Distribution of Drug-Resistant Genes Among Thai Multidrug-Resistant *Mycobacterium Tuberculosis* (MDR-TB) Clinical Isolates

Therdsak Prammananan, Dr. rer. biol. hum.

National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Ministry of Science and Technology, Thailand Science Park, Pathumthani 12120, Thailand.

Siriraj Med J 2011;63:102-105

E-journal: http://www.sirirajmedj.com

One hundred and thirty years after the discovery of tubercle bacilli by Robert Koch, tuberculosis (TB) is still a major cause of illness and death worldwide, particularly in Asia and Africa. It is estimated that there were 9.27 million new cases of TB in 2007 and 1.3 million cases died of TB, of which 0.45 million deaths were in HIV-positive people[1]. The situation will become worse due to the emergence of multidrug-resistant *Mycobacterium tuberculosis* strains. In 2007 multidrug-resistant TB (MDR-TB) occurred in approximately 0.5 million cases. For Thailand, it was reported that there were an estimated 91,000 TB cases annually, of which 39,000 cases were active TB and almost 13,000 died of TB. MDR-TB occurred in 1.7% among new TB cases and 35% of previously treated TB cases[1].

The increasing incidence of MDR-TB and the emergence of extensively drug-resistant tuberculosis (XDR-TB) make the control of TB more difficult. XDR-TB is a MDR-TB strain [resistant at least to rifampicin (RIF) and isoniazid (INH)] that is additionally resistant to any fluoroquinolones (FQs) and to at least one of the three following injectable second-line drugs: amikacin, kanamycin, and capreomycin. Generally, the treatment of drug-susceptible *M. tuberculosis* is achieved by using the "Short-course" regimen, which requires 2 months of treatment with INH, RIF, pyrazinamide (PZA), and ethambutol (EMB), followed by 4 months with INH and RIF. In contrast, treatment of drug-resistant TB needs more drugs and longer time with a minimum of 18-24 months.

One of the most effective controls of MDR-TB is to rapidly identify MDR-TB strains, and provide the appropriate choice of treatment regimen. Understanding the mechanisms of drug resistance is important and can provide basic knowledge of drug action and resistance. Moreover, it can lead to the development of better diagnostic methods and new anti-TB drugs. This article will review drug action and mechanisms of resistance to RIF, INH and PZA in *M. tuberculosis* and provide the prevalence of their resistant genes in Thai MDR-TB clinical isolates.

General mechanisms of drug resistance

Most bacteria become resistant to antibacterial agents by common resistant mechanisms including (1) target modification, (2) target overexpression, (3) reduced uptake or increased efflux, (4) bypass mechanism, (5) the presence of drug-inactivating enzymes, and (6) inactivation of drug-activating enzymes. Since horizontal transfer of resistance determinants has not been reported in *M. tuberculosis*, the most common mechanisms of drug resistance result from chromosomal mutations of genes encoding drug targets. Such a mechanism has been found to confer resistance to RIF, streptomycin (SM), EMB, amikacin (AMK), kanamycin (KAN), and FQs. Inactivation of drug-activation enzymes has been shown to confer resistance only to INH, ethionamide (ETH), and PZA, which need mycobacterial enzymes to convert the prodrug to their active form. Although there have been many genes coding for efflux pumps and drug-inactivating enzymes in the *M. tuberculosis* genome, they have not been reported to confer resistance in clinical drug-resistant isolates.

Mode of action and mechanism of resistance

The chemical structures of most commonly used first- and second-line anti-TB drugs are shown in Fig 1.

Rifampicin

RIF is a semisynthetic derivative of rifamycin and was first used to treat TB in 1968. It is active against various species of mycobacteria, including *M. tuberculosis, M. kansasii, M. avium-intracellulare* complex, and

Correspondence to: Therdsak Prammananan
E-mail: therdsak@biotec.or.th
Received: 18 May 2011
Revised: 6 June 2011

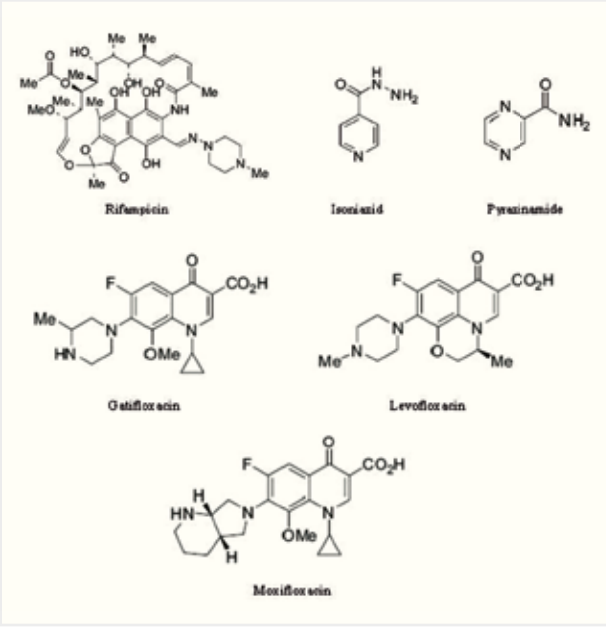

Fig 1. Structures of some first-line and second-line anti-TB drugs.

M. marinum. RIF showed bactericidal activity against *M. tuberculosis* with MIC values of 0.1-1.0 μg/ml. The drug inhibits RNA synthesis by binding to the β subunit of RNA polymerase near the RNA/DNA channel and subsequently blocking the transit of the growing RNA chain. More than 95% of RIF-resistant *M. tuberculosis* strains are caused by mutations of the *rpoB* encoding the drug target β subunit RNA polymerase. Almost all mutations occur in the 81-bp hot-spot region, designated as the RIF-resistant determining region (RRDR). As reported worldwide[2], amino acid substitutions at codon 531, 526, and 516 are the most common mutation sites and confer a high-level of RIF resistance (32 to 256 μg/ml). Some other mutations such as at codon 522 and 533 have also been reported, but they are associated with a low-level of resistance.

Recent study with 143 MDR-TB isolates from Thailand showed a concordant result with earlier studies, but interestingly, all mutations occurred at the *rpoB* with 98.6% located in the hot-spot region, and the remainder in the N-terminal part of the gene[3]. Most mutations were found at codon 531, 526, and 516 with 58%, 25%, and 9%, respectively. Codon 526 showed the most variable mutations, with four different nucleotide substitutions. Substitution of histidine with tyrosine accounted for 52.8% of the mutations at this codon. Deletion was rarely found, whereas no insertions were detected in the study. All mutations are summarized in Table 1.

Isoniazid

INH is a prodrug and is activated to an active form, isonicotinic acid, by catalase-peroxidase enzyme (KatG). The drug is highly active against growing *M. tuberculosis* with the MIC values of 0.02-0.2 μg/ml whereas it has little activity against resting bacteria in a stationary phase or under anaerobic conditions. INH has long been known to inhibit mycolic acid synthesis in the elongation step. A NADH-specific-enoyl-acyl carrier protein reductase (InhA) was the first identified common target of INH and ETH, and has been associated with INH resistance. Recent studies have demonstrated that INH binds with NAD to form INH-NAD adduct which inhibits InhA function[4].

TABLE 1. Distribution and frequency of the *rpoB* mutations among 143 Thai MDR-TB isolates[3].

***rpoB* mutation**	**No. of strains (%)**
Single mutation	**135 (94.4)**
531 TCG>TTG (Ser>Leu)	82 (57.3)
526 CAC>TAC (His>Tyr)	18 (12.6)
526 CAC>CTC (His>Leu)	6 (4.2)
526 CAC>GAC (His>Asp)	5 (3.5)
526 CAC>CGC (His>Arg)	4 (2.8)
516 GAC>GTC (Asp>Val)	11 (7.7)
533 CTG>CCG (Leu>Pro)	3 (2.1)
522 TCG>TTG (Ser>Leu)	1 (0.7)
513 CAA>CCA (Gln>Pro)	1 (0.7)
513 CAA>AAA (Gln>Lys)	1 (0.7)
513 CAA>GAA (Gln>Glu)	1 (0.7)
146 GTC>TTC (Gln>Lys)	2 (1.4)
Double mutations / Deletions	**8 (5.6)**
531 TCG>TTG (Ser>Leu) / 622 TCG>GCG (Ser>Ala)	1 (0.7)
526 CAC>AGC (His>Ser) / 515 ATG>CTG (Met>Val)	1 (0.7)
526 CAC>AGC (His>Ser) / 535 CCC>CAC (Pro>His)	1 (0.7)
526 CAC>TAC (His>Tyr) / 541 GAG>GGG (Glu>Gly)	1 (0.7)
516 GAC>TAC (Asp>Tyr) / 511 CTG>CGG (Leu>Arg)	1 (0.7)
516 GAC>GAG (Asp>Glu) / 522 TCG>TTG (Ser>Leu)	1 (0.7)
Del 513-514 (ATTCATGGA)	1 (0.7)
Del 518-519 (CAA)	1 (0.7)

Several studies from many geographic regions demonstrated that resistance to INH is caused by at least 3 mechanisms including (1) inactivation of INH activating enzyme or KatG, (2) mutations or overexpression of *inhA*, and (3) alteration of the NADH/NAD level inside the cell. The most common mechanism is a mutation of the KatG, which was found in between 20 and 80% of INH-resistant clinical isolates[2]. Mutation of KatG (S315T) is the most common mutation (50-93% of KatG mutations) and does not affect the fitness of the bacteria. The catalase-peroxidase activity of KatG (S315T) is still maintained, even if it is reduced by about 50%, but this mutation affects the ability to bind with the drug, resulting in less activation of INH. Interestingly, a higher percentage of KatG mutation was observed among MDR-TB than non MDR-TB isolates. Overexpression of the inhA gene by mutation in the promoter region or mutation of the inhA structural region has been shown to confer resistance to INH and has been identified in about 15-46% of INH-resistant clinical isolates. There is also an associated mutated allele, which is not causative of INH resistance, the *ahpC* gene encoding antioxidant enzyme alkyl hydroperoxide reductase (AhpC). Overexpression of the *ahpC* gene by mutations of the promoter regions usually occurs as a compensatory mutation in INH-resistant strains having inactive KatG. They have been found in between 10-20% of INH-resistant clinical isolates and can be used as a surrogate marker for detection of INH resistance. However, 10-30% of INH-resistant isolates contain no known mutations, indicating the presence of other unknown mechanisms. Some other resistant determinants have been shown to be associated with INH resistance. One study identified a mutation of the type II NADH dehydrogenase (Ndh-II) coded by a

ndh gene in 9.5% of INH-resistant *M. tuberculosis* clinical isolates. The mycothiol biosynthetic gene, *mshA*, has shown to be involved in INH resistance in *M. smegmatis* and spontaneous mutants of *M. tuberculosis*. However, mutation of this gene has still not been found in INH-resistant clinical isolates.

The study from Thailand with 160 INH-resistant *M. tuberculosis* clinical isolates, consisting of 110 MDR-TB and 50 non MDR-TB isolates, from Thailand revealed the mutations of *katG, inhA, oxyR-ahpC* intergenic region, and ndh in 80.6%, 13.8%, 2.5%, and 0.6%, of cases respectively[5] (Table 2). Almost 98% (126 of 129 isolates) of mutations in the KatG are substitution of serine to threonine at codon 315. Interestingly, a higher percentage of the KatG mutation was found among MDR-TB than non MDR-TB (86.4% vs. 68%). In contrast, mutations of the *inhA* were predominantly found in non MDR-TB compared with the MDR-TB isolates (24% vs. 9%).

Pyrazinamide

PZA is a nicotinamide analog and was recognized for its anti-TB activity in 1952. The drug is active only under an acid environment, but has a high MIC of 50-100 μg/ml even at pH 5.5-6.0 *in vitro*. However, PZA showed high sterilizing activity *in vivo*. Unlike other anti-TB drugs, PZA can kill non-growing persister *M. tuberculosis* more efficiently than actively growing bacilli. The mode of action of PZA has not been clearly understood, but it has been proposed that PZA is a pro-drug and is converted to an active form, pyrazinoic acid (POA), by the pyrazinamidase (PZase)/nicotinamidase enzyme encoded by the *M. tuberculosis pncA*. Once converted, POA exits the cell through passive diffusion and a deficient efflux mechanism of *M. tuberculosis*. Under the acid pH, a small portion of POA outside the cell will become uncharged protonated acid HPOA, which goes back through the membrane into the cell. With the less effective POA efflux than HPOA influx, the HPOA accumulates inside the cell, disrupts the membrane potential, causes cytoplasmic acidification and finally inhibits the major cellular processes[6].

No specific target of PZA has been identified to date. PZA-resistance is usually caused by mutations of the *pncA*, resulting in the loss of PZase activity. Distribution of *pncA* mutations is highly diverse and scattered, but there are three regions of clustered mutations around amino acids 3-17, 61-85, and 132-142[7]. Although 72-97% of PZA-resistant *M. tuberculosis* clinical isolates result from the mutations of the *pncA*, some resistant isolates do not contain such mutations, indicating the undefined resistant mechanisms. There has been very limited information about PZA resistance in Thailand. Only two studies on PZA susceptibility among Thai *M. tuberculosis* isolates have been reported, and the results revealed that the initial PZA resistance was 5.95% and 7.8%, when detected by the PZase assay[8] and by BACTEC 460 TB[9], respectively. Recent study with 100 Thai MDR-TB clinical isolates revealed a strong association of PZA resistance with MDR-TB[10]. PZA-resistant *M. tuberculosis* clinical isolates were found in 49% of MDR-TB. Analysis of the *pncA* sequence revealed that 38 (77.6%) out of 49 PZA-resistant isolates contain mutations in the gene. Twenty-two of 24 mutation types associated with PZA resistance were detected in this study

TABLE 2. Distribution of known mutations among 156 of 160 INH-resistant *M. tuberculosis* Thai isolates[5].

Mutated gene	No. of isolates (%)	
	MDR-TB (n=110)	Non MDR-TB (n=50)
katG	**95 (86.4)**	**34 (68)**
Ser315Thr	9 (8.2)	3 (6)
Ser315Thr / Arg463Leu	83 (75.5)	29 (58)
Ser315Asn / Arg463Leu	1 (0.9)	-
Trp328Ser / Arg463Leu	1 (0.9)	-
Ser315Thr / Arg463Leu / Val248Ile	-	1 (2)
Ser315Thr / Arg463Leu / Ala424Gly	-	1 (2)
Gln352stop	1 (0.9)	-
inhA	**10 (9)**	**12 (24)**
T(-8)C	-	1 (2)
C(-15)T	2 (1.8)	11 (22)
C(-15)T / Ile21Asn	1 (0.9)	-
C(-15)T / Ile21Val	1 (0.9)	-
C(-15)T / Gly40Trp	1 (0.9)	-
C(-15)T / Ser94Ala	1 (0.9)	-
Leu11Val	1 (0.9)	-
Ser94Ala	2 (1.8)	-
Ser94Trp	1 (0.9)	-
***oxyR-ahpC* intergenic region**	**2 (1.8)**	**2 (1.8)**
G(-9)A	1 (0.9)	-
C(-10)A	1 (0.9)	1 (0.9)
C(-10)T	-	1 (0.9)
ndh	**-**	**1 (0.9)**
Thr424Ile	-	1 (0.9)

TABLE 3. Results of *pncA* gene sequencing of 100 MDR-TB Thai isolates[10].

pncA mutation	PZA susceptibility	No. of isolates
No mutation	S	42
Ile31Thr	S	9
No mutation	R	11
A(-11)G	R	1
A(-11)C	R	1
Leu19Arg	R	1
Leu27Pro	R	1
Ile31Ser	R	3
Ile31Thr	R	3
Leu35Pro	R	1
Val45Ala	R	1
Ala46Ser	R	1
Ser67Pro	R	1
His71Asp	R	8
Cys72Tyr	R	1
Gly97Ser	R	3
Ser104Arg	R	2
Gly108Arg	R	1
Val125Phe	R	1
Glu127stop	R	1
Gly132Ser	R	1
G insertion b/w 411-412	R	1
Val139Gly	R	1
Thr142Met	R	1
Ala146Thr	R	1
GG insertion b/w 520-521	R	1
Thr177Ile	R	1

S; susceptible, R; resistant

(Table 3); Ile31Ser and Ile31Thr were also found in PZA susceptible isolates, indicating no association with PZA resistance. There were 5 novel mutation types, consisting of 2 nucleotide substitutions (Leu27Pro and Thr174Ile), 2 nucleotide insertions (G insertion between nucleotide 411 and 412 and GG insertion between nucleotide 520 and 521), and 1 nonsense mutation at Glu127. In agreement with earlier studies, the mutations were diverse and scattered throughout the gene, with the most frequently occurring mutation being His71Asp (8/49 = 16%).

SUMMARY

Almost all mechanisms of resistance to anti-TB drugs are associated with chromosomal mutations either of drug targets, like resistance to RIF, or of drug activating enzymes, such as resistance to INH and PZA. No plasmid-mediated drug resistance has been reported in *M. tuberculosis*. Knowledge of resistance mechanisms can assist not only the development of rapid diagnostic tests based on determining drug-resistant genes but also the idea for development of new anti-TB drugs. However, the prevalence of mutations has been shown to be different for some drugs depending on geographic areas and incidence of MDR-TB. Such differences should be considered for selecting the molecular-based susceptibility tests.

ACKNOWLEDGMENTS

The study was funded by the Drug-Resistant Tuberculosis Research Fund under the Patronage of pass HRH Princess Galyani Vadhana, KromLuang Narathiwas Rachnakarind, Siriraj Foundation, the Siriraj Grant for Research and Development, Faculty of Medicine Siriraj Hospital, and the Thailand Graduate Institute of Science and Technology (TGIST), National Science and Technology Development Agency, Thailand.

REFERENCES

1. World Health Organization. Global tuberculosis control: surveillance, planning, financing: WHO report 2009.
2. Ramaswamy S, Musser JM. Molecular genetic basis of antimicrobial agent resistance in Mycobacterium tuberculosis: 1998 update. Tuber Lung Dis. 1998;79(1):3-29.
3. Prammananan T, Cheunoy W, Taechamahapun D, Yorsangsukkamol J, Phunpruch S, Phdarat P, Leechawengwongs M, Chaiprasert A. Distribution of rpoB mutations among multidrug-resistant Mycobacterium tuberculosis (MDRTB) strains from Thailand and development of a rapid method for mutation detection. Clin Microbiol Infect. 2008 May;14(5):446-53.
4. Timmins GS, Deretic V. Mechanisms of action of isoniazid. Mol Microbiol. 2006 Dec;62(5):1220-7.
5. Boonaiam S, Chaiprasert A, Prammananan T, Leechawengwongs M. Genotypic analysis of genes associated with isoniazid and ethionamide resistance in MDR-TB isolates from Thailand. Clin Microbiol Infect. 2010 Apr;16(4):396-9
6. Zhang Y, Mitchison D. The curious characteristics of pyrazinamide: a review. Int J Tuberc Lung Dis. 2003 Jan;7(1):6-21.
7. Lemaitre N, Sougakoff W, Truffot-Pernot C, Jarlier V. Characterization of new mutations in pyrazinamide-resistant strains of Mycobacterium tuber culosis and identification of conserved regions important for the catalytic activity of pyrazinamidase Pnc. Antimicrob Agents Chemother. 1999 Jul;43(7):1761-3.
8. Rienthong S, Rienthong D, Smithikarn S, Yamnimnual S. Study of initial drug resistance of pyrazinamide in new pulmonary tuberculosis patients before treatment in tuberculosis division by detection of enzyme pyrazinamidase. Thai J Tuberc Chest Dis. 1993; 14: 85-89.
9. Pransucharit V, Chuchottaworn C. Detection of initial drug resistance of pyrazinamide in Thai tuberculosis patients by radiometric method. Thai J Tuberc Chest Dis. 1995; 16: 59-65.
10. Jonmalung J, Prammananan T, Leechawengwongs M, Chaiprasert A. Surveillance of pyrazinamide susceptibility among multidrug-resistant Mycobacterium tuberculosis isolates from Siriraj Hospital, Thailand. BMC Microbiol. 2010 Aug 20;10:223.