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Characterization of mutations in multi- and extensive drug resistance among strains of *Mycobacterium tuberculosis* clinical isolates in Republic of Korea

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ABSTRACT

In order to characterize molecular mechanisms of first- and second-line drug resistance in *Mycobacterium tuberculosis* and to evaluate the use of molecular markers of resistance, we analyzed 62 multidrug-resistant, 100 extensively drug-resistant, and 30 pan-susceptible isolates from Korean tuberculosis patients. Twelve genome regions associated with drug resistance, including *katG*, *ahpC*, and *inhA* promoter for isoniazid (INH); *embB* for ethambutol (EMB), *rpoB* for rifampin (RIF), *pncA* for pyrazinamide (PZA), *gyrA* for fluoroquinolones; *rpsL*, *gidB*, and *rrs* for streptomycin; *rrs* and *eis* for kanamycin (KM); *rrs* and *tylA* for capreomycin (CAP); and *rrs* for amikacin (AMK) were amplified simultaneously by polymerase chain reaction, and the DNA sequences were determined. We found mutations in 140 of 160 INH-resistant isolates (87.5%), 159 of 162 RIF-resistant isolates (98.15%), 127 of 143 EMB-resistant isolates (88.8%), 108 of 123 ofloxacin-resistant isolates (87.8%), and 107 of 122 PZA-resistant isolates (87.7%); 43 of 51 STM-resistant isolates (84.3%), 15 of 17 KM-resistant isolates (88.2%), and 14 of 15 (AMK and CAP)-resistant isolates (93.3%) had mutations related to specific drug resistance. In addition, the sequence analyses of the study revealed many novel mutations involving these loci. This result suggests that mutations in the *rpoB*531, *katG*Ser315Thr, and C-15T in the *inhA* promoter region, and *gyrA*94, *embB*306, *pncA*159, *rpsL*43, and *A*1401G in the *rrs* gene could serve as useful markers for rapid detection of resistance profile in the clinical isolates of *M. tuberculosis* in Korea, with potentials for the new therapeutic benefits in actual clinical practice.

1. Introduction

Mycobacterium tuberculosis is the etiological agent of tuberculosis (TB). It is estimated that about one-third of the world's population is considered to be latently infected with *Mycobacterium tuberculosis*, and 10% of these persons will develop active disease at some point in their lifetimes. Due to the lack of effective vaccine and the current need for the more potent anti-tuberculosis drugs which can shorten the duration of therapy, it is not clear how the disease can ever be controlled in the countries where it is truly endemic. In recent years, the control of TB has become a global challenge due to the emergence of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB). The emergence of multidrug resistant TB (MDR-TB), i.e., *M. tuberculosis* strains, resistant to at least isoniazid (INH) and rifampicin, is of great concern, because it requires the use of second-line drugs that are difficult to procure, have more side effects and are more expensive than the first-line regimen (Espinal et al., 2001). Hence, the detection and treatment of drug-susceptible or single drug-resistant TB is important in optimizing strategies to prevent the emergence of MDR-TB and its transmission (Masjedi et al., 2006). XDR-TB isolates are resistant to isoniazid and rifampicin with additional bacillary resistance to any fluoroquinolone, and to at least 1 of the 3 injectable second-line drugs (amikacin, kanamycin, and capreomycin) (Jassal and Bishai, 2009).

The global increase in drug resistance, particularly MDR-TB, reflects, at least in part, inappropriate use of anti-TB drugs during the treatment course of TB patients with drug susceptible strains (Espinal et al., 2001). Additional factors such as immigration, sex, age, HIV infection, and socioeconomic factors have been shown to be associated with the increased prevalence of MDR-TB (Faustini et al., 2006). The World Health Organization has documented that MDR-TB

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is becoming extensively widespread today. Almost half of the global MDR-TB cases reported from heavily populated areas of China and India (World Health Organization, 2010). The rapid determination of the resistance profile of an isolate can facilitate selection of an appropriate drug regimen and preclude development of additional drug resistances. Rapid detection of resistances can be best achieved with molecular diagnostic approaches, particularly, in developing countries where access to culture facilities is limited. Such strategies require a detailed understanding of the molecular basis of drug resistance.

The present study was undertaken to characterize mutations prevalent in clinical isolates from Korea with respect to various drug resistance target loci. We used DNA sequencing to detect resistance to both the first-line and the second-line anti-tuberculosis drugs. These include isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), streptomycin (SM), ethambutol (EMB), amikacin (AMK), capreomycin (CAP), kanamycin (KAN), and ofloxacin (OFX). Twelve loci were sequenced: *rpoB* (for resistance to RIF); *katG*, *ahpC*, and *inhA* (INH); *pncA* (PZA), *embB* (EMB), *gyrA* (OFX), and *gidB*; and *rpsL*, *rrs*, *eis*, and *tlyA* (SM, KAN, AMK, and CAP). The loci studied were *rpoB* (RNA polymerase B subunit), *katG* (catalase-peroxidase), *inhA* (mycolic acid biosynthetic pathway enzyme), *ahpC* (alkyl hydroperoxide reductase), *rrs* (16S rRNA), *rpsL* (ribosomal protein S12), *eis* (aminoglycoside acetyltransferase), *gidB* (7-methylguanosine methyltransferase), *embB* (arabinosyltransferase), *tylA* (a putative rRNA methyltransferase), *pncA* (pyrazinamidase), and *gyrA* (DNA gyrase A-subunit).

2. Materials and methods

2.1. Mycobacterium tuberculosis clinical strains

M. tuberculosis-resistant strains were selected from sputum cultures of tuberculosis patients registered in public health centers of South Korea from 2009 to 2010. Multiple isolates from the same patient were avoided. A total of 30 pan-susceptible, 100 XDR and 62 MDR strains were used in this study. All strains were obtained from the Korea Mycobacterium Resource Center.

2.2. Drug susceptibility test

The drug susceptibility of the *M. tuberculosis* isolates was determined by the absolute concentration method with Lowenstein-Jensen (LJ) medium (Kim et al., 1997). The drugs used to select MDR and XDR strains and their critical concentrations for resistance were as follows: isoniazid (INH), 0.2 μg/mL; rifampin (RFP), 40 μg/mL; OFX, 2 μg/ml; pyrazinamide (PZA), 100 μg/ml; ethambutol (EMB), 2 μg/mL; SM, 10 μg/mL; kanamycin (KM), 40 μg/mL; amikacin (AMK), 40 μg/mL; and capreomycin (CAP), 40 μg/ml. All tubes were incubated at 37°C for 28 days. The MIC was defined as the lowest concentration of drug resulting in the complete inhibition of growth or growth that constituted <1% of the inoculum.

2.3. Genomic DNA preparation, primer designing, and polymerase chain reaction (PCR) amplification

A loop of fresh *M. tuberculosis* culture on LJ media was suspended in 500 μL of TE buffer [10 mM Tris/HCl (pH 8.0), 1 mM EDTA] in a screw cap microcentrifuge tube. After boiling the mixture for 25 min, the heat-killed bacteria were centrifuged at 13,400*g* for 5 min, and the extracted DNA was analyzed immediately or was stored at −20°C for further use; 5 μL of the supernatant was used for PCR amplification. The following 12 loci were amplified by PCR: *rpoB* (RIF), *katG*, *ahpC*, and *inhA* (INH); *pncA* (PZA), *embB* (EMB), *gyrA* OFX and *rrs* (SM, KAN, CAP, and AMK); *eis* and *gidB* (KAN); *tlyA* (CAP); and *rpsL* (SM). Of the 12 loci, only the drug resistance-determining regions of *embB* (ethambutol resistance-determining region [ERDR]), *rpoB* (rifampicin resistance-determining region [RRDR]), and *gyrA* (quinolone resistance-determining region [QRDR]); the promoters of *ahpC*, *inhA*, and *eis*; and regions with established resistance-associated mutations of *katG* and *rrs* were amplified using locus-specific primers (Table 1). The complete open reading frames of *rpsL*, *gidB*, *tlyA*, and *pncA* were amplified due to the established presence of resistance-associated mutations throughout the loci (Table 1). Each 20-μL PCR mixture contained 10 μL of HotStarTaq master mix, 1.0 μL of the forward, and 1.0 μL of the reverse 5 μM primers; 7 μL of double-distilled H_2O; and 1 μL of genomic DNA. Amplification was carried out for 35 cycles (an initial denaturation step at 94°C for 5 min, followed by denaturation at 94°C for 45 s), various annealing temperatures for different genes at 55–69 °C (*pncA* and *gyrA*: 55 °C; *gidB*, *katG*, *ahpC*, and *inhA*: 60 °C; *embB* and *eis*: 63 °C; *rrs* and *rpoB*: 68 °C and *rpsL* and *tlyA*: 69 °C) for 45 s and elongation at 72 °C for 1 min, with a final elongation step at 72 °C for 7 min.

2.4. DNA sequencing and DNA sequence analysis

The PCR products were used as templates for targeted DNA sequencing. Sequencing of both strands of the PCR product was performed on an ABI373 sequencing instrument according to the protocol supplied by the manufacturer using the Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA, USA). Mutations were determined by comparing with *M. tuberculosis* H37Rv sequence of *rrs*, *rpsL*, *gidB*, *tlyA*, *eis*, *gyrA*, *embB*, *katG*, *pncA*, *rpoB*, *ahpc*, and *inhA* genes (GenBank accession number NC_000962.3, regions *rrs* = 1471846–1473382, *rpsL* = 781560–781934, *gidB* = 4407528–4408202, *tlyA* = 1917940–1918746, *eis* = 2714124–2715477, *gyrA* = 7302-9818, *embB* = 4246514–4249810, *katG* = 2153889-2156111, *pncA* = 2288681–2289241, *rpoB* = 759807–763325, *ahpc* = 2725943–2726780 and *inhA* = 1673223–1675011) from Tuberculist (http://genolist.pasteur.fr/TubercuList/) and the GenBank database (http://www.ncbi.nih.gov/gene).

3. Results

3.1. Mutations in rpoB gene for rifampicin resistance

To identify the mutations associated with rifampicin resistance, we examined the 439 bp region of *rpoB* gene, including the 81-bp, 27-amino acid (codons 507–533) hyper-variable RRDR. The vast majority (159/162; 98.2%) of RFP-resistant isolates harbored at least 1 mutation within the *rpoB* gene, while 3 of the RFP-resistant isolates lacked such a mutation (Table 2). A total of 23 non-synonymous single nucleotide polymorphisms, frame shift mutations causing one 9-bp deletion, and one 10-bp deletion were identified among the study isolates (Table 2); 137 isolates (84.5%) showed a single mutation, while 20 strains (12.3%) had double mutations. Among the 20 double mutants, 4 of the mutations occurred outside the region of RRDR, with resulting amino acid replacements at codons 481 (Thr481Ala), 505 (Phe505Val), 535 (Pro535Ser), and 563 (Thr563Pro). Interestingly, all these codons were found in combination with either Ser531Leu or Asp516Tyr or His526Cys substitution (Table 2). However, the majority (97.4%) of the *rpoB* gene mutations detected in the RFP resistant isolates were found within the boundary of RRDR region. The most common *rpoB* mutations were Ser531Leu, Asp516Val, Asp516Tyr, and His526Tyr (Table 2). Among the 30 pan-susceptible isolates, we could not find any mutations within the *rpoB* gene. In the present study, we were also able to identify 9 novel mutations in the *rpoB* gene region, which had not been previously described (Table 2). Although 505, 509, 512, 515, 516, and 531 codon site mutations were also identified in other studies, previously described mutations possessed different amino acid types.

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