Drug-resistance mechanism, pathogenesis and genomics of tuberculosis: Gene mutations in isoniazid-resistant *Mycobacterium tuberculosis*

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Summary

One of the effective strategies to decrease incidence of tuberculosis (TB) is to find out patients as soon as possible. Therefore, the patients can be treated appropriately and avoid to transmit to other people. Regarding multi-drug resistant (MDR) TB patients, they need longer period of treatment and easily acquire extra drug resistance if they do not get correct regimen. Base on our preliminary study, of the 242 MDR *Mycobacterium tuberculosis* isolates, only 78.5% can be rapidly identified using the GenoType MTBDR*plus* commercial kit. Additional 13.5% of the MDR isolates can be detected by sequencing drug resistance associated genes, including the *rpoB* core-region, *katG*, *inhA*, *inhAr*, and *oxyR-ahpC* genes. The mechanisms of isoniazid (INH) resistant are more complicated than that of rifimpin (RMP) resistance. The INH-associated gene mutations still not yet completely identified. In order to improve the detection rate of MDR *M. tuberculosis* isolates using DNA sequencing, we focus on identification of novel INH resistant-associated genes including the *furA*, *ndh*, *kasA*, *mabA*, *efpA*, *Rv0340*, *iniB*, *iniA*, and *iniC* genes. In this study, we analyzed 57 INH-resistant isolates without mutations in the *katG*, *inhA*, *inhA* regulatory region, and *oxyR-ahpC* genes. The results showed that 14 mutations among 8 new sequenced INH resistant-associated genes (*mabA*, *kasA*, *ndh*, *furA*, *iniB*, *iniA*, *iniC*, and *efpA*) in 33.3% (19/57) of the INH-resistant isolates. Besides the 12-bp deletion of codon 222 in the *iniB* gene, we identified 13 novel mutations that were associated with INH resistance. While, 18 INH-susceptible isolates do not harbored any mutations in those 8 genes sequenced.

I. Purpose

The objective of this study is to identify new mutations of the INH resistant-associated genes to inctease the detection rate of INH-resistant isolates using the DNA sequencing method without waiting for results of time-consuming conventional drug susceptibility testing (DST). Therefore, prompt and proper treatment MDR TB patients can improve the treatment outcome.

II. Methods

Mycobacterium tuberculosis isolates. We received *M. tuberculosis* isolates from clinical mycobacteriology laboratories in Taiwan. One isolates was selected from individual TB case. In this study, 57 MDR *M. tuberculosis* isolates which were INH resistant confirmed by DST but harboring no mutation on the *katG, inhA, inhA* regulatory region, and *oxyR-ahpC* genes were selected to be sequenced. In addition, 18 INH-susceptible isolates were analyzed concordantly as a control.

Drug susceptibility testing. The agar proportion method on either Middlebrook 7H10 or 7H9 (Creative Microbiologicals or Sancordon, Taiwan), and BACTEC^{TM} MGIT^{TM} 960 SIRE Kits (Becton Dickinson Diagnostic Systems, Sparks, MD) with a liquid culture system were used. The critical first-line drug concentrations for the agar proportion method on 7H10 were 0.2 μg/ml and 1.0 μg/ml for INH, 1.0 μg/ml for RMP. Growth on the control medium was compared to growth on the drug-containing medium to determine susceptibility. The tests were validated by comparison to the susceptibility of *M. tuberculosis* H37Rv included in the same DST. MDR was defined as *M. tuberculosis* isolates resistant to at least INH and RMP.

Routine INH-resistant associated gene sequencing. Primer sets were used to analyze the variation at the *katG, inhA, inhA* regulatory region, *and oxyR-ahpC* genes and sizes of the PCR products were listed in Table1. The PCR reactions were performed as follows: 33 cycles at 95°C for 1 min; annealing at 55°C (*katG*) or 60°C (*inhA* and *inhA* regulatory region) for 1 min; and elongation at 72°C for 1 min. Thereafter, the PCR products were analyzed with an ABI Veriti automated sequencer (Applied Biosystems, USA), and the sequence data were assembled and edited using the Sequencher 4.7 Demo software.

Novel INH-resistant associated gene sequencing. Primer sets were used to analyze the variation at the *furA*, *ndh*, *kasA*, *mabA, efpA*, *Rv0340*, *iniB*, *iniA*, and *iniC* genes and sizes of the PCR products were listed in Table 2. The PCR reactions were performed as follows: 30 cycles at 95°C for 30 sec; annealing at 62°C for 30 sec; and elongation at 72°C for 1 min. Thereafter, the PCR products were analyzed with an ABI Veriti automated sequencer

(Applied Biosystems, USA), and the sequence data were assembled and edited using the Sequencher 4.7 Demo software.

III. Results

Gene sequencing analysis

The INH-resistant associated mutations identified in this study were list in Table 3 and Table 4. We identified 14 mutations among 8 additional INH resistant-associated genes (*mabA*, *kasA*, *ndh*, *furA*, *iniB*, *iniA*, *iniC*, and *efpA*) in 33.3% (19/57) of the INH-resistant isolates that did not harbored any mutations in the *katG*, *inhA*, *inhA* regulatory region, and *oxyR-ahpC* genes. Besides the 12-bp deletion of codon 222 in the *iniB* gene, we identified 13 novel mutations that were associated with INH resistance.

IV. Discussion

Based on the sequence analyses of the new INH resistant-associated genes, we found 14 mutation sites in the INH-associated genes in 33.3% (19/57) of the INH-resistant isolates with no mutations in the *katG*, *inhA*, *inhA* regulatory region, and *oxyR-ahpC* genes. These results can be applied in a high throughput system to detect these mutation sites simultaneously. In this study, the results showed that using additional mutations that associated with the INH resistant-associated genes will be helpful in identifying MDR *M. tuberculosis* isolates without waiting for time-consuming conventional DST results. In addition, a rapid molecular diagnosis kit can be as developed with an improved sensitivity.

V. References

1. Huang WL, Chen HY, Kuo YM, Jou R. 2009. Performance assessment of the GenoType MTBDR*plus* test and DNA sequencing in detection of multidrug-resistant *Mycobacterium tuberculosis*. J Clin Microbiol. 47(8):2520-4.
2. Bardou F, Raynaud C, Ramos C, Lanéelle MA, Lanéelle G. 1998. Mechanism of isoniazid uptake in *Mycobacterium tuberculosis*. Microbiology 144 (Pt 9):2539-44.
3. Marrakchi H, Lanéelle G, Quémard A. 2000. InhA, a target of the antituberculous drug isoniazid, is involved in a mycobacterial fatty acid elongation system, FAS-II. Microbiology 146 (Pt 2):289-96.
4. Vilchèze C, Jacobs WR Jr. 2007. The mechanism of isoniazid killing: clarity through the scope of genetics. Annu Rev Microbiol 61:35-50.
5. Cardoso RF, Cooksey RC, Morlock GP, Barco P, Cecon L, Forestiero F, Leite CQ, Sato DN, Shikama Mde L, Mamizuka EM, Hirata RD, Hirata MH. 2004. Screening and

characterization of mutations in isoniazid-resistant *Mycobacterium tuberculosis* isolates obtained in Brazil. Antimicrob Agents Chemother. 48(9):3373-81.

6. Kelley CL, Rouse DA, Morris SL. 1997. Analysis of *ahpC* gene mutations in isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis.* Antimicrob Agents Chemother. 41(9):2057-8.
7. Kim SY, Park YJ, Kim WI, Lee SH, Ludgerus Chang C, Kang SJ, Kang CS. 2003. Molecular analysis of isoniazid resistance in *Mycobacterium tuberculosis* isolates recovered from South Korea. Diagn Microbiol Infect Dis. 47(3):497-502.
8. Lee AS, Teo AS, Wong SY. 2001. Novel mutations in *ndh* in isoniazid-resistant *Mycobacterium tuberculosis* isolates. Antimicrob Agents Chemother. 45(7):2157-9.
9. Hazbón MH, Brimacombe M, Bobadilla del Valle M, Cavatore M, Guerrero MI, Varma-Basil M, Billman-Jacobe H, Lavender C, Fyfe J, García-García L, León CI, Bose M, Chaves F, Murray M, Eisenach KD, Sifuentes-Osornio J, Cave MD, Ponce de León A, Alland D. 2006. Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 50(8):2640-9.
10. Zhang M, Yue J, Yang YP, Zhang HM, Lei JQ, Jin RL, Zhang XL, Wang HH. 2005. Detection of mutations associated with isoniazid resistance in *Mycobacterium tuberculosis* isolates from China. J Clin Microbiol. 43(11):5477-82.
11. Ramaswamy SV, Reich R, Dou SJ, Jasperse L, Pan X, Wanger A, Quitugua T, Graviss EA. 2003. Single nucleotide polymorphisms in genes associated with isoniazid resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 47(4):1241-50.
12. Zahrt TC, Song J, Siple J, Deretic V. 2001. Mycobacterial FurA is a negative regulator of catalase-peroxidase gene *katG*. Mol Microbiol. 39(5):1174-85.
13. Pym AS, Domenech P, Honoré N, Song J, Deretic V, Cole ST. 2001. Regulation of catalase-peroxidase (KatG) expression, isoniazid sensitivity and virulence by furA of *Mycobacterium tuberculosis.* Mol Microbiol. 40(4):879-89.
14. Vilchèze C, Weisbrod TR, Chen B, Kremer L, Hazbón MH, Wang F, Alland D, Sacchettini JC, Jacobs WR Jr. 2005. Altered NADH/NAD+ ratio mediates coresistance to isoniazid and ethionamide in mycobacteria. Antimicrob Agents Chemother. 49(2):708-20.
15. Mdluli K, Slayden RA, Zhu Y, Ramaswamy S, Pan X, Mead D, Crane DD, Musser JM, Barry CE 3rd. 1998. Inhibition of a *Mycobacterium tuberculosis* beta-ketoacyl ACP synthase by isoniazid. Science. 1998. 280(5369):1607-10.
16. Banerjee A, Sugantino M, Sacchettini JC, Jacobs WR Jr. 1998. The *mabA* gene from the *inhA* operon of *Mycobacterium tuberculosis* encodes a 3-ketoacyl reductase that fails to confer isoniazid resistance. Microbiology. 144 (Pt 10):2697-704.

17. Wilson M, DeRisi J, Kristensen HH, Imboden P, Rane S, Brown PO, Schoolnik GK. 1999. Exploring drug-induced alterations in gene expression in *Mycobacterium tuberculosis* by microarray hybridization. Proc Natl Acad Sci U S A. 96(22):12833-8.
18. Alland D, Kramnik I, Weisbrod TR, Otsubo L, Cerny R, Miller LP, Jacobs WR Jr, Bloom BR. 1998. Identification of differentially expressed mRNA in prokaryotic organisms by customized amplification libraries (DECAL): the effect of isoniazid on gene expression in *Mycobacterium tuberculosis*. Proc Natl Acad Sci U S A. 95(22):13227-32.
19. Alland D, Steyn AJ, Weisbrod T, Aldrich K, Jacobs WR Jr. 2000. Characterization of the *Mycobacterium tuberculosis* iniBAC promoter, a promoter that responds to cell wall biosynthesis inhibition. J Bacteriol. 182(7):1802-11.

VI. Publication list for this work

NIL

VIII. Tables

Table 1. Primer sequences used to analyze the variation at the *katG, inhA, inhA* regulatory region, *and oxyR-ahpC* genes

Gene	Primer name	Primer sequence	Size (bp)
katG	katG-F	5′-GTC ACA CTT TCG GTA AGA C-3′	658
	katG-R	5′-TTG TCG CTA CCA CGG AAC G-3′	
inhA	inhA 1713-F	5′-CCG AGG ATG CGA GCT ATA TC-3′	543
	inhA 1713-R	5′-GGC TCG GGT CGA AGT CCA TG-3′	
	inhA 2194-F	5′-AGG CGC TGC TGC CGA TCA TG-3′	456
	inhA 2194-R	5′-CCG AAC GAC AGC AGC AGG AC-3′	
inhA regulatory region	inhA locus-F	5′-AAT TGC GCG GTC AGT TCC AC-3′	453
	inhA locus-R	5′-GTC GGT GAC GTC ACA TTC GA-3′	
oxyR-ahpC	ahpC-F	5′-GCT TGA TGT CGG AGA GCA TCG-3′	701
	ahpC-R	5′-GGTCGC GTA GGC AGT GCC CC-3′	

Table 2. Primer sequences used to analyze the variation at the *furA*, *ndh*, *kasA*, *mabA*, *efpA*, *Rv0340*, *iniB*, *iniA*, and *iniC* genes

Gene	Primer name	Primer sequence	Size (bp)
furA	furA-F	5'-CTCATCGGAACATACGAAGGCT-3'	700
	furA-R	5'-ATTTCATATGACCCACGACGGGAC-3'	
ndh	ndh-1F	5'-GCACGCTGTGGTGTGGCTGATGAC-3'	906
	ndh-1R	5'-CGGAGTCCTTGACGGTGATGCCGT-3'	
	ndh-2F	5'-CCGGCACATCGACTCGACCAA-3'	888
	ndh-2R	5'-GCATTCACCGACGCCATCGACG-3'	
kasA	kasA-1F	5'-GGTGACGTTGTCGCCTACATCC-3'	884
	kasA-1R	5'-CGTCTCGATGAGCATCAGCGCA-3'	
	kasA-2F	5'-CGTCAGATCGTGATGGGCGAC-3'	854
	kasA-2R	5'-GCGATGCCGGTGACGACTACGTA-3'	
mabA	mabA-1F	5'-GCAATTGCGCGGTCAGTTCCA-3'	674
	mabA-1R	5'-CGACCGAATTTGTTGCGCTGC-3'	
	mabA-2F	5'-GCTGTTTGGCGTCGAATGTGAC-3'	685
	mabA-2R	5'-TACCCGTGCGATGTGAAACGCGAT-3'	
efpA	efpA-1F	5'-AACAGACGTTGCGGGCCACCCT-3'	1019
	efpA-1R	5'-CGCTCCACGATGACAAACGCGAC-3'	
	efpA-2F	5'-GCCTTCTCGATCGGTCCTGAA-3'	952
	efpA-2R	5'-GGTGCGCAAGAACAACTCGGACAT-3'	
Rv0340	Rv0340-F	5'-TAATGCGGCCATCCCCTAACG-3'	797
	Rv0340-R	5'-ATCGACGCTATGGATTCCGCCT-3'	
iniB	iniB-1F	5'-GCCGATCCCGATAGGTGTTTGG-3'	981
	iniB-1R	5'-GCATAGCAGCGCCGTTCAAGG-3'	
	iniB-2F	5'-CGCTAGCCAGATCGGTGTCTC-3'	837
	iniB-2R	5'-GCTCGTTTACGCCTCAGATCACG-3'	
iniA	iniA-1F	5'-TCGGTGTTTGACGTCGGTCACGAG-3'	891
	iniA-1R	5'-CAGATGTGCTGCATTGGCATTGAC-3'	
	iniA-2F	5'-CGATGCCGTCTTGGTGGTCAG-3'	800
	iniA-2R	5'-CGAAGTCGGTGCCCATGACGTG-3'	
	iniA-3F	5'-TGTGACCCGACTGCGCATTGG-3'	835
	iniA-3R	5'-CGGTCCAGCTGGCAAAAAACGTCG-3'	
iniC	iniC-1F	5'-CGGAAACCGAGCGGGACAATCG-3'	890
	iniC-1R1	5'-TCAGCGCAAGAAGTCCGGATACC-3'	
	iniC-2F	5'-CTCAAACAGATCGGTGGGCTGGT-3'	1009
	iniC-2R	5'-GCTCGAAAACATGTTCCACCCGGT-3'	

Table 3. Mutations identified in novel INH-resistant associated genes, *mabA*, *kasA*, *ndh*, and *furA* genes

katG, *inhA*, *inhAr*, *oxyR-ahpC*	DST result			*mabA*	*kasA*	*ndh*	*furA*			
				codon 74	codon 253	codon 68	codon 40	codon 40	codon 61	codon 122
				Ala→Thr	His→Tyr	Ile→Thr	Thr→Pro	Thr→Ala	Asp→His	Asp→Lys
wild type	R*	57	mutation	**1**	**1**	**5**	**1**	**1**	**1**	**1**
			wild type	56	56	52	56	56	56	56
	S*	18	mutation	0	0	0	0	0	0	0
			wild type	18	18	18	18	18	18	18

*: R, resistant; S, susceptible

Table 4. Mutations identified in novel INH-resistant associated genes, *iniB*, *iniA*, *iniC,* and *efpA* genes

katG, inhA, inhAr, oxyR-ahpC	DST result			*iniB*		*iniA*			*iniC*	*efpA*
				codon 222	codon 164-175	codon 19	codon 84	codon 634	codon 476	codon 47
				12-bp del	48-bp del	Lys→Asn	Ser→Arg	Arg→Pro	Arg→Cys	Pro→His
wild type	R*	57	mutation	**2**	**1**	**1**	**5**	**1**	**1**	**1**
			wild type	55	56	56	52	56	56	56
	S*	18	mutation	0	0	0	0	0	0	0
			wild type	18	18	18	18	18	18	18

*: R, resistant; S, susceptible