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Detection of Mutations Associated with Isoniazid Resistance in *Mycobacterium tuberculosis* Isolates from China

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Nine structural genes (*furA*, *katG*, *inhA*, *kasA*, Rv0340, *iniB*, *iniA*, *iniC*, and *efpA*) and two regulatory regions (the *oxyR-ahpC* intergenic region and the promoter of *mabA-inhA*) in 87 isoniazid (INH)-monoresistant and 50 INH-susceptible *Mycobacterium tuberculosis* isolates collected from five provinces of China were analyzed by sequencing. Eighty-two (94.3%) INH-resistant isolates had mutations in the *katG* gene, with the *katG* Ser315Thr mutation predominant (55.2%). No mutation at codon 463 of *katG* was detected among the 50 INH-susceptible isolates with different IS*6110* fingerprints. In addition, there were 35 (40.2%) INH-resistant isolates that had a mutation at codon 463 of *katG*. Of the INH-resistant strains, 20 (23.0%) isolates harbored double mutations at two separate loci of *katG*. Mutations in the *inhA* promoter region occurred in 13 (14.9%) isolates; 4.6% of the isolates had *inhA* structural gene mutations, and 11.5% harbored mutations in the *oxyR-ahpC* intergenic region. Drug resistance-associated mutations were detected in the *iniBAC* region and *efpA*.

Tuberculosis (TB) represents one of the world's greatest sources of mortality and morbidity, with approximately 8 million new infections and 2.5 million to 3 million deaths per year. China has one of the highest burdens of TB in the world, with the second-largest total number of TB cases globally (9, 34, 41). Currently, throughout the world, isoniazid (INH) and rifampin (RIF) together represent the backbone of short-course chemotherapy treatment for *Mycobacterium tuberculosis* infections. The rise of multidrug-resistant tuberculosis (MDR-TB), defined as TB showing resistance to at least INH and RIF, is a serious threat to TB control. The World Health Organization estimates that 50 million people worldwide are infected with MDR-TB. The highest percentages of MDR-TB cases that are newly contracted have been found in China (11%) and eastern Europe (7 to 14%) (5, 10, 11).

RIF resistance appears to be mediated by mutations of the β subunit of RNA polymerase, which is encoded by the *rpoB* gene. Previous work indicates that more than 95% of RIF-resistant strains are associated with mutations within an 81-bp region of the *rpoB* gene. Specific mutations, insertions, and deletions have been detected, and this 81-bp region has been termed the rifampin resistance determinant region (7, 13, 42). In contrast, INH resistance is apparently controlled by a more complex genetic system that involves several genes (15, 31). INH, a first-line antituberculosis drug, has a simple chemical structure consisting of a pyridine ring and a hydrazide group. INH is a prodrug that enters actively growing tubercle bacilli by passive diffusion (4). The bifunctional bacterial enzyme catalase-peroxidase (KatG) converts INH to a range of oxygenated and organic toxic radicals that attack multiple targets in the mycobacterial cell (32, 33). The best-characterized target of these radicals is the cell wall mycolic acid, but DNA, carbohydrates, lipids, and DNA metabolism may be targeted as well (6). Reports suggest that an NADH-dependent enoyl acyl carrier protein reductase, encoded by *inhA*, and a β-ketoacyl acyl carrier protein synthase, encoded by *kasA*, are involved in the biosynthesis of mycolic acids and are two potential intracellular enzymatic targets for activated INH (23, 25). Resistance-associated amino acid substitutions have been identified in *katG*, *inhA*, and *kasA* of INH-resistant isolates of *M. tuberculosis* (31, 33). The overexpression of InhA due to an upregulation mutation in the promoter region of *inhA* (preceding the *mabA-inhA* operon) also produces INH resistance via a titration mechanism (29). Mutations in the *oxyR-ahpC* intergenic region, where the putative promoter of *ahpC* is located, are considered to be a compensatory mechanism for the loss of KatG function in resistant strains (16, 38). In addition, mutations in other genes, including *furA*, *iniA*, *iniB*, and *iniC*, were associated with INH resistance but in much lower percentages of strains (31).

A convergence of data indicates that INH-resistant clinical isolates of *M. tuberculosis* from diverse geographic regions have distinct mutation frequencies in the genes *katG*, *kasA*, and *inhA* (regulatory and structural regions) and the *oxyR-ahpC* intergenic region (6, 15, 16, 18, 23, 28, 31, 37). Few of these findings have been applied to studies of Chinese isolates. Therefore, the present study investigated the mutations associated with INH resistance in *M. tuberculosis* isolates from China. As recent findings indicate that *furA* and the *iniBAC* (*iniA*, *iniB*, *and iniC*) region are also related to INH resistance and that the single nucleotide polymorphisms in *efpA* were detected in both INH-resistant and -susceptible isolates (31), we characterized the mutations in these genes by DNA sequencing.

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TABLE 1. *katG* double mutations in 87 INH-resistant *M. tuberculosis* isolates from China

Nucleotide no. for the first mutation in *katG*	Amino acid	Second mutation in *katG*		No. (%) of isolates with mutation	INH MIC range (μg/ml)	Additional mutation(s) (no. of isolates)
		Nucleotide no.	Amino acid			
G→C at position 944	Ser315Thr	G→T at position 1388	Arg463Leu	13 (14.9)	1–>256	*oxyR-ahpC* (2), *inhA* promoter (2), *iniA* (1)
G→A at position 944	Ser315Asn	G→T at position 1388	Arg463Leu	1 (1.1)	16	None
G→C at position 944	Ser315Thr	G→C at position 2053	Gly685Arg	1 (1.1)	2	None
G→C at position 944	Ser315Thr	A insertion at position 1329	Frameshift	1 (1.1)	64	*oxyR-ahpC* (1)
G→T at position 1388	Arg463Leu	G→T at position 1471	Gly491Cys	2 (2.3)	4–8	*inhA* (1)
G→T at position 1388	Arg463Leu	CG→TA at position 1543→1544	Arg515Tyr	1 (1.1)	8	None
G→T at position 1388	Arg463Leu	64-bp insertion at position 1559		1 (1.1)	>256	*oxyR-ahpC* (1), *efpA* (1)

MATERIALS AND METHODS

Clinical *M. tuberculosis* isolates and susceptibility testing. In total, 87 INH-monoresistant and 50 INH-susceptible *M. tuberculosis* isolates were recovered from 137 patients (age range, 16 to 88 years; 71 were male and 66 were female) from five provinces of eastern China (Jiangsu, Zhejiang, Anhui, Fujian, and Jiangxi). Lowenstein-Jensen medium was used for the cultivation of the isolates, and all isolates were initially classified as INH monoresistant or susceptible by use of a BACTEC MGIT 960 instrument (Becton Dickinson, Microbiology Systems, Sparks, MD) and standard procedures with the following critical concentrations: INH, 0.1 μg/ml; RIF, 1 μg/ml; streptomycin, 1 μg/ml; and ethambutol, 5 μg/ml. The MICs were determined by the E test and the proportion method (31). The conventional antibiotic susceptibility testing by the proportion method was performed at the respective institutions (Shanghai Pneumology Hospital and Shanghai CDC TB Laboratory). The E test and the proportion method produced results in good overall agreement.

IS*6110*-based RFLP. All isolates were assessed for their genetic relatedness by IS*6110* restriction fragment length polymorphism (RFLP). PvuI-digested DNA of *M. tuberculosis* was probed with the insertion element IS*6110* according to the standardized method of van Embden et al. (19, 37).

Sequencing strategy. The nine structural genes (*furA*, *katG*, *inhA*, *kasA*, Rv0340, *iniBAC*, and *efpA*) and two regulatory regions (the *oxyR-ahpC* intergenic region and the promoter of *mabA-inhA*) analyzed for a single nucleotide polymorphism were sequenced, after amplification by PCR using KOD polymerase (Toyobo, Co.), with high fidelity according to the manufacturer's instructions. The oligonucleotide primers and PCR conditions have been described previously (31). After purification (QIAquick column PCR purification kit; QIAGEN), the PCR product was sequenced by using an ABI 377 automated DNA sequencer (Applied Biosystems, Inc.). The isolates that showed new nonsynonymous mutations were sequenced again by cloning the PCR products into the T vector. Sequence data were assembled and analyzed by CLUSTAL W.

Nucleotide sequence accession numbers. The sequences with novel mutations were deposited in GenBank under accession numbers DQ056349 to DQ056361.

RESULTS

Among the 137 *M. tuberculosis* isolates examined, 87 were monoresistant to INH. DNA fingerprinting was carried out to distinguish whether the mutations occurred separately in the two populations or whether they happened in one population and then spread to the other region. The IS*6110* RFLP assay of the 137 isolates revealed that the number of hybridizing bands ranged from 8 to 21. Except for two isolates that shared the same print pattern, all other isolates had unique fingerprints. Some isolates with the same mutation had different IS*6110* patterns, a result indicating epidemiologic independence. Two isolates with similar IS*6110* fingerprints had different mutations in *katG*. A total of 56 (64.4%) of the 87 INH-resistant isolates and 34 (68%) of the 50 INH-susceptible isolates had an IS*6110*-based banding pattern characteristic of the Beijing genotype of *M. tuberculosis*.

The DNA sequence of the *katG* gene was determined for 50 INH-susceptible *M. tuberculosis* isolates and 87 INH-resistant isolates. All INH-susceptible isolates possessed the wild-type sequence, whereas resistance-associated mutations within the *katG* gene were found in 82 (94.3%) of the 87 INH-resistant isolates. Mutations at codon 315 occurred in 56 (64.4%) isolates, with a base substitution at nucleotide 944 predominating (Ser→Thr [AGC→ACC], 48 isolates [58.5% of the 82 *katG* mutants]). Other substitution mutations occurred at codon 315 (Ser→Asn [AGC→AAC], $n = 5$; Ser→Gly [AGC→GGC]; $n = 1$; Ser→Arg [AGC→AGG]; $n = 1$; and Ser→Ile [AGC→ATC]; $n = 1$). All of the 50 INH-susceptible isolates had different IS*6110* fingerprints, indicating their epidemiologic independence. No mutation at codon 463 of *katG* was detected in the 50 INH-susceptible isolates. In contrast, 35 (40.2%) of the INH-resistant isolates were found to harbor the Arg463Leu mutation. A characteristic finding was the presence of double mutations occurring at two separate loci of *katG*. Among the 82 *katG* mutants, 20 (24.4%) isolates had double mutations in the *katG* region (Table 1), with Ser315 plus Arg463 double mutations predominating (14 isolates).

Other drug resistance-associated mutations were detected in the *katG* gene (Table 2). Two isolates had complete *katG* deletions, based on the reproducible absence of PCR product; one isolate had termination mutations at codon 90 (TGG→TAG). Except for a single base insertion (insertion of T and A at nucleotide positions 1311 and 1329, respectively) and deletion (deletion of G at position 1559), a 64-bp fragment insertion at position 1559 was detected in only one isolate, in which mutations at other loci occurred, such as the *ahpC* promoter region and *efpA*. The 64-bp insertion sequence was TGCAGCCACAAGTCGGGTGGGAGGTCAACGACCCCGACGGGGATCTGCGCAAGGTCATTCGCAC. Other substitution mutations of *katG* occurred at codons 491 (Gly→Cys [GGC→TGC]; $n = 2$), 685 (Gly →Arg [GGC→CGC]; $n = 1$), and 515 (Arg→Tyr [CGC→TAC]; $n = 1$).

Of the 82 *katG* mutants, 53 (64.6%) had no additional mutations in other genes, whereas 29 (35.4%) had changes in other genes, some of which have not been previously reported (Table 3). A total of 10 isolates (12.2%) had additional mutations in the *inhA* regulatory region, 4 (4.9%) had additional mutations in the *inhA* structure gene, 10 (12.2%) had additional mutations in the *oxyR-ahpC* intergenic region, 4 (4.9%) had additional mutations in *kasA*, 6 (7.3%) had additional mutations in the *ini* region (Rv0340 and *iniBAC*), and 1 (1.2%) had additional mutations in *efpA*. *katG* is cotranscribed with *furA* from a common regulatory region, and reports suggest that *furA* is a negative regulator of *katG* (28, 43). An INH-

TABLE 2. Nonsynonymous mutations in INH-susceptible and -resistant *M. tuberculosis* from China

Gene or gene region	Nonsynonymous mutation	No. of INH-susceptible isolates (n = 50)	No. of INH-resistant isolates with indicated INH MIC range (μg/ml)		
			0.2 ≤ MIC < 1 (n = 24)	1 ≤ MIC < 10 (n = 48)	MIC ≥ 10 (n = 15)
furA	Ser5	0	0	1	1
katG	Completed deletion	0	0	0	2
	Frameshift	0	0	1	2
	Trp90Stop	0	0	1	0
	Ser315	0	9	38	9
	Arg463	0	11	17	7
	Gly491	0	0	2	0
	Arg515	0	0	1	0
	Gly685	0	0	1	0
inhA promoter	−15 C→T	0	2	8	2
	−17 G→T	0	0	0	1
inhA	Ile21	0	0	1	1
	Ile258	0	0	2	0
oxyR-ahpC	−9 G→A	0	0	1	1
	−10 C→T	0	0	1	0
	−12 C→T	0	0	0	2
	−15 C→T	0	0	1	0
	−39 C→T	0	0	1	3
	−48 G→A	0	0	0	1
kasA	Met77	0	0	0	1
	Leu245	0	0	1	0
	Gly269	0	1	0	1
	Gly312	8	2	3	1
	Ser341	2	2	0	1
Rv0340	Thr143	4	1	1	0
	Gly149	5	0	0	0
	Val163	0	0	1	0
iniB	Frameshift	0	1	1	0
	Gly192	2	2	1	1
iniA	Arg537	0	1	0	1
	His481	3	2	1	1
iniC	Frameshift	0	0	1	1
efpA	Ile73	4	3	2	1
	Glu520	0	0	0	1

resistance-associated mutation within *furA* at codon 5 (Ser→Pro [TCC→CCC]) was found in two isolates (2.3%). One of the isolates had additional mutations in *katG*, the *inhA* promoter, and the *oxyR→ahpC* intergenic region, whereas the other had an additional mutation in the *inhA* regulatory region. No resistance-associated mutation was detected in the promoter region of *furA*.

Analysis of the 5′ end of a presumed ribosome binding site in the promoter of *mabA-inhA* revealed nucleotide substitutions at −15 (C→T; n = 12 [13.8%]) and at −17 (G→T; n = 1 [1.1%]). Of these 13 mutants, two (15.4%) isolates had a mutation only in the *inhA* regulatory region, and one (7.7%) isolate had an additional mutation in *furA*, whereas 10 (76.9%) had additional mutations in *katG* (Table 2). The *inhA* structure region mutations occurred in four (4.6%) isolates among the 87 INH-resistant *M. tuberculosis* strains. Two of these harbored a substitution mutation at codon 258 (Ile→Thr [ATC→ACC]), and another two isolates had mutations at codon 21 (Ile→Thr [ATC→ACC], n = 1; Ile→Val [ATC→GTC], n = 1). All four isolates had additional mutations in *katG*.

Nucleotide substitutions in the *oxyR-ahpC* intergenic region were found in 10 (11.5%) resistant isolates. All 10 isolates had additional mutations in *katG*. One isolate had double mutations (−12 C→T plus −39 C→T) in the *ahpC* promoter region, and this isolate had additional mutations in *efpA* and *katG*. Of the 10 isolates with mutations in the *oxyR-ahpC* intergenic region and *katG*, 1 had an additional mutation in *furA*, 1 in the *iniBAC* region, and 1 in *efpA*. There was no drug-associated mutation detected in the structural region of *ahpC*.

Sequence analysis of the *kasA* showed that four (4.6%) isolates had substitution mutations. Two of these isolates had a missense mutation at codon 269 (Gly→Ser [GGT→AGT]), one at codon 245 (Leu→Arg [CTG→CGG]), and one at codon 77 (Met→Ile [ATG→ATC]). All four isolates had additional

TABLE 3. INH-resistant *M. tuberculosis* isolates with mutations in two or more genes

No. of isolates	MIC range (μg/ml)	Mutation(s)[a]									
		furA	*katG*	*inhA* promoter	*inhA*	*oxyR-ahpC*	*kasA*	Rv0340	*iniB*	*iniA*	*iniC*	*efpA*
1	4		1559 C Del[b,e]			−15 C→T						
1	16		1311 T Ins[c]				M771					
1	32		Del			−9 G→A						
1	>256		Del			−12 C→T						
5	0.5–32		S315T	−15 C→T								
1	1		S315T		I21T				198 T Ins[e]			
1	2		S315T			−9 G→A						
1	2		S315T			−39 C→T						
1	2		S315T								79 T Ins	
1	16		S315T	−17 G→T								
1	16		S315T		I21V							
1	16		S315T				G269S				98 A Ins[e]	
1	0.5		R463L									
1	1		R463L				L245R					
1	2		R463L		I258T[e]		G269S			R537H		
1	4		R463L					V1631				
1	16		R463L	−15 C→T								
1	16	S5P	R463L	−39 C→T[e]		−39 C→T						
2	1–2		S315T; R463L	−15 C→T								
1	2		S315T; R463L			−10 C→T						
1	32		S315T; R463L			−48 G→A				R537H		
1	64		S315T; 1329 A Ins			−39 C→T						
1	>256		R463L; 1559 Ins of 64 bp[d,e]			−12 C→T; −39 C→T						E520V[e]
1	4		R463L; G491C[e]		I258T[e]							
1	2	S5P		−15 C→T								

[a] Amino acid abbreviations: S, Ser; T, Thr; R, Arg; L, Leu; P, Pro; G, Gly; C, Cys; I, Ile; V, Val; M, Met; H, His; E, Glu. Nucleotide abbreviations: A, adenine; C, cytosine; G, guanine, T, thymidine.
[b] Deletion (Del) of nucleotide C at position 1559.
[c] Insertion (Ins) of nucleotide T at position 1311.
[d] Insertion (64 bp) at position 1559.
[e] Novel mutation.

mutations in *katG*, and two isolates had additional mutations in the *iniBAC* region as well as in *katG*. Polymorphisms at codon Gly321 and Ser341 were found in both INH-resistant and -susceptible isolates (Table 2).

The *ini* region has four genes designated Rv0340, *iniB*, *iniA*, and *iniC*; of these genes, *iniB*, *iniA*, and *iniC* are organized as an operon. The Rv0340 gene is located upstream of the *iniBAC* operon and is transcribed in the same orientation (2, 3, 8, 31). Sequence analysis showed that the four genes had mutations associated with drug resistance, and there were seven (8.0%) isolates that harbored mutations in this region. One isolate had a mutation at codon 163 of Rv0340 (Val→Ile [GTT→ATT]), and this isolate harbored an additional mutation in *katG*. Two isolates had insertion (insertion of T at nucleotide 198) and deletion (deletion of A at nucleotide 211) mutations in *iniB*, respectively. Of the two *iniB* mutants, the insertion mutant harbored additional mutations in *katG* and *inhA*, in contrast to the deletion mutant, which had no additional mutations at the other loci. Two isolates had a substitution mutation at codon 537 of *iniA* (Arg→His [CGC→CAC]). Of the two *iniA* mutants, one harbored additional mutations in *katG* and the *oxyR-ahpC* intergenic region, whereas the other had additional mutations in *katG*, *inhA*, and *kasA*. Frameshifts occurred in two *iniC* mutants, with insertion of A and T at nucleotide positions 98 and 79, respectively. The two *iniC* mutation isolates harbored an additional *katG* 315 mutation.

e*fpA*, which encodes an efflux protein, was induced by INH (31). Sequence analysis showed one isolate had a drug resistance-associated mutation at codon 520 (Glu→Val [GAG→GTG]). This isolate had an additional mutation in the *oxyR-ahpC* intergenic region and double mutations in *katG*.

DISCUSSION

All of the 87 INH-resistant isolates from China had mutations in the nine structural genes (*furA*, *katG*, *inhA*, *kasA*, Rv0340, *iniB*, *iniA*, *iniC*, and *efpA*) or two regulatory regions (the *oxyR-ahpC* intergenic region and the promoter of *mabA-inhA*). Thirty-nine distinct mutations were identified. Among these, mutations in the *katG* gene were predominant, as expected; *katG* mutations were found in 82 (94.3%) isolates. Geographical differences in the frequencies of the *katG* codon 315 mutations were apparent in the analysis of data from other studies: mutations in *katG* codon 315 were detected in only 34.6% of the INH-resistant isolates from Madrid (18) but were detected in a substantial 93.6% of isolates from northwest Russia (26). Therefore, the documented information regarding the frequencies and types of mutations in one country or geographical region may not apply generally to other regions. We found mutations in *katG* codon 315 in 64.4% of the INH-resistant isolates from five provinces of China, which is different from the data (51%) from Hong Kong (22). Previous work

found these mutations in 34.6 to 62.2% of INH-resistant isolates, for which MICs of INH were at least 0.2 μg/ml (12, 27, 35), whereas other investigators found these mutations in 61.9 to 97.4% of INH-resistant isolates, for which MICs of INH were at least 1 μg/ml (6, 1, 17, 24). These findings suggest that mutations in the *katG* codon 315 are associated with high levels of INH resistance. This was also observed in our study (Table 2). Of the 24 isolates exhibiting low levels of INH resistance (0.2 μg/ml ≤ MIC < 1 μg/ml), 9 (37.5%) isolates showed this mutation. In contrast, 38 (79.2%) and 9 (60%) isolates showing intermediate (1 μg/ml ≤ MIC < 10 μg/ml) and high (MIC ≥ 10 μg/ml) levels of INH resistance harbored the *katG* codon 315 mutation, respectively.

Previous findings indicated that *katG* codon 463 was a polymorphic site and that the *katG* codon 463 mutation (Arg→Leu) was not associated with INH resistance (14, 22, 36). In our study, no mutation at codon 463 of *katG* was detected in 50 INH-susceptible isolates with different IS*6110* fingerprints, but 35 (40.2% of the 87 INH-resistant *M. tuberculosis* isolates) INH-resistant isolates had a mutation at codon 463 of *katG*. Among the 35 Arg463Leu mutants, 11 (31.4%) (0.2 μg/ml ≤ MIC < 0.5 μg/ml) isolates had no additional mutations at other loci. A total of 24 (68.6%) (MIC ≥ 1 μg/ml) isolates harbored additional mutations at *katG* or other genes. The *katG* Arg463Leu mutation was associated with low levels of INH resistance among the clinical isolates from eastern China.

Double point mutations in two separate loci of *katG* were detected in 20 isolates from China, with substitutions at codons 315 and 463 being predominant (Table 1). Regardless of the mutations in other genes, four (12.5%) isolates of the 32 *katG* mutants harboring the single mutation of Ser315Thr exhibited high levels of INH resistance (MIC ≥ 10 μg/ml). And three (17.6%) isolates of the 17 *katG* mutants harboring the single mutation of Arg463Leu exhibited high levels of INH resistance. Of the 13 isolates harboring double mutations of Ser315Thr and Arg463Leu in *katG*, 3 (23.1%) isolates exhibited high levels of INH resistance. Piatek et al. suggest that isolates develop resistance to INH by a stepwise accumulation of mutations, which may be important for achieving the higher levels of resistance or maintaining virulence in a human host (27). In our study, the association between high levels of INH resistance and the accumulation of mutations in *katG* supports such a hypothesis. However, this hypothesis does not exclude the possibility that full resistance can also develop in a single step, as has been observed in laboratory mutants. This possibility will be explored in future studies.

Five novel mutations within *katG* were found in this investigation. They were mutations Gly→Cys (GGC→TGC) at codon 491, Arg→Tyr (CGC→TAC) at codon 515, and Gly→Arg (GGC→CGC) at codon 685, deletion of G at position 1559, and a 64-bp fragment insertion at position 1559. The 64-bp nucleic acid came from duplication of a fragment of *katG* (from positions 1496 to 1559), and this insertion mutation did not create a frameshift or termination mutation in *katG*. The isolate harboring the 64-bp fragment insertion had additional mutations in the *oxyR-ahpC* intergenic region and *efpA*. The additional mutation in *efpA* was Glu→Val (GAG→GTG) at codon 520, which has not been previously described. Ramaswamy et al. reported that *efpA* had no INH resistance-associated mutation (31). Additional studies of INH-resistant *M. tuberculosis* isolates from global populations are needed to see whether the mutation in this gene plays a role in INH resistance.

Previous studies indicate that mutations in the upstream region of *inhA* result in an increase of InhA expression, thereby elevating the drug target levels and producing INH resistance via a titration mechanism (29). Previous investigators found *inhA* promoter mutations in 10 to 34.2% of the INH-resistant isolates (18, 12, 17, 20, 35). In the current study, the mutations in the promoter region of *inhA* were found in 14.9% of the INH-resistant isolates from China. A novel substitution mutation at position −17 (G→T) was observed. The mutations identified in the structural region of *inhA* result in INH resistance due to the reduced binding affinity of the INH-NAD^+ adduct for enoyl reductase (35). Our results suggest that the substitution frequency (4.6%) of *inhA* was very low. Two isolates harbored a novel substitution mutation at codon 258 (Ile→Thr).

Mutations in the *oxyR-ahpC* region occurred in 10 (11.5%) isolates. One isolate harbored double mutations (−12 C→T plus −39 C→T) in this region, and they have not been described previously. Previous reports found mutations of the *oxyR-ahpC* region in 4.8 to 24.2% of the INH-resistant isolates (6, 16, 21). In our study, all 10 isolates had an additional mutation in *katG*, and the MICs were higher than 2 μg/ml. Genetic and biochemical studies have shown that certain mutations of the *ahpC* promoter region result in overexpression of *ahpC* as a compensatory mechanism for the loss of catalase activity due to *katG* mutations (16, 38).

The *iniBAC* operon encodes genes that are induced by a broad range of antibiotics, including INH and ethambutol (3). Mutations in the *ini* genes were identified in INH-resistant *M. tuberculosis* isolates as well as in ethambutol-resistant isolates (30, 31). One isolate harbored a frameshift mutation with an insertion of T at nucleotide position 198 in *iniB*. One isolate had an insertion of A at 98 in *iniC*. These results have not been previously discovered.

Analysis of the nine structural genes (*furA*, *katG*, *inhA*, *kasA*, Rv0340, *iniBAC*, and *efpA*) and two regulatory regions (*ahpC* and *inhA*) of 87 *M. tuberculosis* clinical isolates from China was performed, and a variety of mutations were identified. The present findings contribute substantially to our knowledge of the spectrum of gene mutations that may participate in INH resistance and provide clues for elucidating the drug-resistance mechanisms of *M. tuberculosis*. Rapid detection of the *M. tuberculosis* complex is critical to enable the appropriate antimycobacterial therapy for patients to commence promptly as well as to control the spread of this pathogen. Therefore, more information regarding these mutations would be beneficial for the development of novel molecular diagnostic methods such as the DNA line probe and DNA microarrays (39, 40).

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