MULTIPLE MUTATIONS IN *KATG* AND *INHA* IDENTIFIED IN THAI ISONIAZID-RESISTANT *MYCOBACTERIUM TUBERCULOSIS* ISOLATES

Dhruba Kumar Khadka[1], Boonchuay Eampokalap[2], Jarinee Panitchakorn[1], Pongrama Ramasoota[3] and Srisin Khusmith[1]

[1]Department of Microbiology and Immunology, [2]Department of Social and Environmental Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok; [3]Department of Microbiology, Bamrasnaradura Infectious Disease Institute, Ministry of Public Health, Nonthaburi, Thailand

Abstract. A total of 29 Thai multi-drug-resistant/isoniazid-resistant *Mycobacterium tuberculosis* isolates were analyzed for mutations in *katG* from codons 254 to 549, *inhA* promoter and *inhA* open reading frame by DNA sequencing and single strand conformation polymorphism. Twenty-five multi-drug resistant isolates exhibited single point mutations (17 isolates at Ser315Thr plus Arg463Leu, 1 at Thr308Pro plus Arg463Leu, 7 at either Ser315Thr or Arg463Leu) while the other 4 isoniazid-resistant isolates had single point mutation only at Arg463Leu. Seven of 25 multi-drug-resistant isolates [4 at C(-15)T, 1 at T(-8)C; 1 at C(-15)T plus Ser94Ala and 1 at Ile21Val] and 2 of 4 isoniazid-resistant isolates [1 at C(-15)T, 1 at C (-15)T plus Ile21Thr] had mutations in *inhA* promoter and open reading frame, while the other 20 isolates had no mutation at any position. No frame shift mutation was observed in any tested isolates. This is the first report of two mutations, Trp308Pro of *katG* and T (-8)C of *inhA* in *Mycobacterium tuberculosis* isolates.

INTRODUCTION

Isoniazid (INH) together with rifampin (RIF) form the cornerstone of short course chemotherapy for tuberculosis and resistance to either drug hampers the complete cure of patients. In Thailand, a nationwide survey of drug resistant *Mycobacterium tuberculosis* strains revealed that 10-12% of the isolates are resistant to INH while 5% of the isolates are resistant to RIF. Among these, more than 95% RIF-resistant isolates are associated with mutations of *rpoB* (Paca-uccralertkun and Chuchottaworn, 1997).

The determination of drug resistance in *M. tuberculosis* using conventional culture methods routinely takes 6 to 10 weeks. In order to shorten this period, targeted molecular approaches have been developed. Genomic mutations frequently associated with resistance to each of the primary anti-tuberculosis drugs have been identified. Resistance to INH is associated with a variety of mutations that effect several genes including those encoding catalase-peroxidase *(katG)* that converts INH to an active form and enoyl-acyl carrier protein reductase (enoyl-ACP-reductase) (*inhA*) that is involved in mycolic acid biosynthesis in mycobacterial cell wall formation (Haas *et al*, 1997). Enoyl-ACP-reductase binds nicotinamide adenine dinucleotide to which catalase peroxidase activated INH forms a complex and allowing biosynthesis of mycolic acid to be inhibited (Whitney and Wainberg, 2002). *KatG* is the most commonly altered, with the majority of mutations occurring in

Correspondence: Prof Srisin Khusmith, Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, 420/6 Ratchawithi Road, Bangkok 10400, Thailand.
Tel: 66 (0) 2354-9100-2, Fax: 66 (0) 2643-5583
E-mail: tmskm@mahidol.ac.th

codon 315 (Musser *et al*, 1996; Haas *et al*, 1997). Mutations in the *katG* codon 315 and the promotor region of *inhA* have been identified in INH resistant, but not INH susceptible *M. tuberculosis* isolates (Dobner *et al*, 1997; Kiepiela *et al*, 2000). In Thailand, only the alterations in *katG* gene have been reported in isonoazid resistant *M. tuberculosis* (INH^r-*Mtb*) comprising 26% base deletion, 26% base insertion and 47% point mutation (Pacauccralertkun and Chuchottaworn,1995; Imwithaya *et al*, 2001).

The purpose of this study was to identify mutation in *katG* and *inhA* in multidrug/INH resistant *M. tuberculosis* (MDR INH^r-*Mtb*) isolates from Thailand using DNA sequencing and PCR-single strand conformation polymorphism (PCR-SSCP).

MATERIALS AND METHODS

Mycobacterium tuberculosis strains

A total of 29 multidrug/INH resistant *M. tuberculosis* (MDR/INH^r-*Mtb*) (25 MDR and 4 INH^r) isolates identified by conventional culture method from Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok (10 isolates) and from Chest Disease Institute, Ministry of Public Health, Nonthaburi (19 isolates) were obtained with informed consent and approved by the Ethics Committee of the Faculty of Medicine, Siriraj Hospital and Ministry of Public Health. An isoniazid susceptible (INH^s) *M. tuberculosis* strain obtained from the Department of Microbiology, Faculty of Science, Mahidol University (H37Ra reference strain ATCC 25177) was used as control. The isolates were stored at -80ºC until use.

Polymerase chain reaction (PCR) amplification and sequencing

M. tuberculosis cultures were grown in Lowenstein-Jensen medium and one loopful was suspended in 0.5 ml of sterile distilled water and mixed briefly by vortexing. The cell suspension was then frozen at -80ºC for 30 minutes before incubation at 95ºC for 5 minutes. The steps of freezing and thawing were repeated 3 times. Mycobacterial cells suspended in lysis buffer (0.5% SDS, 20 mg/ml proteinase-K and Tris-EDTA, pH 8.0) were incubated at 37ºC overnight followed by sodium chloride and cetyl tri-methyl ammonium bromide (NaCl/CTAB pre-warmed at 65ºC for 5 minutes) treatment. The suspension was incubated at 65ºC for 10 minutes and centrifuged at 5,000*g* for 5 minutes after adding chloroform/isoamyl alcohol (24:1). DNA was purified by phenol:chloroform extraction and 95% ethanol precipitation and dissolved in 50 µl of sterile distilled water (stock DNA) and the concentration was measured at 260 nm (1 A_{260} = 50 ng/µl) using spectrophotometer (UV-160A, SHIMAZDU, Japan). The purity of DNA was determined by A_{260}/A_{280} (optical density of DNA measured at 260 and 280 nm).

Primer pair sequences were designed on the basis of the published *katG* 894 bp sequence (*katG*- for 5′-TTTCGGCGCATGGCCA TGA-3′ and *katG*-Rev 5′-ACAGCCACCGA GCACGAC-3′) *katG* (Haas *et al*, 1997) and *inhA* sequences (Kiepiela *et al*, 2000; Morlock *et al*, 2003) to amplify the 248 bp promoter region (*inhA*-1 for 5′-CCTCGCTGCCCAGAAA GGGA-3′ and *inhA*-1Rev 5′-ATCCCCCGGTTT CCTCCGGT-3') and 516 bp ORF (*inhA*-2 for 5′-AGGTCGCCGGGGTGGTCAGC-3′ and *inhA*-2 Rev 5′-AGCGCCTTGGCCATCG AAGCA-3′). Aliquots of purified DNA from MDR/INH^r-*Mtb* and INH^s-*Mtb* isolates were amplified in a final volume of 75 µl reaction mixture containing 1x *Taq* PCR buffer [(75mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20)], 2.5 mM of $MgCl_2$ for *katG* and *inhA*II and 7.5 mM of $MgCl_2$ for *inhA* I, 0.5 µM of each primer, 0.4 mM of each of the four deoxynucleoside triphosphates and 1 U of *Taq* polymerase (Fermentas, Life Sciences, USA) using DNA thermocycler (Perkin-Elmer 2400, Gene Amp PCR System, USA). The PCR (6 steps) was carried out at 95ºC for 5 minutes

of initial denaturation and enzyme activation, then at 95ºC, 64ºC and 72ºC (30 seconds each) for 2 cycles; 95ºC, 63ºC and 72ºC (30 seconds each) for 2 cycles; 95ºC, 62ºC and 72ºC (30 seconds each) for 2 cycles; 95ºC, 61ºC and 72ºC (30 seconds each) for 2 cycles; 95ºC, 60ºC and 72ºC (30 seconds each) for 30 cycles; a final 7 minutes extension at 72ºC for 1 cycle. The PCR started with a high annealing temperature for the first primer-annealing step and then the annealing temperature was reduced by 1ºC for each later cycle in order to reduce nonspecific amplification and to improve specificity and product yield (Hecker and Roux, 1996). The amplicons were stained with Gel Star [4:96 Gel Star (BMA, Rockland, ME, USA): DMSO] followed by addition of loading dye (0.09% bromophenol blue, 0.09% xylene cyanol FF, 60% glycerol and 60mM EDTA) and were separated by 1.5% agarose gel electrophoresis (SeaKem® LE agarose, Rockland, ME, USA). Any sample showing negative result was repeated at least twice. The amplicons for DNA sequencing were purified to remove unincorporated nucleotides and primers by filtration with NucleoSpin® Extract II kit (MACHEREY-NAGEL GmbH & Co. KG, Germany). The purified PCR products and forward primers (*katG, inhA* 1and *inhA* 2) were sent to Macrogen (World Meridian Center, Kumchun-ku Seoul, Korea) for DNA sequencing.

Single strand conformation polymorphism (SSCP) analysis

PCR-SSCP was performed according to the method described previously with some modification (Bassam *et al,* 1991). In brief, 8 µl of each purified PCR product was mixed with 2 volumes of formamide loading dye (95% formamide, 0.5 M EDTA, 0.05% bromophenol blue and 0.05% xylene-cyanol FF) followed by denaturation at 95ºC for 15 minutes and immediately cooled on dry ice. Electrophoresis was then carried out in 10% polyacrylamide gel (29:1 polyacrylamide:bisacrylamide gel) in 0.5% Tris-borate EDTA buffer (53 mM Tris, 53 mM boric acid, 1.5 mM disodium-EDTA) at 4ºC. The electrophoresis was conducted at 40 mA for 2.5 hours for 248 bp of *inhA* promoter fragment and 8 and 9 hours for *katG* and *inhA*-ORF, respectively. Gels were fixed with 10% ethanol and 160 mM HNO_3, rinsed twice with distilled water and followed by silver staining based on the protocol of manufacturer (AmpliFLO™ D1S80 PCR Amplification Kit, Perkin-Elmer, USA). The gel was stained with 0.2% $AgNO_3$ for 20 minutes and followed by chilled developer (3% sodium carbonate, 0.05% formaldehyde). The reaction was stopped by addition of 10% glacial acetic acid and the gel was dried after rinsing twice with distilled water.

Statistical analysis

Mutations in *katG* and *inhA* genes were analysed by Fisher's exact test using Epi Info version 6 (EPI6.EXE) software, with $p < 0.05$ considered as statistically significant.

RESULTS

Amplification of all 29 multidrug/isoniazid resistant *M. tuberculosis* (MDR/INH^r-*Mtb*) by PCR using three different pairs of primers revealed that each pair of primer produced the expected PCR products of 894 bp of *katG*, 248 bp of *inhAI* and 516 bp of *inHII* (data not shown).

When the DNA sequences of all the 29 MDR/INH^r-*Mtb* isolates were compared with the INH^s- *Mtb* sequence of H37Rv (Gen Bank Accession *X68081)* for *katG* and CDC1551 (Gen Bank Accession *AE000516)* for *inhA*, 25 MDR-*Mtb* isolates exhibited single point mutations, of which 17 isolates had mutation at position Ser315Thr plus Arg463Leu, 1 isolate at position Thr308Pro plus Arg463Leu, 6 isolates at position Arg463Leu, and 1 isolate at position Ser315Thr, whereas all 4 INH^r-*Mtb* isolates had mutation at position Arg463Leu only (Table 1). The frequency of *katG* muta-

Table 1

Mutations in *katG* and *inhA* of 29 MDR/INH^r-*Mtb* isolates from tuberculosis patients in Thailand.

Isolate	Drug resistance	Position of mutation	
		katG	*inhA*
1(CCI)	HRSKO	Ser315Thr (AGC-ACC) Arg463Leu (CGG-CTG)	WT
2(CCI)	HRSO	Ser315Thr (AGC-ACC) Arg463Leu (CGG-CTG)	WT
3(CCI)	HRSO	Ser315Thr (AGC-ACC) Arg463Leu (CGG-CTG)	WT
4(CCI)	HRSO	Ser315Thr (AGC-ACC) Arg463Leu (CGG-CTG)	WT
5(CCI)	HO	Arg463Leu (CGG-CTG)	WT
6(CCI)	HRO	Ser315Thr (AGC-ACC) Arg463Leu (CGG-CTG)	C(-15)T
7(CCI)	HRO	Ser315Thr (AGC-ACC) Arg463Leu (CGG-CTG)	WT
8(CCI)	HRSEO	Ser315Thr (AGC-ACC) Arg463Leu (CGG-CTG)	WT
9(CCI)	HRSO	Ser315Thr (AGC-ACC) Arg463Leu (CGG-CTG)	WT
10(CCI)	HRSO	Ser315Thr (AGC-ACC) Arg463Leu (CGG-CTG)	T(-8)C
11(CCI)	HRSEO	Ser315Thr (AGC-ACC) Arg463Leu (CGG-CTG)	WT
12(CCI)	HRSO	Ser315Thr (AGC-ACC) Arg463Leu (CGG-CTG)	WT
13(CCI)	HRSO	Ser315Thr (AGC-ACC) Arg463Leu (CGG-CTG)	WT
14(CCI)	HRSEO	Arg463Leu (CGG-CTG)	WT
15(CCI)	HRSKO	Ser315Thr (AGC-ACC) Arg463Leu (CGG-CTG)	WT
16(CCI)	HO	Arg463Leu (CGG-CTG)	WT
17(CCI)	HRSKO	Ser315Thr (AGC-ACC) Arg463Leu (CGG-CTG)	Ile21Val (ATC-GTC)
18(CCI)	HRO	Ser315Thr (AGC-ACC) Arg463Leu (CGG-CTG)	WT
19(CCI)	HSO	Arg463Leu (CGG-CTG)	C(-15)T
20(SIMI)	HRSO	Arg463Leu (CGG-CTG)	WT
21(SIMI)	HRSO	Arg463Leu (CGG-CTG)	WT
22(SIMI)	HRSEO	Thr308Pro (ACC-CCC) Arg463Leu (CGG-CTG)	C(-15)T
23(SIMI)	HRSEO	Ser315Thr (AGC-ACC) Arg463Leu (CGG-CTG)	WT
24(SIMI)	HRSEO	Ser315Thr (AGC-ACC) Arg463Leu (CGG-CTG)	WT
25(SIMI)	HRSEO	Ser315Thr (AGC-ACC)	WT
26(SIMI)	HRSEO	Arg463Leu (CGG-CTG)	C(-15)T
27(SIMI)	HO	Arg463Leu (CGG-CTG)	C(-15)T, Ile21Thr (ATC-ACC)
28(SIMI)	HRSEO	Arg463Leu (CGG-CTG)	C(-15)T, Ser94Ala(TCG-GCG)
29(SIMI)	HREO	Arg463Leu (CGG-CTG)	C(-15)T

CCI = Central Chest Disease Institute, Ministry of Public Health; SIMI = Siriraj Hospital, Department of Microbiology, Faculty of Science, Mahidol University; H = Isoniazid, R = Rifampicin, S = Streptomycin, E = Ethambutol, K = Kanamycin, O = Ofloxacin; WT = Wild type

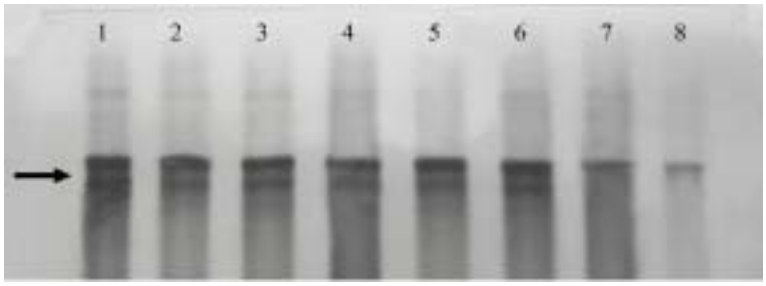

Fig 1–PCR-SSCP pattern of 248 bp fragment of *inhA* promoter region of MDR/INH^r-*Mtb* isolates. The single point mutation was detected by single strand conformation polymorphism (SSCP). The differentiation of mutant and wild type PCR product was observed based on the varied migration or SSCP patterns of ssDNA on polyacrylamide gel electrophoresis (PAGE). Lanes 1-6, 248 bp fragment of *inhA* promoter containing single nucleotide transition C(–15)T of presumed ribosome binding site; lane 7, PCR product of MDR/INH^r-*Mtb* isolate without mutation; lane 8, PCR product of INH^s-*Mtb* isolate without mutation.

tions at two positions among MDR-*Mtb* isolates was significantly higher than the mutation at one position in INH^r-*Mtb* isolates ($p < 0.05$). Seven of 25 MDR-*Mtb* isolates (4 at position C(-15)T, 1 at position T(-8)C, 1 at position C(-15)T plus Ser94Ala and 1 at position Ile21Val and 2 of 4 INH^r-*Mtb* isolates (1 at position C(-15)T, 1 at position C(-15)T plus Ile21Thr) had mutations either in one or both positions of *inhA* promoter and *inhA* open reading frame (Table 1). The other 20 isolates had no mutation at any position.

PCR-SSCP patterns of 248 bp fragment of *inhA* promoter containing single nucleotide transition C(–15)T from 6 MDR/INH^r-*Mtb* isolates were clearly different from those of control strain and INH^r-*Mtb* isolate without mutation at this position (Fig 1) However, the MDR/INH^r-*Mtb* isolates with mutations at various positions of *katG* and *inhA* as well as H37Ra control strain did not show any bands in SSCP gel.

DISCUSSION

Mycobacterial *katG* and *inhA* genes play crucial roles in activation of INH leading to drug resistance. Gene sequencing analysis of INH^r-*Mtb* isolates is a gold standard for identifying the root cause of drug resistance. In the present study, point mutations were identified in *katG* and *inhA* by DNA sequencing in 29 Thai MDR/INH^r-*Mtb* isolates. The frequency and pattern of *katG* mutations at Ser315Thr and Arg463Leu either singly or combined were similar among Thai MDR/INH^r-*Mtb* strains and those observed in 27% of 41 American, Russian and Brazilian strains (Morlock *et al*, 2003) and 59% of 71 Korean strains (Kim *et al*, 2003). However, a previous study in Thailand (Paca-uccaralertkun and Chuchotoaworn, 1997) reported *katG* gene mutations in 19 INH^r-*Mtb* Thai isolates having 26% base deletions, 26% base insertion and 47% single pair mutations. But a subsequent report revealed that 6 (55%) of 11 Thai INH^r-*Mtb* isolates have *katG* mutation at position 315 (Imwithaya *et al*, 2001).

Similarly, the frequency and patterns of *inhA* mutations at upstream position C(-15)T, either singly or combined with point mutations in *inhA*-ORF (Ile21Val/Thr, Ser94Ala) in 29 Thai MDR/INH^r-*Mtb* strains were also similar to that of 24% of 71 Korean strains (Kim *et al*, 2003) and 16% of 104 Lithuanian strains (Bakonyte *et al*, 2003). Out of 41 American, Russian and Brazilian strains 66% had base substitution at C(-15)T and 22% had point mutations in *inhA*-ORF (5 at Ile21Val/Thr, 3 at Ser94Ala 1 at Leu44Leu,). No frame shift mutations in *inhA* were found in Thai MDR/INH^r-*Mtb* strains.

SSCP patterns of 6 MDR/INH^r-*Mtb* isolates using 248 bp fragment of *inhA* promoter having single nucleotide transition C(–15)T was informative, whereas 894 bp *katG* and 516 bp *inhA*-ORF as well as H37Ra control strain

showed no bands on SSCP gel. At least 80% to 90% of all point mutations are detectable when fragments are maintained at approximately 200 bp or less than 400 bp (Hayashi, 1992). Longer DNA fragment should be trimmed down by the cleavage using specific restriction enzymes.

The *katG* and *inhA* gene mutations identified in 29 Thai MDR/INH^r-*Mtb* strains was associated with isoniazid resistance, due to mutations of *katG* at Ser315Thr, and Arg463Leu and single nucleotide substitution at upstream position C(-15)T of *inhA* promoter region. However, mutations in genes other than *katG* and *inhA, eg* ketoacyl acyl carrier protein synsthase (*kasA*), alkyl-hydroperoxide reductase (*ahpC*), NADH dehydroenase (*ndh*), have been reported (Ramaswamy and Musser, 1998; Lee *et al*, 2001). Since the present study has been conducted on only 29 isolates, the results might not represent the status of isoniazid resistance in all Thai stains. Further study on larger samples collected from different geographical regions in the country should be continued. Although the present study involved only a small sample size, the results obtained from the specific gene alterations (*katG* and *inhA*) could be a model to predict the status of isoniazid resistance in Thai *M. tuberculosis* isolates. The *katG* mutation at Ser315Thr plus Arg463Leu in the same isolate (17 of 29 isolates) is new among Thai MDR/INH^r-*Mtb* isolates. There could be several factors influencing such results, of which a noteworthy fact is that the majority of these isolates were collected from MDR or chronic tuberculosis patients. The longer *M. tuberculosis* remains in a treated patient, the greater number of gene mutations can be accumulated for drug resistance (Ramasoota, 2000). Furthermore, this is the first time that the two mutations, Trp308Pro of *katG* and T(-8)C of *inhA* promoter, were identified in MDR/INH^r-*Mtb* isolates. Identifying molecular mutation of target genes will be a great help in manufacturing more appropriate drugs. This in turn will be a part of an effective therapy management, improvement of patient's survival and reduction in disease transmission.

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