RESEARCH ARTICLE

Genetic and phenotypic characterizations of drug-resistant *Mycobacterium tuberculosis* isolates in Cheonan, Korea

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Background: *Mycobacterium tuberculosis* (MTB) causes tuberculosis (TB), which is a fatal disease. Cases of drug-resistant MTB have increased in recent years. In this study, we analyzed 7 sites of MTB DNA sequences, including the *rpoB* and *inhA* gene, to investigate the relationship between gene mutations and drug resistance in MTB.

Methods: *Mycobacterium tuberculosis* liquid culture samples (197 specimens from 74 cases) were collected between June 2015 and May 2016 and sequenced. The results were compared with those obtained from antibiotic susceptibility tests.

Results: In 65 (87.8%) cases, the antibiotic-resistant phenotype was consistent with genotyping results, whereas in 9 (12.2%) cases, there was no match. Eight mutations were detected in the *rpoB* gene, which showed the highest mutation rate. Sequencing results indicated that these mutations were present in 12 cases.

Conclusion: Previously published data on antibiotic resistance genes are insufficient for effective prevention of multidrug- or extensive drug-resistant TB. Additional studies are needed to characterize the complement of antibiotic resistance genes in MTB.

KEYWORDS

antimicrobial susceptibility test, drug resistance, multidrug resistance, sequencing test, tuberculosis

1 | INTRODUCTION

Although the incidence of tuberculosis (TB) in Korea has decreased over the last 50 years, the number of new cases increased from 35 000 in 2007 to 40 000 in 2011.^{1,2} Although bacterial culture and population surveillance are performed to prevent the spread of drug-resistant strains, the nationwide incidence of multidrug-resistant (MDR) *Mycobacterium tuberculosis* (MTB) has exceeded the controllable limit.^{3,4}

As such, preventing the spread of MDR bacteria and monitoring and controlling large-scale infections is a priority for the medical community and has important implications for public health policy.⁵ Diagnosing TB infection in hospitals requires the culture of MTB, for which the US Centers for Disease Control and Prevention and WHO recommend liquid medium.⁶⁻⁹ The Korean Institute of Tuberculosis uses the absolute concentration method with Löwenstein-Jensen (LJ) medium to determine the minimum drug concentration that can inhibit the growth of susceptible wild-type

MTB strains isolated from treatment-naïve patients as well as the resistance of 20 colonies to the same drug concentration as the standard.¹⁰

The transmission of drug-resistant MTB between individuals is a major challenge for healthcare professionals. The anti-TB susceptibility test can detect drug-resistant MTB strains, thus hastening patient diagnosis and treatment aside from preventing the development and spread of infection.¹¹

2 | MATERIALS AND METHODS

2.1 | Sample collection

In this study, the clinical samples were obtained from cases that were referred to Dankook University Hospital between June 2015 and May 2016. The samples included ascites fluid, bronchial aspirate, cerebrospinal fluid, gastric juice, pleural fluid, pus, sputum, stool, tissue, and urine. For this study, we retrospectively analyzed the results using

these samples. Samples used for sequencing were collected from liquid culture.

2.2 | Culture

For solid cultures, 3% Ogawa medium (Eiken, Tokyo, Japan) was used. Subsequently, examinations were conducted once every week, and the samples were cultured for up to 8 weeks to be confirmed as negative. If proliferation was observed, characteristics such as the color and shape of the colonies and their numbers were noted. The cultured strains were smear tested to confirm that they were acid-fast bacilli (AFB).

For liquid cultures, 7-mL Mycobacteria Growth Indicator Tube (MGIT; Becton Dickinson, Sparks, MD, USA) was used. The barcode of the inoculated MGIT tube was scanned using MGIT 960, and the tube was placed in the designated area with green lights and incubated for 42 days. The presence of AFB was confirmed via Ziehl-Neelsen staining; if AFB were observed, then we determined whether they were MTB or not.

2.3 | Antituberculosis drug susceptibility test

The suitable sample was sent to the Korean Institute of Tuberculosis laboratory for antituberculosis drug susceptibility test. Fresh MTB grown on solid medium for <15 days was used for the sensitivity test if at least 10 colonies were present. Drug susceptibility was evaluated based on the LJ medium absolute concentration method using an

M-kit (The Korean Institute of Tuberculosis, Osong, Korea). The drug susceptibility test was performed by the Korean National Tuberculosis Association through the Seoul Clinical Laboratories for the following 15 antibiotics: isoniazid (INH), rifampicin (RIF), streptomycin (SM), ethambutol (EMB), kanamycin (KM), capreomycin (CPM), amikacin (AMK), prothionamide, cycloserine (CS), para-aminosalicylic acid, ofloxacin (OFX), moxifloxacin (MOX), levofloxacin (LEV), rifabutin, and para-nitrobenzoic acid (Table 1).

2.4 | Target gene selection and sequencing

We investigated the genetic basis for drug resistance in MTB by analyzing 7 genomic DNA sequences in 197 specimens collected from 74 cases. Seven genes known to harbor antibiotic resistance mutations, namely, *ahpC*, *eis promoter*, *gyrA*, *inhA*, *katG*, *rpoB*, and *rrs*, were analyzed via sequencing using TB-positive samples of liquid culture. Genomic DNA was extracted using an ExiPrep Dx Mycobacteria Genomic DNA Kit (Bioneer, Daejeon, Korea) according to the manufacturer's protocol using an ExiPrep16 Dx instrument (Bioneer). The DNA was stored at -70°C until use. Polymerase chain reaction (PCR) was performed using an AccuPower ProFi Taq PCR PreMix (Bioneer) according to the manufacturer's protocol on a MyGenie 96 Gradient Thermal Block instrument (Bioneer). An AccuPrep PCR Purification Kit was used for PCR product purification. Sequencing was performed using a BigDye Terminator v.3.1 sequencing kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol on

M-kit	1	2	3	4	5	6	7
A	Control	INH 0.1	INH 0.2	RIF 10	RIF 40	SM 4	EMB 2
B	Control	INH 1.0	INH 0.2	RIF 20	RIF 40	SM 10	EMB 2
C		KM 40	CPM 40	PTH 40	CS 30		RBT 20
D	PAS 1	OFX 2	MOX 2	AMK 40	LEV 2	PNB 500	RBT 40

TABLE 1 Drugs and concentrations used in M-Kit susceptibility tests ($\mu\text{g/mL}$)

FIGURE 1 Number of drug-resistant *Mycobacterium tuberculosis* detected by antituberculosis drug susceptibility test between June 2015 and May 2016. AMK, amikacin; CPM, capreomycin; CS, cycloserine; EMB, ethambutol; INH, isoniazid; KM, kanamycin; LEV, levofloxacin; MOX, moxifloxacin; OFX, ofloxacin; PTH, prothionamide; RBT, rifabutin; RIF, rifampicin; SM, streptomycin

TABLE 2 Distribution of antibiotic resistance-related mutations and cross-comparison of mutations (n = 74)

	Locus	Antibiotic susceptibility test results				Mutation ratio, %	Mutation
		Resistant		Susceptible			
		With mutations	Without mutations	With mutations	Without mutations		
INH	<i>katG</i>	3	6	2	63	6.8	1, Ser315Thr (ACA) 3, Ser315Thr (ACC) 1, Ile317Leu
	<i>inhA</i>	0	9	0	65	0.0	
	<i>ahpC</i>	0	9	0	65	0.0	
RIF	<i>rpoB</i>	6	0	2	66	10.8	1, His526Leu 1, His526Tyr 1, His526Thr 4, Ser531Leu 1, Lys593Glu
OFX, LEV	<i>gyrA</i>	1	1	1	71	2.7	2, Asp94Ala
MOX	<i>gyrA</i>	0	2	1	71		
Injectable drug (KM, CPM, AMK)	<i>rrs</i>	0	1	0	73	0.0	
	<i>eis promoter</i>	0	1	0	73	0.0	

AMK, amikacin; CPM, capreomycin; INH, isoniazid; KM, kanamycin; LEV, levofloxacin; MOX, moxifloxacin; OFX, ofloxacin; RIF, rifampicin.

an ABI3730XL instrument (Thermo Fisher Scientific). Sequencing primer sequences are listed in Table S1.

2.5 | Data analysis

The statistical program R (Version 3.3.3, Comprehensive R Archive Network; <https://www.r-project.org>) was used to assess the relationship between genotype and phenotype. Sequencher (version 5.1, Gene Codes; <https://www.genecodes.com>) was used to compare and analyze gene sequences.

2.6 | Ethics

This study did not require ethical approval because it is a retrospective analysis of the results of examinations for routine medical care. The study was conducted in conformance with the Declaration of Helsinki.

3 | RESULTS

3.1 | Drug susceptibility testing

We found that 13 of 74 (17.6%) of cases showed resistance to at least 1 of the 13 antibiotics tested. Most of the cases were resistant to INH (9 cases), followed by SM and RIF (7 and 6 cases, respectively) (Figure 1).

3.2 | Sequencing results

Of the 74 cases tested, 13 were positive in the drug susceptibility test, and 12 (16.2%) were mutation positive. Genotype-phenotype comparisons revealed that 65 cases (87.8%) were matched in terms of antibiotic resistance, while 9 (12.2%) were not.

3.3 | Analysis of drug susceptibility and sequence data

We analyzed the *ahpC*, *eis promoter*, *gyrA*, *inhA*, *katG*, *rpoB*, and *rrs* genes in the 74 cases to identify drug resistance-related mutations. The sequencing results indicated that these mutations were present in 12 cases. We found mutations in *gyrA*, *katG*, and *rpoB* in 2, 5, and 8 cases, respectively (Table 2). An Asp94Ala mutation (GAC→GCC) in *gyrA* was detected in 2 cases. For *katG*, Thr308Ser (ACC→TCG, TCC) and Ile317Leu (ATC→CTC) were found in 1 case, while Ser315Thr (AGC→ACC, ACA) was present in 4 cases. For *rpoB*, His526Leu (CAC→CTC), His526Tyr (CAC→TAC), and His526Thr (CAC→ACC) mutations were observed in 1 case each; Ser531Leu (TCG→TTG) in 4 cases; and Lys593Glu (AAG→GAG) in 1 case (Table 2).

Antibiotic resistance was evaluated in relation to each mutated gene. Four cases harbored a Ser315Thr mutation in *katG*, and 3 (75.0%) of these were resistant to INH. Six (8.7%) of the 69 cases lacking mutations in *katG* and *inhA* were resistant to INH. His526Leu, His526Tyr, and His526Thr mutations in the *rpoB* gene were associated with resistance to RIF in 1 case each. Additionally, 3 (75.0%) of the 4 cases with the Ser531Leu mutation were RIF-resistant. However, RIF resistance was not observed in the 66 cases lacking mutations in the *rpoB* gene.

We also examined mutations in the *gyrA* gene that confer resistance to antibiotics of the fluoroquinolone (FQ) family. One of the 2 cases with an Asp94Ala mutation was resistant to OFX and LEV but not to MOX. One of the 72 (1.4%) cases did not harbor any mutations in *gyrA*, but was resistant to all 3 drugs. No mutations were found in the *rrs* and *eis promoter* genes—which are associated with resistance to the injectable antibiotics KM, CPM, and AMK—among the 74 cases, although 1 case showed resistance to these 3 drugs (Table S2).

4 | DISCUSSION

This study investigated the current status of drug-resistant MTB in Korea by analyzing 7 genes (ie, *ahpC*, *eis promoter*, *gyrA*, *inhA*, *katG*, *rpoB*, and *rrs*) associated with antibiotic resistance¹²⁻¹⁴ in 74 TB cases.

The *katG* gene product converts INH into a biologically active form; mutations in *katG* are associated with INH-resistant MTB.¹⁵ In this study, 5 cases collectively harbored mutations at 3 loci (ie, 1 case of Thr308Ser and Ile317Leu mutations and 4 cases of Ser315Thr mutation). The Ser315Thr mutation was ACA or ACC. Three of the 5 cases (60%) were resistant to INH. Of the 69 cases lacking INH resistance mutations in *katG*, 6 (8.7%) nonetheless showed INH resistance, suggesting that this phenotype is associated with genes other than *katG*. On the other hand, *katG* has been linked to resistance to SM, RIF, and EMB in addition to INH.¹⁶ The *ahpC* gene of MTB encodes the antioxidant enzyme catalase-peroxidase that causes mutations in *katG* and is known to be associated with resistance to INH, an anti-TB drug.^{12-14,17} However, *ahpC*-associated mutations were not detected in this study.

Using the MGIT, Rigouts et al¹⁸ reported that all strains harboring Asp516Val or Asp516Phe mutations in *rpoB* were resistant to RIF, but 1 of the 6 strains with the Asp516Tyr mutation showed RIF resistance, and only at 0.5 µg/mL. Sun et al¹⁹ reported that in 76.3% of patients with TB who received antibiotic treatment for 18 months, Leu533Pro mutation was replaced by the His526Tyr mutation in the *rpoB* gene, while His526Leu (n = 3), His526Tyr (n = 2), His526Thr (n = 1), and Ser531Leu (n = 2) mutations were also reported. In the present study, of the 74 cases tested for drug resistance, 8 (10.8%) showed RIF resistance; of these, 6 (75%) cases showed a concordant phenotype, whereas 2 (25%) did not. Based on the detection of His526Leu, His526Tyr, and His526Thr mutations, we conclude that codon 526 is a site that is frequently mutated in RIF-resistant TB.

Mycobacterium tuberculosis exhibiting resistance to FQs has been reported in many regions in Korea.^{20,21} In this study, of the 13 cases showing antibiotic resistance, 2 harbored *gyrA* gene mutations (ie, at Asp94Ala). One of these was resistant to both OFX and LEV but not to MOX. A study reported a case in which the concurrence between genotype and phenotype was associated with antibiotic resistance—that is, resistance to OFX and MOX was observed at high rates when mutations were present in the *gyrA* gene.²² In addition, 1 case showed resistance to 3 drugs without mutation in *gyrA*. If there is resistance to FQ without *gyrA* mutation, *gyrB* mutation may be present.²³

The *eis promoter* gene encodes aminoglycoside acetyltransferase, which plays a role in cell survival.²⁴ Mutations in the *eis promoter* have been linked to low-level resistance to KM.²⁴ Of the 74 MTB-positive cases analyzed in the present study, 1 case each showed resistance to KM, CPM, and AMK, although none of these had *eis promoter* mutations. *rrs* and *eis promoter* can cover approximately 80%-90% of second-line injectable drug resistance.²⁵

A previous study reported that among 35 INH-resistant strains, 33 (94%) strains exhibiting high resistance to INH harbored the Ser315Thr mutation in the *katG* gene, whereas those with low INH resistance (n = 2 strains, 6%) had the *inhA* C15T mutation.²⁶ However, we did not observe any relationship between *inhA* mutations and INH

resistance. Mutations in nucleotides 1401, 1402, and 1484 of the *rrs* gene were found to be associated with resistance to aminoglycosides, AMK, and KAN, respectively.²⁷ In addition, these authors reported that the *eis promoter* mutation C-14T, which was also detected in this study, was associated with low-level resistance to KAN, with some cases exhibiting a slight increase in AMK resistance. However, we did not detect any *rrs* mutations in our study.

Genetic mutations did not match the resistance phenotype of the isolate in 9 (12.2%) of the 74 cases examined. Given that in many instances, the resistance phenotype cannot be explained by known gene mutations, further research is needed to identify and characterize novel mutations to contain the spread of MDR strains of MTB.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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