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ORIGINAL ARTICLE

The mutations of *katG* and *inhA* genes of isoniazid-resistant *Mycobacterium tuberculosis* isolates in Taiwan

Shu-Ting Tseng [a], Chun-Hsi Tai [a], Chia-Ru Li [a], Chin-Fu Lin [c], Zhi-Yuan Shi [a,b,*]

[a] *Division of Infectious Diseases, Department of Internal Medicine, Taichung Veterans General Hospital, Taichung, Taiwan*
[b] *School of Medicine, National Yang-Ming University, Taipei, Taiwan*
[c] *Microbiology Section of the Medical Laboratory, Taichung Veterans General Hospital, Taichung, Taiwan*

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Background/purpose: The isoniazid (INH) resistance of *Mycobacterium tuberculosis* is caused by mutations in the *katG* and *inhA* genes encoding for catalase-peroxidase and inhA, respectively. Sequences of the *katG* and *inhA* gene of 70 isolates were analyzed to identify the mutations and to compare the mutations with their related susceptibilities.
Methods: Sequences of the *katG* and *inhA* genes and the resistance profiles were analyzed for the 70 *M. tuberculosis* isolates, collected from nine hospitals in Taiwan during the period from 1999 to 2011.
Results: Fifteen alleles were identified in the *katG* gene and two alleles were identified in the *inhA* gene. Among the 15 alleles identified in the *katG* gene, 14 alleles were found in isolates resistant to isoniazid, while only three alleles were found in isolates susceptible to isoniazid. The mutations of the *katG* gene and their frequencies of 41 INH-resistant isolates were Arg463Leu (51%), Ser315Thr (29%), Ser315Asn (9.8%), and other loci (22%). The sensitivity and specificity of the Ser315Thr mutation for the detection of INH-resistant isolates were 29% and 100%, respectively. The frequency of *inhA* gene mutation was low (2.44%) in the 41 INH-resistant isolates.
Conclusion: The diverse alleles of the *katG* gene associated with INH resistance are present in the *M. tuberculosis* isolates in Taiwan. These data may be applied to develop new probes for various alleles associated with INH resistance in order to increase the sensitivity for the

* Corresponding author. Section of Infectious Diseases, Taichung Veterans General Hospital, No. 1650, Section 4, Taiwan Boulevard, Taichung 40705, Taiwan.
E-mail address: zyshi@vghtc.gov.tw (Z.-Y. Shi).

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detection of genetically diverse *M. tuberculosis* isolates in different geographic areas. The diversity of mutations can also provide information for investigating the evolutional lineages of *M. tuberculosis* isolates.

Introduction

Mycobacterium tuberculosis (TB) infection has been an emerging global health issue. According to the World Health Organization (WHO) Global Tuberculosis Report 2012,[1] there were an estimated 8.7 million incident cases of TB in 2011. There were also 1.4 million deaths from TB (990,000 deaths among HIV-negative individuals and 430,000 among HIV-positive patients). There were an estimated 12 million prevalent cases of TB in 2011, equivalent to 170 cases per 100,000 population. Globally, 3.7% (2.1—5.2%) of new cases and 20% (13—26%) of previously treated cases are estimated to have MDR-TB (defined as resistance to at least isoniazid and rifampicin). In 2011, there were an estimated 630,000 cases of MDR-TB among the world's 12 million prevalent cases of TB. According to the Taiwan Tuberculosis Control Report 2012,[2] the annual incident cases of TB were 12,634 (54.5 cases per 100,000 population) in 2011, and the estimated rates of MDR-TB among new cases and previously treated cases were 1.0% and 6.7%, respectively. In a study conducted by a tertiary medical center in northern Taiwan in 2010, the resistance patterns of *M. tuberculosis* isolates from 326 patients were 12.0% for isoniazid, 2.8% for rifampicin, 1.5% for ethambutol, and 7.1% for streptomycin.[3]

Isoniazid is a prodrug that is activated by the catalase-peroxidase enzyme encoded by the *katG* gene. Activated isoniazid disrupts the synthesis of essential mycolic acids by inhibiting inhA, the NADH-dependent enoyl-ACP reductase enzyme encoded by *inhA* gene.[4] Mutations of the *katG* gene and *inhA* gene lead to reduction or loss of the activity of catalase-peroxidase and structural change of the target of isoniazid, and both result in resistance to isoniazid.[5,6]

The GenoType MTBDR*plus* (Hain Lifescience GmbH, Nehren, Germany) is a test that uses PCR and DNA strip hybridization for the rapid detection of isoniazid and rifampicin resistance. The probes are designed to recognize mutations of the *katG* (S315T) and *inhA* (C15T A16G, T8C, and T8A) genes. A previous report in 2009 compared the performance of GenoType MTBDR*plus* test and DNA sequencing of the aforementioned loci in Taiwan.[7] The GenoType MTBDR*plus* test was less sensitive than DNA sequencing (81.8% vs. 93.4%) for the detection of isoniazid resistance because only limited codons were analyzed. The authors suggest that new alleles of INH-resistant genes are required to improve the sensitivity of GenoType MTBDR*plus* test.[7] Thus far, there has been no reports on the detailed single nucleotide mutations of the *katG* and *inhA* genes associated with resistance to isoniazid for *M. tuberculosis* isolates in Taiwan. Hence, we conducted this study to identify the detailed mutated sequences of the *katG* and *inhA* genes of 70 *M. tuberculosis* isolates collected in Taiwan during the period from 1999 to 2011. Sequences of their *katG* and *inhA* genes and resistance profiles of these isolates were analyzed and compared with the mutations of isoniazid-resistant strains from different geographic regions.

Materials and methods

Collection of *M. tuberculosis* isolates

Seventy *M. tuberculosis* isolates were collected for this study during the period from 1999 to 2011. Forty-six isolates were resistant to at least one of the four drugs, including isoniazid (INH), rifampicin (RMP), ethambutol (EMB), and streptomycin (SM). Forty-one of the 46 isolates were resistant to isoniazid. Among these 46 resistant isolates, 34 isolates were collected from Taichung Veterans General Hospital (TCVGH) and 12 isolates were collected from the other eight hospitals in northern Taiwan. Twenty-four isolates were susceptible to all the four drugs. Among these 24 susceptible isolates, 12 isolates were collected from TCVGH, and 12 isolates were collected from the other eight hospitals in northern Taiwan. The sources of specimens included sputum (62 isolates, 88.6%), cerebrospinal fluid (5, 7.1%), pleural effusion (2, 2.9%), and abscess (1, 1.4%).

Susceptibility test

The antimycobacterial susceptibility tests were performed according to the CLSI M24-A2 modified agar proportion method.[8] The procedure is performed by inoculating equal quantities of 100× and 10,000× dilutions of a standardized inoculum (0.5w1 McFarland) onto 7H10 agar medium (Bio Star, Taichung, Taiwan) with and without the test drug and then incubating them at 35°C, 10% CO_2 for 21 days. The number of colony-forming units growing on the drug-containing medium compared with the number of those growing on the drug-free medium is then determined and expressed as a percentage. Resistance is defined as growth on drug-containing agar greater than 1% of the number of colonies that grow on the drug-free agar. The concentrations of antituberculosis drugs were as follows: isoniazid, 0.2 mg/mL; streptomycin, 2.0 mg/mL; rifampicin, 1.0 mg/mL; and ethambutol, 5.0 mg/mL. Multidrug resistance was defined as isolates resistant to at least isoniazid and rifampicin.[8]

DNA sequencing

The DNA was extracted by 10 mg/mL of proteinase K and 5% Tween 20 in 200-mM Tris-HCl solution (pH = 8.3) at 60°C

Table 1 Primers, annealing temperature, and sequence length of sequencing

Gene (Accession no. of reference strain)	Nucleotide position of primer	Sequence	Annealing temp (°C)	Sequence length (bp)
katG (X68081)	202 (forward)	GTCGCGACCATCGACGTTGA	65	1710
	780 (reverse)	GACGTCGTTCATGGCCATGC		
	673 (forward)	ATGGGGCTGATCTACGTGA	65	
	1010 (reverse)	TACAGGATCTCGAGGAAACT		
	892 (forward)	TTGGGCTGGAAGAGCTCGTA	65	
	1460 (reverse)	TCGCTACCACGGAACGACGA		
	1398 (forward)	ATTGACTGTCTCACAGCTAG	65	
	2002 (reverse)	AGGTGATACCCATGTCGAG		
InhA (AF106077)	Upstream of *inhA* (forward)	TGGTCAGCTTCCTGGCTTCC	55	810
	489 (reverse)	GACCGTCATCCAGTTGTAG		
	272 (forward)	TGGTGCATTCGATTGGGTTC	55	
	Downstream of *inhA* (reverse)	GGTAACGTTCTCCAGGAAC		

overnight. The polymerase chain reaction (PCR) was performed in a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, CT, USA) using the primer pairs listed in Table 1. The PCR began with an initial 4-minute denaturation at 94°C, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 2 minutes, extension at 72°C for 2 minutes, and a final extension step at 72°C for 5 minutes. The sequence reactions were performed by adding fluorescent terminators (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) and the primers used in the initial

Table 2 Mutations of the *katG* gene and resistance profiles of 70 *M. tuberculosis* isolates

Alleles	Codon, amino acid, and nucleic acid change	No. of isolates	Resistance profiles (*n*)
Allele 1[a]	No mutation	23	INH (5); RMP (2); EMB (2); INH + SM (1); INH + EMB (2); INH + SM + EMB (1); Susceptible to all four drugs (10)
Allele 2	506 GAG (Glu) → TAG (stop)	1	INH + RMP (1)
Allele 3	202 GCC (Ala) → GCA (Ala); 463 CGG (Arg) → CTG (Leu)	1	Susceptible to all four drugs (1)
Allele 4	463 CGG (Arg) → CTG (Leu)	23	INH (1); INH + RMP (2); RMP + SM (1); INH + RMP + SM (1); INH + SM + EMB (4); Resistant to all four drugs (1); Susceptible to all four drugs (13)
Allele 5	315 AGC (Ser) → AAC (Asn); 463 CGG (Arg) → CTG (Leu)	3	INH + RMP + SM (2); Resistant to all four drugs (1)
Allele 6	315 AGC (Ser) → ACC (Thr)	6	INH (1); INH+EMB (1); INH+RMP+SM (3); Resistant to all 4 drugs (1)
Allele 7	127 CAG (Gln) → CCG (Pro)	1	INH+RMP+SM (1)
Allele 8	315 AGC (Ser) → ACC (Thr); 463 CGG (Arg) → CTG (Leu)	5	INH (1); INH + RMP (1); INH + RMP + SM (2); INH + SM + EMB (1)
Allele 9	315 AGC (Ser) → AAC (Asn)	1	INH + RMP (1)
Allele 10	463 CGG (Arg) → CTG (Leu); 518 GGTC insertion	1	INH + RMP (1)
Allele 11	203 ACC (Thr) → ACT (Thr); 463 CGG (Arg) → CTG (Leu)	1	INH (1)
Allele 12	202 GCC (Ala) → GCA (Ala); 315 AGC (Ser) → ACC (Thr); 463 CGG (Arg) → CTG (Leu)	1	INH + RMP (1)
Allele 13	352 CAA (Gln) → GAA (Glu)	1	INH + SM (1)
Allele 14	98 TAC (Tyr) → TGC (Cys); 379 GCC (Ala) → ACC (Thr); 463 CGG (Arg) → CTG (Leu)	1	Resistant to all four drugs (1)
Allele 15	269 GGT (Gly) → CGT (Arg)	1	INH (1)

[a] Allele 1 is identical to the reference sequence X68081.
EMB = ethambutol; INH = isoniazid; RMP = rifampicin; SM = streptomycin.

Table 3 Mutations of the *inhA* gene and resistance profiles of 70 *M. tuberculosis* isolates

Alleles	Codon, amino acid, and nucleic acid change	No. of isolates	Resistance profiles (*n*)
Allele 1[a]	No mutation	69	INH (10); RMP (2); EMB (2); INH + EMB (3); INH + RMP (7); INH + SM (2); RMP + SM (1); INH + RMP + SM (9); INH + SM + EMB (6); Resistant to all four drugs (3); Susceptible to all four drugs (24)
Allele 2	74 ATC (Ile) → ACC (Thr)	1	Resistant to all four drugs

[a] Allele 1 is identical to the reference sequence, AF106077.
EMB = ethambutol; INH = isoniazid; RMP = rifampicin; SM = streptomycin.

PCR. The sequences were determined with an ABI PRISM 310 and 3100 automated DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA). The sequences obtained from all 70 isolates were compared. Each unique mutated DNA fragment was defined as an allele.

Results

The mutations of the *katG* and *inhA* genes and their resistance profiles are listed in Table 2 and Table 3, respectively. Fifteen alleles were indentified in the *katG* gene, and two alleles were identified in the *inhA* gene. Allele 1 represented the original sequence without mutation for both the *katG* and *inhA* genes. The other alleles demonstrated mutated sequences. Some of the alleles are listed in GenBank under accession numbers AY155347 to AY155354, AY178906, and AY155363.

Twenty-three isolates were assigned as allele 1 in the *katG* gene, including 14 isolates susceptible to isoniazid, and nine isolates resistant to isoniazid. Among the 15 alleles identified in the *katG* gene, 14 alleles were found in isolates resistant to isoniazid (alleles 1, 2, and 4—15), while only three alleles were found in isolates susceptible to isoniazid (alleles 1, 3, 4). The findings indicated that genetic polymorphisms in the *katG* gene could be associated with isoniazid resistance. The common mutations, Arg463Leu, Ser315Thr, and Ser315Asn, were identified in 8, 3, and 2 alleles, respectively. The mutations of the *katG* gene and their frequencies of the 41 INH-resistant isolates were Arg463Leu (51%), Ser315Thr (29%), Ser315Asn (9.8%), and other loci (22%). For the *inhA* gene, 69 isolates were assigned as allele 1, including 29 isolates susceptible to isoniazid and 40 isolates resistant to isoniazid. Only one isolate was assigned as allele 2 (Ile25Thr mutation), and it was resistant to all the four drugs.

The statistical data, including sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), of the *katG* gene mutations for detection of INH-resistance are listed in Table 4. The sensitivity, specificity, PPV, and NPV of mutated codon 315 (Ser315Thr and Ser315Asn) were 39%, 100%, 100%, and 54%. Although the sensitivity for each of the other seven mutated codons (Tyr98Cys, Gln127Pro, Gly269Arg, Gln352Glu, Ala379Thr, Glu506stop, and insertion in the codon 518) was low (2.4%), the specificity and PPV for the detection of INH-resistance were 100%. Identify these eight mutated codons (315, 98, 127, 269, 352, 379, 506, and 518) in *M. tuberculosis* isolates may be helpful for the detection of INH-resistance because of the high specificity and PPV.

Discussion

This study demonstrated the relationship between the resistance profiles and the mutations of the *katG* and *inhA* genes of *M. tuberculosis* isolates in Taiwan over a 12-year period. There were significant genetic polymorphisms in the *katG* gene, especially in isolates that were resistant to isoniazid. The most common mutations among the

Table 4 Sensitivity, specificity, PPV, and NPV of the *katG* gene mutations to predict the resistance to isoniazid

Mutated codon	No. of isolates	Sensitivity (%)[a]	Specificity (%)	PPV (%)	NPV (%)
Ser315Thr	12	29	100	100	50
Ser315Asn	4	9.8	100	100	44
Arg463Leu	36	51	48	58	41
Ala202Ala[a]	2	2.4	97	50	41
Thr203Thr[a]	1	2.4	100	100	42
Tyr98Cys	1	2.4	100	100	42
Gln127Pro	1	2.4	100	100	42
Gly269Arg	1	2.4	100	100	42
Gln352Glu	1	2.4	100	100	42
Ala379Thr	1	2.4	100	100	42
Glu506stop	1	2.4	100	100	42
518 GGTC insertion	1	2.4	100	100	42

[a] Ala202Ala was the silent mutation GCC202GCA. Thr203Thr was the silent mutation ACC203ACT.
NPV = negative predictive value; PPV = positive predictive value.

Table 5 Frequency of the *katG* gene mutations in isoniazid-resistant *M. tuberculosis* isolates from different geographic regions

Geographic area	Country	No. of isolates resistant to INH	Frequencies of mutations among isolates resistant to INH (%)		
			S315T	*KatG* mutations other than S315T	No mutation
Southeastern Asia	This study (Taiwan)	41	29.3	48.8	21.9
	Taiwan[7]	242	50.4	43	6.6[a]
	Shanghai, China[11]	242	62.8	26.5	10.7[a]
	Bangladesh[12]	218	83.9	10.6	5.5[a]
	Myanmar[13]	96	57.3	8.3	34.4
	Mumbai, India[14]	104	21.15	NP	NP
Northeastern Asia	Korea[15]	52	23.1	53.8	23.1[a]
Central Asia	Pakistan[16]	50	74	10	16
Northern Asia	Russia[17]	24	91.7	8.3	0
Africa	Sierra Leone[18]	32	71.9	15.6	12.5
Europe	Swizerland[19]	101	65.6	28.6	5.8[a]
South America	Brazil[20]	16	25	25	50
North America	New York, USA[21]	33	36.4	15.1	48.5[a]

[a] No mutation detected in *inhA* regulatory region and *katG* gene.
INH = isoniazid; NP = not performed.

41 isoniazid-resistant isolates were Arg463Leu (51%), Ser315Thr (29%), and Ser315Asn (9.8%). The Ser315Thr (S315T) mutation has been the most widely studied mutation of the *katG* gene worldwide and has been used in the GenoType MTBDR*plus* as a molecular target to detect isoniazid resistance. Enzymatic kinetic study demonstrated that the Ser315Thr mutation triggered the least catalase peroxidase activity among the 23 *katG* gene mutations tested,[9] which explained the high level of isoniazid resistance of the S315T mutation.[10] The frequency of Ser315Thr mutation in INH-resistant isolates varies in different geographic regions in the world, as shown in Table 5.[7,11–21] In this study, the frequency of Ser315Thr mutation (29%) was comparable to the results of previous studies from the Mumbai (21.2%),[14] Korea (23.1%),[15] and Brazil (25%).[20] However, a higher frequency of Ser315Thr mutation in the *katG* gene was reported in other countries, such as Shanghai, China (62.8%),[11] Switzerland (65.6%),[19] Africa (71.9%),[18] and Russia (91.7%).[17] About 8.3% to 53% of mutations are located in the *katG* gene but not the Ser315Thr mutation.

The finding that the Ser315Thr mutation in the *katG* gene occurs less frequently in this study in Taiwan has a direct impact on the application of molecular techniques for rapid detection of MDR-TB. The probes used in the GenoType MTBDR*plus* are designed to recognize the mutations of the *katG* (S315T) and *inhA* (C15T A16G, T8C, and T8A) genes. By the GenoType MTBDR*plus* test, S315T mutation in the *katG* gene were found in 122 (50.4%) of 242 INH-resistant isolates collected in Taiwan.[7] Among the 41 INH-resistant isolates in this study, the *katG* mutations associated with resistance were S315T (29%) and other loci (48.8%). The sensitivity of GenoType MTBDR*plus* for detection of the mutated *katG* gene appears to be low for the isolates in this study. Therefore, it is reasonable to develop new probes of the *katG* genes for detection of the novel mutations which were selected under therapeutic pressure or evolved in different geographic areas.

The sensitivity and specificity of Arg463Leu mutation for the detection of INH resistance were only 51% and 48% in this study. In a previous study, the proportions of isolates from the Netherlands with Arg463Leu mutation were similar in INH-susceptible isolates and INH-resistant or INH-intermediate isolates (32% and 29%, respectively).[22] Site-directed mutagenesis experiments demonstrated that Arg463Leu mutation did not alter the activity of the catalase-peroxidase of *M. tuberculosis*.[23] These data suggested that the Arg463Leu mutation in the *katG* gene of *M. tuberculosis* did not confer resistance to INH. All of the isolates with the *katG* gene mutation were resistant to INH, except allele 3 and some of the isolates of allele 4. Allele 3 possessed the mutations of GCC202GCA and Arg463Leu. GCC202GCA is a silent mutation, and the Arg463Leu mutation does not confer to isoniazid resistance. Allele 4 contained Arg463Leu mutation alone. It was reasonable that allele 3 and some of the isolates of allele 4 were susceptible to INH.

Sreevatsan et al reported that the variants at *katG* codon 463 and *gyrA* codon 95 were present at high frequency.[24] These two mutation sites are apparently not related with antibiotic resistance and can be used as genetic markers to trace the history of evolutional divergence.[24] In the present study, nine isolates with the mutations of the *katG* gene located in codon 463 combined with other loci (315 and 463; 463 and 518; 203 and 463; 202, 315, and 463; 98, 379, and 463) were multidrug resistant. Further surveillance of the codon 463 and these mutation loci in *M. tuberculosis* isolates will be helpful for tracing the evolutional lineage of these resistant clones.

In contrast to the high frequencies of the *katG* gene mutations, the frequency (1 of 41 INH-resistant isolates) of the *inhA* gene mutation was low in this study. The only isolate with the *inhA* gene mutation also possessed Arg463Leu mutation in the *katG* gene. Among the 41 INH-resistant isolates, 32 (78%) isolates had mutations in the *katG* gene, and nine isolates (22%) had neither *inhA* nor *katG* gene mutation. One of the possible mechanisms of the drug resistance of *M. tuberculosis* isolates with normal structural sequences of the *inhA* and *katG* gene may be mutation in the *inhA* regulatory region,[25] although it was

not demonstrated in this study. However, the frequency of isoniazid resistance without mutations in the *inhA* regulatory region or the *katG* gene still ranged from 5.5～48.5% worldwide, as shown in Table 5. According to the studies performed by Ramaswamy et al[10,26,27] and Zhang et al,[28] approximately 10% to 25% of INH-resistant strains do not contain mutations in any known gene targets for INH resistance. Although mutations in at least 16 other genes have been reported to be associated with INH resistance in clinical isolates, their mechanisms of INH resistance remain unclear.[10,26,27] These results indicate that further investigations are needed in this issue.

Hazbón et al[29] indicated that most studies examined relatively small numbers of isolates or failed to include sufficient numbers of drug susceptible controls to demonstrate statistically significant associations between specific mutations and INH resistance.[6,17,23,26–35] The collection of drug-resistant isolates is often limited by the relatively low prevalence rate of drug-resistant *M. tuberculosis* isolates. This bias is also present in the research of frequency of *rpoB* gene mutations in *M. tuberculosis* isolates.[36] The limitation of this study is that small number of INH-resistant isolates and an insufficient amount of INH-susceptible isolates would result in bias in the proportion of resistant isolates among the collection. Therefore, we focused on the frequency of each point mutation in the resistant isolates rather than the proportion in all of the isolates. The drug-resistant isolates were collected from nine hospitals in Taiwan during a 12-year period to avoid sampling from a small region and in short period of time. The *katG* gene mutations have demonstrated the variations that can provide basic information for further investigations. Another limitation is that analysis of the regulatory region of the *inhA* gene was not performed because of the limited budget. Further investigation of the regulatory region of the *inhA* gene is promising for the detailed analysis of mutations related to INH-resistance.

In conclusion, the diverse alleles of the *katG* gene associated with INH-resistance are present in the *M. tuberculosis* isolates in Taiwan. These data may be applied to develop new probes for various alleles associated with INH-resistance, in order to increase the sensitivity for detection of genetically diverse *M. tuberculosis* isolates in different geographic areas. The diversity of mutations can also provide information for investigating the evolutional lineages of *M. tuberculosis* isolates.

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