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Molecular characterization of drug-resistant and -susceptible *Mycobacterium tuberculosis* isolated from patients with tuberculosis in Korea☆

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Abstract

We investigated the causal relationship between genotype and phenotype of drug-resistant *Mycobacterium tuberculosis* isolates obtained from patients with pulmonary tuberculosis (TB) in Korea. Of 80 isolates tested, 17, 20, 1, and 7 isolates were mono-resistant to ethambutol (EMB), isoniazid (INH), pyrazinamide (PZA), and rifampicin (RFP), respectively, and 31 isolates (38.8%) were multidrug-resistant (MDR). Sequencing analysis showed that 78% (32/41) of RFP-resistant strains had mutations in the rifampicin resistance–determining region (RRDR) of *rpoB*, and the mutation at *rpoB*531 (59.4%) was most abundant. In 52 INH-resistant strains, mutations were found mostly at C-15T (n = 21, 40.4%) in the *inhA* promoter region as well as at *katG*315 (n = 12, 23.1%). Mutations at *embB*306 were mostly found in 26.7% (12/45) of EMB-resistant isolates. New mutations found here in MDR isolates include *rpoB*523 (Gly523Glu) and *embB*319 (Tyr319Ser). Consequently, mutations in the *rpoB531*, C-15T in the *inhA* promoter region, *embB306*, and *katG315* would be a useful marker for rapid detection of MDR *M. tuberculosis* isolates in Korea.

Keywords: Drug resistant; Korea; Mutation analysis; *Mycobacterium tuberculosis*; Tuberculosis

1. Introduction

Tuberculosis (TB) is a global public health problem that results in the death of millions of humans every year worldwide. Despite optimal TB control programs, it has been estimated that more than 50 million people die from TB between 1998 and 2020 (Brewer and Heymann, 2005). The threat posed by TB is further complicated by the increasing appearance of multidrug-resistant (MDR) *Mycobacterium tuberculosis*, defined as resistance to both rifampicin (RFP) and isoniazid (INH). In Korea, TB also remains a major public health threat. A survey in 2001 reported that 10.6% of new pulmonary TB cases were resistant to at least 1 drug and 2.2% of them were MDR (Espinal et al., 2001). However, from the 2004 survey, a statistically significant increase has been observed in any drug resistance (12.8%) and MDR (2.7%), suggesting that the overall prevalence of drug-resistant TB has been steadily increasing in Korea (Bai et al., 2007).

Generally, the detection of *M. tuberculosis* drug resistance has been performed by using mycobacterial culture and drug susceptibility test (DST) on liquid or solid media. However, they are laborious and time-consuming procedures that can take several weeks to months. Therefore, an efficient and rapid detection of drug-resistant TB is essential for the prevention and control of drug-resistant TB transmission.

Drug resistance of TB is known to be associated with mutations in several genes that encode either the target

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proteins of the drug or enzymes that are involved in drug activation (Somoskovi et al., 2001). The action mechanism of RFP is to inhibit mycobacterial transcription by targeting RNA polymerase. The development of resistance to RFP is due to mutations in the rifampicin resistance–determining region (RRDR), an 81-bp hotspot region (codons 507–533), of the *rpoB* gene encoding the beta subunit of RNA polymerase (Miller et al., 1994; Telenti et al., 1993). Furthermore, more than 90% of the RFP-resistant strains contain mutations within this RRDR of *rpoB* (Musser, 1995). Ethambutol (EMB), a frontline anti-TB drug, targets the mycobacterial cell wall, and mutations in the *embB* gene lead to resistance to EMB in *M. tuberculosis* (Telenti et al., 1997). The EMB resistance is primarily associated with missense mutations within the conserved ethambutol resistance–determining region (ERDR) of *embB*, and the most commonly found mutations occur at *embB* codon 306 in 50% to 70% of EMB-resistant clinical isolates (Plinke et al., 2006).

In contrast, resistance to INH is more complex. Many INH-resistant strains have mutations in the *katG* gene encoding catalase–peroxidase that result in altered enzyme structure. These structural changes apparently result in decreased conversion of INH as a pro-drug to a biologically active form. Some INH-resistant strains also have mutations in the presumed regulatory region of the *inhA* locus or *kasA* gene encoding an enoyl-acyl carrier protein reductase or a beta-ketoacyl-acyl carrier protein synthase, respectively (Musser, 1995; Ramaswamy and Musser, 1998). Pyrazinamide (PZA) is also a pro-drug that requires conversion into its active form, pyrazinoic acid (POA), by the bacterial enzyme pyrazinamidase (PZase) encoded by *pncA* (Scorpio and Zhang, 1996; Zhang and Mitchison, 2003), for activity against *M. tuberculosis*. Since mutations in *pncA* result in lost or reduced PZase activity, such mutations are thus considered to be the primary mechanism of PZA resistance in *M. tuberculosis* (Hirano et al., 1997). However, some PZA-resistant isolates retain PZase activity, suggesting that there are other mechanisms of resistance (Mestdagh et al., 1999).

Since the frequency of mutations in genes related to TB drug resistance varies widely around the world, molecular analysis of regional isolates is an essential step to developing molecular-based detection methods for local TB resistance. Because drug resistance in *M. tuberculosis* is mostly correlated with mutations in the relatively conserved regions of several of the genes (*embB, inhA, katG, pncA*, and *rpoB*) described above, therefore, gene sequence-based analysis, including direct DNA sequencing, is the most widely used and reliable technique for detection of both known and novel mutations worldwide. Despite the increasing number of any drug resistance and MDR TB cases in Korea, however, relatively few studies have determined the prevalence of different drug resistance–conferring mutations among drug-resistant and MDR clinical isolates (Cho et al., 2009; Nam et al., 2008; Park et al., 2005).

In this study, for measuring the link between phenotype and gene mutations in drug-resistant *M. tuberculosis* isolates in Korea and for finding critical mutation sites that can be used to prospectively and rapidly screen isolates to detect drug-resistant TB in Korea, we sequenced and analyzed the partial *embB* and *rpoB* genes including the ERDR and the 81-bp RRDR, respectively, and the complete *inhA* and *pncA* loci including the promoter region, as well as the region surrounding *katG*315 in 80 clinical *M. tuberculosis* culture isolates obtained from patients with pulmonary TB. The results of sequence analysis were compared with those of drug susceptibility analysis.

2. Materials and methods

2.1. Sample collection and drug susceptibility test

For measuring the link between phenotype and gene mutations in drug-resistant *M. tuberculosis* isolates, a total of 80 TB isolates were deliberately selected for this study based on their phenotypic drug resistance characteristics. All isolates were obtained from specimens that were stored anonymously at Yonsei University College of Medicine in Korea between 2005 and 2008. They comprised 49 isolates resistant to at least one of the primary TB drugs (EMB, INH, PZA, and RFP) and 31 MDR isolates.

The drug susceptibility of *M. tuberculosis* isolates was determined by the absolute concentration method using Löwenstein–Jensen medium (Kim et al., 1997). The drugs and their critical concentrations for resistance were as follows: 0.2 μg/mL of INH, 40 μg/mL of RFP, and 2 μg/mL of EMB. PZA susceptibility was determined by PZase activity assay using the modified PZase agar method (Singh et al., 2007), with slight modifications previously described as the Wayne (1974) method. The PZA-susceptible *M. tuberculosis* strain H37Rv was used as a positive control for the PZase assay. The PZase assay was performed at least twice.

2.2. DNA extraction and PCR amplification of drug-resistant genes

Genomic DNA from each clinical isolate was prepared using a modified cetyltrimethylammonium bromide method (Murray and Thompson, 1980). Genotypic mutations were evaluated by polymerase chain reaction (PCR) amplification followed by DNA sequencing. For targeting the hotspot regions of *rpoB*, *katG, embB*, and the complete *inhA* and *pncA* loci including the regulatory region, the oligonucleotide primers described in Table 1 were used for PCR amplification. The PCR amplification was performed in a 20 μL reaction mixture containing 8 pmol of each primer, 0.5 U of HotStar Taq DNA polymerase (Qiagen, USA), and 2 μL of each genomic DNA. Amplification was performed with the MJ Mini thermal cycler (Bio-Rad, USA) using the following protocol: initial activation at 95 °C for 5 min, 35 cycles each of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s,