB* gene among MDR TB isolates from four Asian countries. A total of 66 rifampin-resistant (Rif^r) MDR TB isolates from China (n = 25), Japan (n = 3), Korea (n = 18), and Taiwan (n = 20) were provided by the following institutions: Central Reference Laboratory, National Tuberculosis Control Center, Beijing, China; Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, Tokyo, Japan; Department of Microbiology, Yonsei University, Seoul, Korea; and Taiwan Provincial Chronic Disease Control Bureau, Taipei, Taiwan. Only one isolate per patient was tested. Drug susceptibility testing was performed by the institutions providing the samples, and resistance was determined by the absolute concentration method. We previously classified the isolates by spoligotyping as belonging to the nine-spacer signature of the Beijing family or as non-Beijing family isolates and by the IS*6110* DNA fingerprinting method (12, 23, 30).

Two oligonucleotide primers described by Williams et al. (31), rpo105 (5′-CGT GGA GGC GAT CAC ACC GCA GCA GTT-3′) and rpo293 (5′-AGT GCG ACG GGT GCA CGT CGC GGA CCT-3′), were used to amplify a 215-bp fragment of the *rpoB* gene containing the 81-bp hypervariable region. The PCR product was sequenced using a Prism 377 automated DNA sequencer (Applied Biosystems Inc., Foster City, Calif.). Sequence data were assembled and analyzed by Clustal W (http://www.clustalw.genome.ad.jp).

Although the genotype of *rpoB*, as a marker for MDR and epidemiology of *M. tuberculosis* from different geographic regions has been reported by several groups in the last decade (4, 5, 11, 12, 16, 17, 22, 25–29, 32), we have expanded this information by adding data from China, Japan, Korea, and Taiwan. Our analysis was also influenced by the dominance of the

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TABLE 1. Sequence analysis of rifampin-resistant *M. tuberculosis* isolates from four East Asian countries and regions

Amino acid substitution	No. (%) of isolates	Origin of isolates (no. of isolates)
Gln513Leu	1 (1.5)	China (1)
Gln513Lys	3 (4.5)	China (3)
514-Phe ins.[a]	1 (1.5)	Taiwan (1)
Asp516Ala	6 (9.1)	China (1), Korea (3), Taiwan (2)
Asp516Tyr	5 (7.6)	China (3), Korea (1), Taiwan (1)
His526Arg	1 (1.5)	Taiwan (1)
His526Asn	1 (1.5)	Korea (1)
His526Leu	2 (3.0)	Korea (2)
His526Tyr	3 (4.5)	China (1), Japan (1), Taiwan (1)
Ser531Gln	1 (1.5)	Taiwan (1)
Ser531Leu	33 (50.0)	China (14), Japan (2), Korea (10), Taiwan (7)
Leu533Pro	2 (3.0)	Taiwan (2)
None	7 (10.6)	China (2), Korea (1), Taiwan (4)

[a] Phenylalanine insertion.

Beijing family in these regions (Qian et al., Abstr. 101st Gen. Meet. Am. Soc. Microbiol. 2001). Twelve distinct mutations affecting six codons in the 81-bp region, those encoding Gln-513, Phe-514, Asp-516, His-526, Ser-531, and Leu-533, were found among the 66 Rif^r isolates (Table 1). None of the isolates had more than one mutation. No novel mutations or double or multiple mutations were exhibited beyond the 23 mutations described in earlier studies (33). The three most prevalent mutations (accounting for 79% of the total) were missense mutations at the positions Ser-531, His-526, and Asp-516. This finding is comparable to the results of early studies demonstrating the *rpoB* mutation frequencies in isolates from the United States (13, 32), European and African countries (28), Japan (21, 27), and Asian countries (11). A total of 58 isolates (88%) demonstrated a point (missense) mutation. A 3-nucleotide insertion, resulting in the addition of a phenylalanine residue between Phe-514 and Met-515, was found in one Rif^r isolate (2%). In addition, 7 (11%) of the 66 Rif^r isolates contained no mutations in the 215-bp sequenced region. It was not possible to retest these isolates to reconfirm the resistance.

The frequency of mutations in isolates from the four countries is shown in Table 2. The most frequent mutations, regardless of the geographic origins described by other Asian investigators, occur at the codons encoding Ser-531 (42 to 53%), His-526 (17 to 34%), and Asp-516 (7 to 14%) (11, 14, 16, 21, 27). We found that the most prevalent mutation occurred at the codon encoding Ser-531 (52%). However, our results differed from the others in that in our study the mutation with the second highest frequency was Asp-516 (17%) rather than His-526 (8%). Earlier investigations revealed that mutant alleles particularly affecting either position 531 or 526 were associated with high-level resistance to rifampin (MIC $>$ 64 μg/ml) (3, 21) and high-level cross-resistance to all rifamycins (33), while mutation at the codon encoding Asp-516 was found to cause medium-level rifampin and rifapentine resistance (MIC = 32 μg/ml) but susceptibility to rifabutin. Based on these reports, our finding might indicate that using different analogs of rifamycin in different geographic regions results in a selective pressure for mutations at different *rpoB* codons. For example, it is known that rifabutin is the drug of choice for treatment of tuberculosis in some areas of China.

A missense mutation at the codon encoding Gln-513 was only observed in 4 (16%) of the 25 isolates from China. However, this mutation has been observed in isolates from other countries, but at lower frequencies (11, 14, 28, 32). Two isolates with the Leu533Pro missense mutation in this study were from Taiwan but were also reported to be present at low frequencies in isolates from the United States (28, 32) and the Philippines (32). A single isolate with an insertion mutation, adding a phenylalanine residue between positions 514 and 515, was from Taiwan. Of the seven Rif^r isolates showing no mutation in the 81-bp region, four were from Taiwan; these made up 20% of the Taiwanese isolates.

We compared the mutation profiles of the isolates from the four regions, although we were limited to three isolates from Japan. A genotype encoding the His526Tyr mutation was found in one of the three isolates from Japan in our study. This differed from previous findings that isolates from Japan did not possess this mutation (21, 32). In addition, we observed that less frequent mutations, at Gln-513, the 514-Phe insertion, and at Leu-533, occurred mostly in isolates from China and Taiwan. In terms of resistance to other first-line drugs, earlier reports showed that strains with the same *rpoB* genotype but from different geographic locations had similar patterns of drug resistance (19, 33). However, our data differed from the earlier data in that the isolates with the same *rpoB* genotypes tended to have the same resistance patterns to other drugs (isoniazid, streptomycin, and ethambutol) only within their geographic areas. In other words, the drug resistance patterns were not shared among the isolates of different geographic origin, although these isolates had the same *rpoB* genotypes (data not shown). This is, again, probably the result of selective pressure created by the implementation of different therapeutic regimens and control policies in different regions.

Based on the recent information that the Beijing family has emerged in many countries and regions where the drug resistance of *M. tuberculosis* was high, it is thought that the Beijing family might be associated with increased acquisition of drug resistance (1, 8, 15). We found that the most frequent mutation in both Beijing family and non-Beijing family isolates occurred at Ser-531 (51 and 50%, respectively) (Table 3). However, the Beijing family had higher mutation rates than the non-Beijing isolates at Asp-516 (18 and 12.5%, respectively) and His-526 (12.0 and 6.3%, respectively). On the other hand, we observed that the less frequently encountered mutations, at Leu-533 and the Phe-514 insertion, were found only in the non-Beijing

TABLE 2. Frequency of *rpoB* mutations in 66 rifampin-resistant *M. tuberculosis* isolates from four regions

Position of mutation	No. (%) of isolates with substitution			
	China (n = 25)	Japan (n = 3)	Korea (n = 18)	Taiwan (n = 20)
Gln-513	4 (16.0)			
514-Phe ins.[a]				1 (5.0)
Asp-516	4 (16.0)		4 (22.2)	3 (15.0)
His-526	1 (4.0)	1 (33.3)	3 (16.7)	2 (10.0)
Ser-531	14 (56.0)	2 (66.7)	10 (55.6)	8 (40.0)
Leu-533				2 (10.0)
None	2 (8.0)		1 (5.6)	4 (20.0)

[a] Phenylalanine insertion.

TABLE 3. Frequency of *rpoB* mutations among Beijing family and non-Beijing family rifampin-resistant *M. tuberculosis* isolates

Position of mutation	No. (%) of isolates with substitution	
	Beijing (n = 50)	Non-Beijing (n = 16)
Gln-513	4 (8.0)	
514-Phe ins.[a]		1 (6.3)
Asp-516	9 (18.0)	2 (12.5)
His-526	6 (12.0)	1 (6.3)
Ser-531	26 (52.0)	8 (50.0)
Leu-533		2 (12.5)
None	5 (10.0)	2 (12.5)

[a] Phenylalanine insertion.

family isolates. These findings, however, could be biased due to the small number of the non-Beijing family isolates (n = 16). Limited data are available on the comparison of the Beijing family with drug resistance rate in an area, mainly because the numbers of Beijing family isolates and non-Beijing family isolates were usually unequal in a selected area. However, we found, in a recent study (J. Douglas, L. Qian, W. Guo, H. Chien, M. Yu, and T. Ping, Abstr. 99th Gen. Meet. Am. Soc. Microbiol. 1999, p. 438, 1999) that the Beijing family accounted for about 50% of isolates from Taiwan and that the drug resistance rate in this family was significantly higher than that in the non-Beijing family isolates (44 and 21%, respectively.) Furthermore, a study of the *M. tuberculosis* W family, originally isolated in New York City, where coinfection with *M. tuberculosis* and human immunodeficiency virus was high, revealed that this family was highly related to MDR (2). Interestingly, the W family has similar IS*6110* fingerprinting patterns to those of the Beijing family (2, 30), which strongly suggested that the two families had a common ancestor in recent evolutionary history. We expected that when resistance occurred in the Beijing family, it would be with the *rpoB* mutations which were related to high-level rifampin resistance as described in previous reports (3, 33). However, our data demonstrated that Beijing family and non-Beijing family isolates both acquire high-level rifampin resistance (Ser-531 mutation) at similar frequencies (52 and 50%, respectively), although differences were observed in gaining the secondary mutations (His-526 and Asp-516), with the Beijing family showing higher frequencies.

In summary, our data on *rpoB* mutation frequencies in isolates from the four countries or regions supported the common notion that rifampin resistance genotypes with mutations at critical codons, i.e., those encoding Ser-531, His526 and Asp516, were the most frequently found in *M. tuberculosis* populations regardless of geographic origin. Trend of mutations on *rpoB* appeared to be independent of their spoligotyping patterns. The Beijing family, which obviously had certain selective advantages in these geographic regions, did not show significant differences in frequency of occurrence of the most common mutations in *rpoB* compared with isolates of non-Beijing family genotypes. Regional differences in the selection of mutations may be associated with the types of treatment and choice of rifamycin analogs.

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