

Original Article

Molecular Characterization of Drug-Resistant *Mycobacterium tuberculosis* Isolates in Guangdong, China

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SUMMARY: The aim of this study was to characterize 160 clinical *Mycobacterium tuberculosis* isolates from Guangdong with respect to their drug susceptibility phenotypes to three common anti-tuberculosis drugs, isoniazid (INH), rifampin (RIF) and streptomycin (SM), and with respect to genetic mutations in the most commonly corresponding resistance genes (*katG*, *rpoB* and *rpsL*). The drug susceptibility profiles were evaluated by the absolute concentration method, and genetic mutations in the corresponding resistance genes were identified by DNA sequencing. Among these isolates, 33.1% (53/160) were drug-resistant. The percentages of isolates resistant to INH, RIF and SM were 21.9% (35/160), 16.9% (27/160) and 15.6% (25/160), respectively. Twenty-five of 35 (71%) INH-resistant isolates, 22 of 27 (81.5%) RIF-resistant isolates and 19 of 25 (76%) SM-resistant isolates were found to have mutations in the analyzed *katG*, *rpoB* and *rpsL* gene fragments. The most frequent mutation patterns for the three drugs were as follows: INH, Ser315Thr (68.6%) in *katG*; RIF, Ser531Leu (55.6%) in *rpoB*; and SM, Lys43Arg (72%) in *rpsL*. These findings provide useful data on the mutation types of drug-resistant genes in *M. tuberculosis* isolates from Guangdong province in China.

INTRODUCTION

Tuberculosis (TB) is a major cause of illness and death worldwide, especially in Asia and Africa, with 9.2 million new cases and 1.7 million deaths occurring in 2006 globally (1). China is one of the high burden countries, ranking second only to India in the total number of new TB cases (1). In China, treatment of *Mycobacterium tuberculosis* infection relies primarily on the use of four first-line anti-tuberculosis drugs: isoniazid (INH), rifampin (RIF), streptomycin (SM) and ethambutol (EMB). Currently, national and regional studies indicate that the real level of *M. tuberculosis* drug resistance in developing countries has been increasing in recent years (2). Treatment of TB is compromised worldwide by the emergence of multiple drug-resistant strains of *M. tuberculosis*.

The genetic basis of antibiotic resistance in clinical drug-resistant *M. tuberculosis* isolates has been widely studied and is generally believed to be caused by point mutations in several key resistance genes within the *M. tuberculosis* genome, including *katG* (encoding catalase-peroxidase) for INH, *rpoB* (encoding RNA polymerase β -subunit) for RIF, and *rpsL* (encoding the ribosomal protein S12) for SM (3). The most frequent mutation patterns of INH-, RIF- and SM-resistant strains occurred at codon 315 (55-90%) of *katG* (4-11); codon 531 (40-60%) and codon 526 (10-30%) within the RIF-resistance-determining region (RRDR, codons 507 to 533) of *rpoB* (12-20); and codon 43 (47-79%) of *rpsL* (21-26).

Despite the abundance of data regarding hot-spot mutations in drug-resistant *M. tuberculosis* isolates, most studies suggest that mutations in various gene loci which contribute to drug resistance display geographic variation. In Guangdong

province in China, we lack a comprehensive analysis of the drug resistance phenotypes and their corresponding genetic profiles among the local *M. tuberculosis* isolates, as well as the degree of correlation between them. In this study, we used the absolute concentration method to characterize the drug resistance phenotypes to three common anti-tuberculosis drugs (INH, RIF and SM) of 160 clinical *M. tuberculosis* isolates from Guangdong between January 2007 and March 2008, and then used the PCR direct sequencing method to determine the frequency of the common genetic mutations occurring within the *katG*, *rpoB* and *rpsL* target gene regions. Through analyzing the drug susceptibility and the mutation profile of the corresponding drug-resistant genes of these local *M. tuberculosis* isolates, we hoped to obtain geographic information about the mutation patterns conferring resistance to INH, RIF and SM in our region.

MATERIALS AND METHODS

Clinical isolates and drug susceptibility testing: One hundred sixty clinical isolates of *M. tuberculosis* were collected from patients (including previously infected and new patients) suffering from pulmonary TB between January 2007 and March 2008 in Guangdong province in China. All isolates were cultured on Löwenstein-Jensen slants and were characterized by conventional biochemical testing. Preliminary epidemiological data and patient information suggested that there were no familial ties among the 160 *M. tuberculosis* isolates. The drug susceptibility profiles of the 160 *M. tuberculosis* isolates were evaluated by the absolute concentration method as recommended by the Chinese Antituberculosis Association (27); this method has been used extensively (28,29) and is among those approved by the World Health Organization (30). The drug concentrations for susceptibility testing were as follows: INH, 1.0 and 10 μ g/mL (Lo-INH and Hi-INH); RIF, 50 and 250 μ g/mL (Lo-RIF and Hi-RIF); and SM, 10 and 100 μ g/mL (Lo-SM and Hi-SM). Strain H37Rv was

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Table 1. Oligonucleotide primers used for PCR amplification and DNA sequencing

Gene (accession no.)	Primer	Sequence (5'-3')	Nucleotide position (nt)	Product size (bp)
<i>katG</i> (X68081)	katG-F	GTCGGCGGTCACACTTTC	2777-2794	660
	katG-R	GCTACCACGGAACGACGAC	3418-3436	
<i>rpoB</i> (L27989)	rpoB-F	TACGGTCGGCGAGCTGATCC	2201-2220	411
	rpoB-R	TACGGCGTTTCGATGAACC	2593-2611	
<i>rpsL</i> (X70995)	rpsL-F	AGTAAGGTCAAGACCGCG	49-66	267
	rpsL-R	CTGCGTATCCAGCGAACC	298-315	

included as a control in each susceptibility test. Isolates that grew on the low concentration of the tested drug were considered to be resistant to that drug.

DNA extraction procedures: Genomic DNA was extracted as described previously (31). Briefly, *M. tuberculosis* from the Löwenstein-Jensen medium was treated with a 300- μ L lysis solution (4.5 g/L Tween-20, 4.5 g/L NP-40, 0.2 g/L proteinase K and 1 \times PCR buffer), incubated at 55°C for 3 h and then heat-killed at 95°C for 10 min. Cell lysates were extracted by the phenol/chloroform method. The purified DNA pellet was allowed to air-dry and was finally dissolved in sterile double-distilled water and stored at -20°C until use.

PCR amplification: The extracted DNA was amplified with the oligonucleotide primers listed in Table 1. These primers were designed by using Primer Premier 5.0 and were denoted at the end by either “F” or “R” to indicate forward or reverse primers, respectively. PCR was performed in a 50- μ L final volume containing 5 μ L 10 \times *Ex Taq* Buffer, 0.2 mM of each dNTP, 0.25 μ M of each primer, 1.25 U TaKaRa *Ex Taq* DNA polymerase (TaKaRa, Dalian, China) and 100 ng genomic DNA as a template. The conditions used for amplification were as follows: initial denaturing at 94°C for 5 min; 30 cycles of amplification with 1 min at 94°C, 1 min at 55°C and 1 min at 72°C; and then a final extension for 10 min at 72°C. The PCR products were electrophoresed in 1.5% agarose gel containing ethidium bromide and were visualized under UV light.

DNA sequencing and sequences analysis: PCR products that appeared as a unique size when visualized on the gel were purified using a high pure PCR purification kit (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer’s protocol and then sequenced directly with both forward and reverse primers using the Bigdye-Terminator kit and an ABI Prism 3730 DNA sequencer (Perkin-Elmer, Wellesley, Mass., USA). Sequences obtained were compared with previously published *katG*, *rpoB* and *rpsL* gene sequences of strain H37Rv in the NCBI database. The alignment of amino acid sequences was performed using ClustalX 1.81 software.

RESULTS

Resistance profiles: The results are listed in Table 2. Among the 160 *M. tuberculosis* isolates included in this study,

53 (33.1%) strains exhibited resistance to at least one common anti-tuberculosis drug. The resistant rates to one, two and three test drugs were 18.1% (29/160), 8.8% (14/160) and 6.3% (10/160), respectively. The percentages of *M. tuberculosis* isolates resistant to INH, RIF and SM were 21.9% (35/160), 16.9% (27/160) and 15.6% (25/160), respectively. Detailed analysis of the resistant isolates revealed that 62.9% (22/35) of isolates were resistant to Hi-INH, 70% (19/27) of isolates were resistant to Hi-RIF, and 88% (22/25) of isolates were resistant to Hi-SM (Table 3).

INH-resistant mutation profile: The DNA sequence of the *katG* fragment was determined for 10 INH-susceptible *M. tuberculosis* isolates and 35 INH-resistant isolates. All INH-susceptible isolates were found to harbor no detectable mutations, while 25 of 35 (71.4%; the 95% confidence interval [CI] was 55.7-87.2%) INH-resistant isolates were found to have mutations in the analyzed *katG* gene fragment, including 7 Lo-INH- and 18 Hi-INH-resistant isolates. The frequency of mutations in the *katG* gene of Hi-INH-resistant isolates (81.8%, 18/22) was slightly higher than those of Lo-INH-resistant isolates (53.8%, 7/13), without statistical significance ($P = 0.17$). We detected three different mutation patterns in the *katG* gene fragment of the 35 INH-resistant strains, and the mutation patterns were determined to be as

Table 2. Prevalence of each of the possible combinations of resistance phenotypes among 160 clinical *M. tuberculosis* isolates

Resistance phenotype	Frequency of recovery
Susceptible	107 (66.9%)
INH	13
RIF	6
SM	10
Subtotal	29 (18.1%)
INH + RIF	9
INH + SM	3
RIF + SM	2
Subtotal	14 (8.8%)
INH + RIF + SM	10 (6.3%)
Total	160

INH, isoniazid; RIF, rifampin; SM, streptomycin.

Table 3. Summary and correlation of phenotypic and genetic characteristics of 160 clinical *M. tuberculosis* isolates

Drug/ resistance gene	Resistance to specific drug (%)			Resistant isolates that harbor mutations (%)			Mutations exhibiting resistance (%)	Susceptible strains that harbor mutations (%)
	Lo & Hi	Lo	Hi	Lo & Hi	Lo	Hi		
INH/ <i>katG</i>	35/160 (18.4)	13/35 (37.1)	22/35 (62.9)	25/35 (71.4)	7/13 (53.8)	18/22 (81.8)	25/25 (100)	0/155 (0)
RIF/ <i>rpoB</i>	27/160 (14.2)	8/27 (29.6)	19/27 (70.3)	22/27 (81.5)	5/8 (62.5)	17/19 (89.5)	17/17 (100)	0/163 (0)
SM/ <i>rpsL</i>	25/160 (13.2)	3/25 (12)	22/25 (88)	19/25 (76)	2/3 (66.7)	17/22 (77.3)	19/19 (100)	0/165 (0)

Lo, low concentration of drug; Hi, high concentration of drug.

Table 4. The prevalence and patterns of mutations in *katG*, *rpoB* and *rpsL* gene regions among INH, RIF and SM resistant isolates

Strain	No. of strain (%)	Gene/codon	Nucleotide change	Amino acid change
INH-resistant isolates (35)	20 (57.1)	<i>katG</i> /315	AGC→ACC	Ser→Thr
	4 (11.4)	<i>katG</i> /315 & 463	AGC→ACC & CGG→CTG	Ser→Thr & Arg→Leu
	1 (2.9)	<i>katG</i> /315	AGC→GGC	Ser→Gly
	10 (28.6)	ND	ND	ND
RIF-resistant isolates (27)	6 (22.2)	<i>rpoB</i> /526	CAC→CTC	His→Leu
	15 (55.6)	<i>rpoB</i> /531	TCG→TTG	Ser→Leu
	1 (3.7)	<i>rpoB</i> /533	CTG→CCG	Leu→Pro
	5 (18.5)	ND	ND	ND
SM-resistant isolates (25)	18 (72)	<i>rpsL</i> /43	AAG→AGG	Lys→Arg
	1 (4)	<i>rpsL</i> /88	AAG→AGG	Lys→Arg
	6 (24)	ND	ND	ND

ND, mutations not detected in the analyzed gene region.

follows: AGC→ACC (Ser→Thr) at codon 315 in 24 (68.6%) isolates, AGC→GGC (Ser→Gly) at codon 315 in 1 (2.9%) isolate, and CGG→CTG (Arg→Leu) at codon 463 in 4 (11.4%) isolates which also had the Ser315Thr mutation (Table 4). In 10 (28.5%) phenotypically INH-resistant isolates, no nucleotide changes were detected at the analyzed *katG* gene fragment.

RIF-resistant mutation profile: The DNA sequence of the *rpoB* fragment was determined for 10 RIF-susceptible *M. tuberculosis* isolates and 27 RIF-resistant isolates. All RIF-susceptible isolates were found to harbor no detectable mutations, while 22 of 27 (81.5%, the 95% CI was 65.8-97.1%) RIF-resistant isolates were found to have a mutation within the *rpoB* RRDR, including 5 Lo-RIF- and 17 Hi-RIF-resistant isolates. The frequency of mutations in the *rpoB* gene of Hi-RIF-resistant isolates (89.5%, 17/19) was slightly higher than those of Lo-RIF-resistant isolates (62.5%, 5/8), without statistical significance ($P = 0.27$). We detected three different patterns of mutations, and the most affected codon was found to be codon 531. The mutation patterns of the 27 RIF-resistant strains were determined to be as follows: TCG→TTG (Ser→Leu) in 15 (55.6%) isolates at codon 531; CAC→CTC (His→Leu) in 6 (22.2%) isolates at codon 526; and CTG→CCG (Leu→Pro) in 1 (3.7%) isolate at codon 533 (Table 4). In 5 (18.5%) phenotypically RIF-resistant isolates, no nucleotide changes were detected at the analyzed *rpoB* gene fragment.

SM-resistant mutation profile: The DNA sequence of the *rpsL* fragment was determined for 10 SM-susceptible *M. tuberculosis* isolates and 25 SM-resistant isolates. All SM-susceptible isolates were found to harbor no detectable mutations, while 19 of 25 (76.0%; the 95% CI was 58.0-94.0%) SM-resistant isolates were found to have a mutation in the *rpsL* gene fragment, including 2 Lo-SM- and 17 Hi-SM-resistant isolates. The frequency of mutations in the *rpsL* gene of Hi-SM-resistant isolates (77.3%, 17/22) was slightly higher than those of Lo-SM resistant isolates (66.7%, 2/3), without statistical significance ($P = 0.27$). We detected two different patterns of mutations, and the most affected codon was found to be codon 43. The mutation patterns of the 25 SM-resistant strains were determined to be as follows: AAG→AGG (Lys→Arg) in 18 (72%) isolates at codon 43 and AAG→AGG (Lys→Arg) in 1 (4%) strain at codon 88 (Table 4). In 6 (24%) phenotypically SM-resistant isolates, no nucleotide changes were detected at the analyzed *rpsL* gene fragment.

DISCUSSION

The present study is the first investigation involving molecular characterization of clinical drug-resistant *M. tuberculosis* isolates from Guangdong province between January 2007 and March 2008. In this study, 53 drug-resistant isolates (35 INH-resistant, 27 RIF-resistant and 25 SM-resistant isolates) and 30 susceptible isolates were assessed by DNA direct sequencing. Mutations in *katG*, in particular the Ser315Thr substitution, are responsible for INH resistance in a large proportion of *M. tuberculosis* isolates. The frequency of the Ser315Thr mutation (68.6%) was not as high as those in northwestern Russia (93.6%) (32), Germany (86.4%) (6) and Lithuania (85.7%) (7), but was in concordance with other results in Mexico (67.6%), Poland (66.3%), Australia (65.4%) and Vietnam (71%) (8-11). One case had a Ser315Gly amino acid change, which was found in Guangdong for the first time but was reported previously in Vietnam and New York (11,33). Four isolates, in addition to having the *katG* Ser315Thr mutation, also had the *katG* 463 polymorphism, which is not associated with INH resistance (34). Our findings of mutations in the *katG* loci are similar to those reported in other parts of the world, especially the common mutations pattern, which reflects a global pattern.

Among the 27 RIF-resistant isolates, 81.5% were found to have point mutations within the *rpoB* RRDR, which is slightly lower than in previous studies (up to 90%) (12-20). The most frequently detected *rpoB* mutation pattern in this study was Ser531Leu (55.6%), which was higher than the frequencies detected in Poland (37.5%) (9), Vietnam (40.4%) (11) and Germany (41.7%) (12), but was in concordance with the high mutant ratios of this codon in India (59%), Italy (56.7%) and other areas of China, including Beijing (59.2%), Shanghai (53.8%), Shandong (55%) and Sichuan (56.7%) (13-18). The frequency of His526Leu (22.2%) was consistent with those reported elsewhere, namely 22% in India (13), 23.5% in Singapore (19), 23.1% in Shanghai (16) and 20.5% in Hong Kong (20). These two mutation types together accounted for 95% of all *rpoB* mutations while the Leu533Pro mutation played a minor role. Locally, we did not detect any mutation at codons 516, 522 or 513 of *rpoB*, which had been previously reported in various findings (12-20).

With regard to the 25 SM-resistant isolates, 76% had mutations in the *rpsL* gene, and the most predominant mutation pattern was Lys43Arg (72%), while the Lys88Arg mutation played a minor role (4%). These results are closely consistent with data published by Wu et al. (21) and Shi et al. (22), who found the Lys43Arg mutation (72 and 70%, respectively)

and Lys88Arg mutation (7 and 6%, respectively) in resistant strains in the Beijing province of China. The rate of the Lys43Arg mutation (72%) was much higher than those in Germany (40%) (23), Japan (36.7%) (24), Poland (30.7%) (25), and Mexico (24%) (8), and the rate of the Lys88Arg mutation was slightly lower than that found in Japan (10.2%) (24), but a few studies did not find any mutation at codon 88 (8,26), which indicated a different geographic distribution of the *rpsL* mutation among SM-resistant isolates.

In our study, all of the mutations were characterized as single nucleotide changes, although deletions or insertions were found in INH- and RIF-resistant strains in previous reports (35,36). All isolates with point mutations were found to be resistant to the corresponding drugs. However, a small proportion of phenotypically resistant isolates were found to have no mutations in the genes *katG*, *rpoB* and *rpsL*. A similar situation has also been reported in previous findings (4-11,17-19,21-26). This finding suggested that the correlation between the INH, RIF and SM resistance phenotypes and *katG*, *rpoB* and *rpsL* mutations is not absolute. Mutations in other resistance genes such as *inhA*, *ahpC*, *oxyR* (INH) and *rrs* (SM), along with some undefined genetic mutations or resistance mechanisms may be responsible for the discrepancy.

Although the frequency of the targeted gene mutations in Lo- and Hi-resistance was not statistically significant, the tendency for Hi-resistant strains to have a higher frequency of targeted gene mutations than Lo-resistant strains was consistent with a previous report (37). However, Brzostek et al. (25) showed a clear distinction between Hi-resistant strains, in which *rpsL* mutations were present, and Lo-resistant strains, in which *rpsL* mutations were absent. There are two possible reasons for this discrepancy. It may be attributed to the use of different drug susceptibility tests to evaluate Lo- and Hi-resistance (Brzostek used the proportion method while we used the absolute concentration method), or it may be due to the intervention of other mutations that were not examined in our study, such as *rrs* (SM).

In conclusion, the high percentage of common mutations in *katG* 315, *rpoB* 531 and *rpsL* 43 demonstrate the importance of these codons for the development of INH, RIF and SM resistance in Guangdong province. This investigation of drug resistance to INH, RIF and SM and the frequency of mutations occurring at the common target loci provides useful information regarding our geographical region, enhancing our understanding of the geographical distribution of resistant alleles. This understanding will be valuable for the development of rapid and reliable molecular approaches to the detection of drug-resistant *M. tuberculosis* in our restricted region.

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