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katG Mutation of Isoniazid-Resistant Isolated from Tuberculosis Patients

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ABSTRACT

Background: The emergence of drug-resistant strains of *Mycobacterium tuberculosis* (MTB) is an increasing problem in developed and developing countries. The aims of the present study were to identify various types of mutations in *katG* region from 28 MDR strains isolated from sputum of tuberculosis patients.

Materials and Methods: Twenty-eight rifampin-resistant strains isolated from sputum of patients with active pulmonary tuberculosis were obtained from various geographic regions of Iran. Drug susceptibility was determined by using the BACTEC system. DNA extraction, standard PCR identification, *katG* gene amplification, DNA sequencing and analysis were done.

Results: There was no mutation in 2 strains. In 20 strains, mutation was shown to be in codon 315. Three types of mutations were detected consisting of AGC→ACC (Ser→Thr) (80%), AGC→AGG (Ser→Arg) (5%) and AGC→AAC(Ser→Asn) (15%). Furthermore, one type of mutation was seen in codons 311, 299, and 323. Twelve strains showed one mutation in codon 315 (46%), 7 strains 2 mutations (27%), 5 isolate 3 mutations (19%) and in 2 strains 4 mutations (8%) were observed in different codons. Nine silent mutations was demonstrated in codon 311(GAC→TAC).

Conclusion: This research demonstrated that mutations were mostly seen in codons 315 and 299 indicating resistance to isoniazide. (*Tanaffos* 2006; 5(1):31- 36)

Key words: Tuberculosis, *katG*, Mutation, MDR-TB

INTRODUCTION

The emergence of drug-resistant strains of *Mycobacterium tuberculosis* (MTB) is an increasing

problem in developed and developing countries (1, 2, 3). Rifampicine (RIF) and isoniazid (INH) are important chemotherapeutic agents for treatment of multidrug resistant *M. tuberculosis* infection. Several studies have evaluated genomic region of MTB

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involved in development of resistance to isoniazid. In the United States, about 13% of isolates from new tuberculosis cases are resistant to one or more of the first-line anti-tuberculosis drugs, and 1.6% of cases are resistant to both isoniazid and rifampicine, defined as multi-drug resistant tuberculosis (4, 5, 6, 7). Since its discovery five decades ago (8, 9, 10, 11), isoniazid has been commonly used to treat and prevent tuberculosis. Despite its importance, only recently its insight detail has been described with molecular mechanism of isoniazid action. It is now understood that isoniazid is a prodrug (12, 13) which is converted into a biologically active form by *M. tuberculosis* catalase-peroxidase, KatG (14, 15, 16, 17). Two enzymes involved in the biosynthesis of mycolic acids have been suggested to be the targets of KatG-activated isoniazid: the NADH-dependent enoyl-acyl carrier protein reductase (InhA) (15, 18) and the-ketoacyl-acyl carrier protein synthase (designated KasA) (6, 15, 19, 20). Resistance to isonizide in CDC1551 and H37RV strains, has been shown to correspond with catalyse peroxides enzyme in region of 2153889 to 2156111 (H37RV) 2151180 to 2153402 (CDC 1551) and Catalase peroxides T in 75186 to 77408 DNA molecule (Blast/pubMed-Gene bank).

The aims of the present study, were to identify various type of mutations in *katG* region from 28 MDR strains isolated from sputum of tuberculosis patients in the southern endemic region of Iran (Afghanistan border, Zabol).

MATERIALS AND METHODS

Mycobacterial strains and Susceptibility

From total 91 strains, 28 isoniazid-resistant strains were isolated from sputum of patients with active pulmonary tuberculosis, from July to September 2005. All strains were cultured on Lowenstein–

Jensen solid medium and identified to the species level using TCH (2-thiophene carboxylic acid) and PN99B (paranitrobenzoic acid) selective media or by standard biochemical procedures (21).

Drug susceptibility testing was performed by BACTEC system and CDC procedure (isoniazid 1 µg/mL, rifampin 40 µg/mL, streptomycin 10 µg/mL, or ethambutol 2 µg/mL) (21), using absolute concentration method on slants with H37RV strain of *M. tuberculosis* as positive control. Resistance was defined as growth on solid media containing graded concentrations of drugs with more than 20 CFU at a specific drug concentration. The breakpoints for INH were 1µg/ml on Lowenstein-Jensen medium and 0.1 µg/ml on the BACTEC system; for RIF, 40.0 µg/ml on Lowenstein-Jensen medium and 2.0 µg/ml on the BACTEC system.

Standard PCR identification and *katG* gene amplification.

DNA extraction was done by Fermentase kit (K512), and DNA purification by Fermentase kit (k513).

Twenty-nine isoniazid-resistant isolates were collected and DNA extraction was done (kit manufacture procedure). DNA isolated from *M. tuberculosis* CDC1551 and *Mycobacterium H37RV* strains was used as control. A 209 bp segment of the *katG* gene was amplified by PCR with the following synthetic oligonucleotide primers: *katG* F 5'-GAAACAGCGGCGCTGGATCGT-3, *katG* R 5'-GTTGTCCCATTTTCGTCGGGG-3. The following thermocycler parameters were used: initial denaturation at 94°C for 5 min; 42 cycles of denaturation at 94°C for 1 min; primer annealing at 57°C for 1 min; extension at 72°C for 1 min; and a final extension at 72°C for 10 min. The products were checked and purified on the gel electrophoresis (FIG. 1) and purified *katG* segment were amplified. The resultant DNA amplifications were used for sequencing.

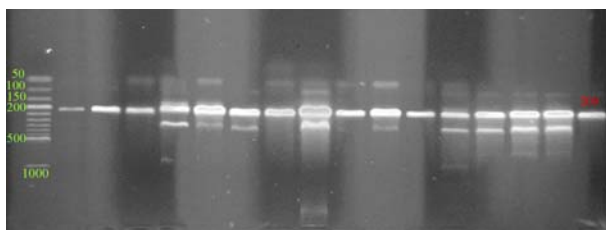


Figure 1. 209-bp fragment of the *katG* segment amplified for purification and sequencing.

| 309 | 311 | 314 | 315 | 316 | 322 | 324 | 328 |
|---|-----|-----|-----|-----|-----|-----|-----|
| AAG GAC GCG ATC ACC AGC GGC ATC GAG GTC GTA TGG ACG AAC ACC CCG ACG AAA TGG GGT | | | | | | | |
| GTT | TAC | | ACC | | ATC | GCG | |
| | | | AGC | | | | |
| | | | AAC | | | | |

Figure 2. Location, type, frequency of mutations in *kat G* gene in region of 309-328 bp (29 strains of *M. tuberculosis*)

DNA sequencing

A 209-bp fragment of *katG* gene was amplified by PCR using primers *katG* R 5'-GTTGTCCCATTTTCGTCGGGG-3'. Polymerase chain reaction (PCR) was carried out in 50 µl containing 2 µl KCl, 2 µl Tris (pH 8.0), 1.5 µl MgCl₂, 5 µl dNTP, 1U *Taq* polymerase, 27 µl water (molecular grade), 20 pmol of each primer and 6-10 µl of DNA template. For sequencing, the same primers with PCR parameters were used; 33 cycles of denaturation at 94°C for 30min; primer annealing at 48°C for 45 sec; and extension at 60°C for 4 min. The *katG* gene fragments of tuberculosis strains were sequenced using the Amersham auto sequencer and Amersham Pharmacia DYEnamic ET Terminator Cycle Sequencing Premix Kits.

Analyzing of DNA sequencing

Alignment of the DNA fragments (*katG*) was carried out with the help of MEGA and DNAMAN software (Blast/PubMed-Gene bank) and the samples were compared with standard strains of CDC 1551, H37RV and *M. tuberculosis* 210.

RESULTS

Mycobacterial strains and susceptibility

All 28 samples were cultured and identified as *M. tuberculosis* by PCR method. 48 mutations were detected in all stains.

PCR and DNA sequencing analysis

From 91 strains, 4 isolates were identified as Non-Tuberculous Mycobacteria (NTM) indicating different pattern in PCR and sequencing. From 28 INH-r, 2 strains showed no mutation and in 20 strains mutations were observed in codon 315, revealing three types of mutations consisting of AGC→ACC (Ser→Thr) (80%), AGC→AGG (Ser→Arg) (5%) and AGC→AAC (Ser→Asn) (15%). One type of mutation detected in codon 299 indicating GGC→AGC and changes in amino acid Gly → Ser. In codon 311 (*katG*) only one base change was obtained Tyr → Tyr (GAC → TAC) in nine strains demonstrating a none sense mutation. Furthermore, only one mutation was observed in codons 311, 299 and 322, and in 12 strains one mutation in codon 315 (46%), in 7 strains 2 mutations (27%), in 5 strains 3 mutations (19%) and in 2 isolates 4 mutations (8%) were obtained respectively. No mutations were demonstrated in two *M. tuberculosis* INH-r resistant strains.

DISCUSSION

Most of hot mutations of *M. tuberculosis* have been reported in codon 463 (CGG→CTG) and 315 (Thr→ser) and a fewer number were observed in other codons (1, 9, 10, 17, 22). In this research most mutations in 209 bp fragment were observed in codon 315, indicating three types of mutations AGC→ACC, AGC→AGG, AGC→AAC in 20 strains, which reflect 75% of all Iranian isolates. A fewer number of mutations were detected in codon 299, 311, and 322 (table 1 and 2).

Table 1. DNA sequencing data for *katG* mutations in INH-r *M. tuberculosis* strains from Iran

| Codon | Nucleotide changes(Number) | Aminoacid changes | No. of strains with mutation |
|-----------|----------------------------|-------------------|------------------------------|
| 315(n=20) | AGC→ACC | Ser→Thr | 16 |
| | AGC→AGG | Ser→Arg | 1 |
| | AGC→AAC | Ser→Asn | 3 |
| 299(n=8) | GGC→AGC(n=8) | Gly→ser | 8 |
| 311(n=9) | GAC→TAC (n=9) | Tyr→Tyr | 9 |
| 309(n=2) | GGT→GTT (n=2) | Gly→Val | 2 |
| 322(n=6) | AAC→ATC (n=6) | Asn→Ile | 6 |
| 324(n=3) | CCG→GCG (n=3) | Pro→Ala | 3 |

Table 2. Various number of mutations and related codons for *katG* mutations in INH-r *M. tuberculosis* strains from Iran

| Mutation | Many | Number codon | Many | Isolate number |
|-------------|------|-----------------|------|---|
| 1 Mutation | 12 | 315 | 12 | 3542-600-98(1384)-9-10-19-3548-441-48-3708-1619(mac)-29 |
| | | | | 633-12 |
| | | | | 663 |
| 2 Mutations | 7 | 315-309 | 2 | 108-303(281) |
| | | 315-323 | 1 | |
| | | 315-325 | 2 | |
| | | 299-311 | 1 | |
| | | 311-315 | 1 | |
| 3 Mutations | 5 | 315-299-311 | 1 | 159 |
| | | 311-299-323 | 3 | 163-167-165 |
| | | 299-311-325 | 1 | 161 |
| 4 Mutations | 2 | 299-311-315-323 | 1 | 290 |
| | | 299-311-323-315 | 1 | 710 |

Other authors reported following nucleotide changes in codon 315 in some other countries, consisting of (AGC→ACC, ACA, ACT, ATC,

AAC) (17, 22, 23, 24). In contrast, our data indicate higher nucleotide changes as AGC→ACC. Furthermore, we observed one rare mutation as AGC→AGG which has not yet been reported. Mutation in codon 311 (9 strains) revealed a silent mutation which has no effect on resistance. Nucleotide changes observed in codon 322 AAC→ATC (n=6) and 299 GGC→AGC (n=8) resulted in aminoacid changes as Asn→Ile and Gly→ser respectively, which also have not yet been reported and may play a role in emergence of resistance due to changes in cell wall or preplasmic protein. Our data reveal observation of two INH-r resistant strains with no mutation indicating phenotypic resistant. In addition to mutation in catalyse peroxides gene (*katG*) in the codon 315, 463 other mutations have also been reported in codon 279 (Poland) 88 and 155 (Russia) which have not been demonstrated in our study. This study also demonstrated that 65% of the 28 INH-r strains had similar genetic pattern (*katG*) with *Mycobacterium tuberculosis* 210(Beijing strain), when compared with standard strains H37RV, CDC1551 and 210. These results may indicate that different types of mutation observed in codons 315, 299 and 322 may reflect geographic and epidemiologic position in the southern endemic region of Iran (Afghanistan border, Zabol). Changes in codon 299 and 322 were also associated with INH-r resistance in Iran. Other studies showed that there were additional genes responsible for INH resistance, such as *inhA*, *ahpC*, *oxyR* and *kasA* genes (6, 25), that had not been studied in this research. Further investigation is needed to find out whether any changes in other genes may affect INH resistance.

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