

Isoniazid MIC and *KatG* Gene Mutations among *Mycobacterium tuberculosis* Isolates in Northwest of Iran

¹Seed Reza Moaddab, ^{*2,3,4}Safar Farajnia, ³Davood Kardan, ⁴Sajad Zamanlou, ⁵Mohammad Yousef Alikhani

Abstract

Objective(s)

Isoniazid (INH) is one of the main first line drugs used in treatment of tuberculosis and development of resistance against this compound can result in serious problems in treatment procedures. Resistance to INH is mediated mainly by mutation in *KatG* gene that is coded for the catalase enzyme. The proportional method for detection of INH-resistance is time consuming due to the slow growth rate of *Mycobacterium tuberculosis*. In this study, we used PCR-RFLP approach for screening of common mutations in *KatG* gene for detection of INH resistance, and compared the results to minimal inhibitory concentration (MIC) in *M. tuberculosis* isolates.

Materials and Methods

Fifty *M. tuberculosis* isolates were subjected to study of which, 25 strains were INH-resistant and 25 strains were INH-sensitive.

Results

Of 25 INH-resistant strains, the mutation was identified in 56% and 20% in the *KatG*315 and *KatG*463 loci, respectively. In 24% of INH-resistant strains, no mutation was observed in the studied loci. INH MIC was <0.2 µg/ml in all sensitive strains whereas among 25 INH -resistant isolates, INH MIC was higher than 0.2 µg/ml ranged from 0.2 to 3.2 µg/ml.

Conclusion

Our findings revealed that PCR-RFLP is capable to identify INH-resistance in more than 76% of INH-resistant *M. tuberculosis* strains, and could be used for rapid identification of INH resistance. High levels of INH MIC were observed in the strains which had mutation in the *KatG* gene in position 315.

Keywords: Isoniazid Resistance, *Kat G*, *Mycobacterium tuberculosis*, PCR, RFLP

1- Tuberculosis and Lung Research Centre and Paramedical Faculty, Tabriz University of Medical Sciences, Tabriz, Iran

2- Biotechnology Research Centre, Tabriz University of Medical Sciences, Tabriz, Iran

*Corresponding author: Tel: 09143018589; Fax: 04113363231; email: farajnia@gmail.com

3- Infectious and Tropical Disease Research Centre, Tabriz University of Medical Sciences, Tabriz, Iran

4- Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

5- Microbiology department, Faculty of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran

Introduction

Although tuberculosis is regarded as a historical disease, in the recent years, the medical community has increasingly worried about the spread of drug resistant *Mycobacterium tuberculosis* strains. Isoniazid (INH) and rifampin are two first line drugs against tuberculosis and strains display resistance against these drugs are considered as multi drug Resistant (MDR) *M. tuberculosis* (1-3). The control program of tuberculosis has been affected seriously by the spread of MDR strains and so, rapid identification of resistant strains has a special impact on the treatment and control of dissemination of MDR strains in the communities (4, 5). INH is a pro-drug that enters into the bacillus via passive diffusion, changes into an active form by enzyme catalase-peroxidase, and then the free radicals attack different targets in the microorganism. The previous observations have showed that tuberculosis bacillus lose their acid fastness property after exposure to INH (6- 9).

Drug resistance in bacterial population takes place randomly by mutations. Resistance to INH is associated with a variety of mutations in several genes like *KatG*, *inhA*, *oxyR-aphC*, *kasA* and *ndh* (4, 8, 10). The *KatG* gene encodes enzyme catalase-peroxidase and mutation in this gene decreases or blocks the enzyme activity. Mutation in the *katG* gene is the main mechanism of INH-resistance in most strains (11).

There are various methods for detection of INH-resistant *M. tuberculosis* strains including conventional and molecular methods. The conventional methods based on culture and proportional assay of the bacterial sensitivity are time consuming and need several weeks for bacterial growth. In the recent years, rapid molecular techniques such as PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) have been proposed for detection of resistance to INH. This method reduces the time of diagnosis to few hours (1, 3, 4, 11). Since the frequency and type of mutations in the *KatG* gene are

varied in different geographic regions, this study aimed to determine the prevalence of mutations in the *KatG* gene and INH minimal inhibition concentration (MIC) in *M. tuberculosis* strains in Northwest of Iran.

Materials and Methods

The bacteria included in this study were 50 isolates of *M. tuberculosis* which were recovered from pulmonary tuberculosis patients. Out of 50 strains, 25 were susceptible and 25 were resistant to INH. Bacterial culture was accomplished in Lowenstein-Jensen (LJ) medium and the isolates were identified by standard microbiological methods such as Ziehl-Neelson staining, morphology of colony, pigment and niacin production, nitrate and catalase tests. For susceptibility testing the proportional method was employed (12-14). The MICs of INH (0.2, 0.4, 0.8, 1.6 and 3.2 µg/ml) was determined on LJ medium for INH- resistant strains. For all experiments, *M. tuberculosis* HRV37 was used as standard.

DNA Extraction

For bacterial DNA extraction, the grown colonies of *M. tuberculosis* on LJ medium was resuspended in 300 µl TE buffer (Tris 10 mM, pH 8; EDTA 1 mM) and inactivated for 40 minutes at 80 °C. Then the DNA of isolates was extracted by SDS and proteinase-K method (4, 15).

PCR-RFLP

The coding region of *KatG* gene was amplified by primers *KatG* 904: 5'-AGCTCGTATGGC ACCGGAAC3' and *KatG* 1523: 5'-TTGA CCTCCCACCCGACTTG3' as described previously (16). The PCR reactions was prepared in a 25 µl volume included 10 pM of primers (1 µl of each forward and reverse primers), 2 mM MgCl₂ (1.5 µl), 0.2 mM dNTPs (0.5 µl) and 2 unit (0.25 µl) of Taq DNA polymerase (Fermentas). The PCR program was initial denaturation at 94 °C for 4 minutes followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 63 °C for 1min and extension at 72 °C for 1 min with a

final extension at 72 °C for 5 min. In order to characterize the mutations in the *KatG* gene, the PCR products were purified with PCR product purification columns (Qiagen, Germany) and digested by *MspI* restriction enzyme (Fermentas). Finally, the reaction mixture was electrophoresed on 1% agarose gel and visualized on UV light after staining with ethidium bromide (16, 17).

Results

Totally, 50 *M. tuberculosis* strains were isolated from pulmonary tuberculosis patients among which 28 (56%) were males and 22 (44%) were females. The age range of patients was 23 to 63 years and the median age was 42.92 years. Out of 50 *M. tuberculosis* strains, 25 strains were resistant and 25 strains were found to be sensitive to INH by proportional method. We studied the INH MIC among 25 resistant isolates and we also aimed to determine the probability of relationship between MIC ranges and type of mutations in the *KatG* gene. According to the protocol of proportional method, strains with MIC 0.2 µg/ml and higher was considered as INH resistant. The results indicated that among 25 INH-resistant strains, the MICs were 0.4 µg/ml in one (4%) isolate, 0.8 µg/ml in one (4%) isolate, 1.6 µg/ml in 3 (12%) isolates and 3.2 µg/ml in 4 (16 %) isolates. In the remaining 16 (64%) resistant isolates, the MICs were 0.2 µg/ml. The MICs of *M. tuberculosis* strain HRV37 (standard strain) and all INH-sensitive isolates, were less than 0.2 µg/ml.

PCR amplification of *KatG* gene was positive in all isolates and resulted in a 620 bp product in agarose gel electrophoresis. After *MspI* digestion of *KatG* amplicon, four distinct RFLP patterns were observed (16) (Table and Figure 1).

Our findings showed that 25 INH-sensitive *M. tuberculosis* strains had no mutation in the *KatG* gene. Among 25 INH-resistant isolates, 14 strains (56%) showed mutation in the *KatG*315 and 5 strains (20%) showed mutation

in the *KatG* 463 loci. Six resistant isolates (24%) had no mutation in the studied loci. Mutation in both 315 and 463 codons were not found in any isolates. *M. tuberculosis* strain HRV37 susceptible to all anti-tuberculosis drugs was used for quality control. This strain was negative for mutation in the *KatG* gene in PCR-RFLP. For confirmation of RFLP results, the PCR products from 10 stains were submitted for sequencing (MWG, Germany). The sequencing results were in agreement with PCR-RFLP results for both INH-resistant and INH-sensitive strains (Table 2).

Table1. Predicted fragment sizes of *MspI* digest of *KatG* amplicone.

KatG mutation	DNA fragments (bp)			
Wild type	65	137	153	228
at codon 315 (S→T)	65	132	137	228
at codon 463 (R→L)			153	220 228
at codons 315 and 463		132		220 228

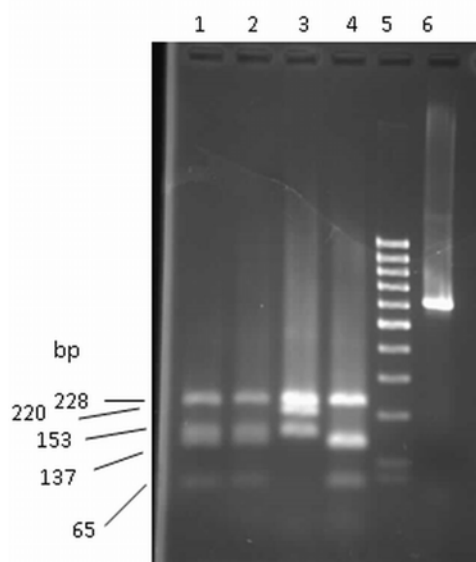


Figure 1. PCR RFLP pattern of INH-sensitive and INH-resistant *M. tuberculosis* isolates. Lanes 1 and 2, INH sensitive wild type; lane 3, R-463-L mutant; lane 4, S-315-T mutant isolates and lane 6, PCR amplified *KatG* gene before restriction digestion. Lane 5 is related a 100 bp DNA ladder.

Table 2. DNA fragments sizes of PCR products after digestion with MspI.

Mutation	No. of strains	Fragments (bp)						Observed phenotype
		65	132	137	153	220	228	
No mutation	31 (6 INH resistant, and 25 INH sensitive isolates)	+		+	+		+	Wild type
At codon 315	14	+	+	+			+	Type 1
At codon 463	5				+	+	+	Type 2

Discussion

The spread of INH and rifampin resistant *M. tuberculosis* strains, which are known as MDR strains, have caused a major health problem in global community (18). Rapid detection of MDR strains would help efficient treatment of patients and prevents widespread distribution of these isolates (1-5). This study aimed to survey the use of PCR-RFLP in comparison with proportional method for rapid detection of INH resistant *M. tuberculosis* strains.

The resistance to INH was screened by proportional method in LJ medium and then INH MIC was determined in INH-resistant isolates. Analysis of mutation in two *KatG* gene loci was carried out by PCR- RFLP. The results indicated that PCR-RFLP was able to detect INH- resistance in 76% of 25 resistant strains. Mutation was identified at codon 315 in 56% of strains whereas codon 463 contained mutation in 20% of resistant strains.

Twenty five INH- sensitive *M. tuberculosis* strains as well as standard strain (*M. tuberculosis* H37RV) showed no mutation in the studied codons. These findings are consistent with some reports about the rate of mutation in the *KatG* gene in other parts of Iran. Zaker Bostanabad *et al* (19) reported that 61% of INH-resistant *M. tuberculosis* isolates in the east region of Iran contained mutation in the *KatG* gene. In another study carried out on the strains of Isfahan and Tehran, the mutation rate was 78% for Isfahan and 61% for Tehran (20). In other countries, the results varied in different part of the world. For instance, in Turkey, Cavusoglu *et al* (21) reported the mutation rate of 73% and in another study taken place in east of Turkey; the rate of mutation in the *KatG* was reported as 63% (22). Among countries in the southern

boundary of Iran, the percentage of mutation in the *KatG* gene was reported 65% in Kuwait, 64% in Dubai, 35% in Beirut and 47% in Egypt (23-26). In the other countries, the mutations in the *KatG* have been reported as 98% in Russia, 84% in Lithuania, 71% in Vietnam, 65% in Australia, 60% in South Africa, 60% in China, 60% in Philippines, 59% in Spain, 46% in Switzerland, and 55% in India (1, 3, 21, 25, 26).

In the present study, 20% of INH- resistant strains had mutation in the *KatG* codon 463 and no simultaneous mutation was observed in either codons 315 and 463. Zhang *et al* (27) reported mutation at codon 463 in 40% of INH-resistant *M. tuberculosis* strains. In 24% of our INH-resistant strains (6 strains) no mutation was identified in the *KatG* gene in the study loci. In a study in India, in 27% of 120 INH-resistant strains, no mutation was detected in the *KatG* gene (4). These findings show that mutations in other codons of *KatG* gene or mutations in other genes such as *inhA*, *oxyR-aphC*, *kasA* and *ndh* might be involved in INH-resistance in these isolates (3, 4, 8, 19, 23, 25).

Analysis of INH MIC among 25 INH-resistant strains demonstrated that the MIC was 0.2 µg/ml in 16 strains, 0.4 µg/ml in one strain, 1.6 µg/ml in 3 strains, and 3.2 µg/ml in 4 strains. The results also indicated that the MIC of most strains with mutation at codon 315 was >2 µg/ml. These findings are consistent with reports on correlation of INH MIC and type of mutation in *KatG* gene (28- 31). It has been reported that the level of INH MIC is correlated to the type and extent of changes in the *KatG* gene. Hass *et al*, (28) reported INH MIC>50 µg/ml in the strains which have lost their *KatG* totally on their

chromosome whereas, in 83% of INH-resistant strains with mutation at codon 315, the INH MIC have been $>2 \mu\text{g/ml}$. Our findings in accordance with previous results showed that minor changes in the *KatG* gene were associated with decreased catalase activity and low INH MIC (28).

Conclusion

The result of this study revealed that mutation at codons 315 and 463 of *KatG* gene is

responsible for INH resistance in 76% of *M. tuberculosis* strains in northwestern of Iran. The result also indicated that PCR-RFLP method can be used for rapid diagnosis of INH-resistant tuberculosis in most cases.

Acknowledgment

This work was supported by Tuberculosis and Lung Research Centre, Tabriz University of Medical Sciences, Tabriz, Iran.

References

1. Simon S, Listiawan I. Drug resistance in *Mycobacterium tuberculosis*: a molecular perspective. J Indonesian Med Assoc 2003; 4:26-35.
2. Abbadi SH, Sameaa GA, Morlock G, Cooksey RC. Molecular identification of mutations associated with anti-tuberculosis drug resistance among strains of *Mycobacterium tuberculosis*. Int J Infect Dis 2009; 13:673-678.
3. Abe C, Kobayashi I, Mitarai S, Wada M, Kawabe T, Takashima T, *et al*. Biological and molecular characteristics of *Mycobacterium tuberculosis* clinical isolates with low-level resistance to Isoniazid in Japan. J Clin Microbiol 2008; 46:2263-2268.
4. Mathuria JP, Nath G, Samaria JK, Anupurba S. Molecular characterization of INH-resistant *Mycobacterium tuberculosis* isolates by PCR-RFLP and multiplex-PCR in North India. Infect Genet Evol 2009; 9:1352-1355.
5. Ghebremichael S, Petersson R, Koivula T, Pennhag A, Romanus V, Berggren I, *et al*. Molecular epidemiology of drug resistant tuberculosis in Sweden. Microbes Infect 2008; 10: 699-705.
6. Timmins GS, Deretic V. Mechanisms of action of isoniazid. Mol microbial 2006; 65:1220-1227.
7. Ramaswamy SV, Reich R, Dou SJ, Jasperse L, Pan X, Wanger A, *et al*. Single nucleotide polymorphisms in genes associated with isoniazid resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 2003; 47:1241-12450.
8. Hristea A, Otelea D, Paraschiv S, Macri A, Baicus C, Moldovan O, *et al*. Detection of *Mycobacterium tuberculosis* resistance mutations to rifampin and isoniazid by real-time PCR. Indian J Med Microbiol 2010; 28:211-216
9. Narvskaya O, Otten T, Limeschenco E, Sapozhnikova N, Grashchenkova O, Steklova L, *et al*. Nosocomial outbreak of multidrug-resistant tuberculosis caused by a strain of *Mycobacterium tuberculosis* W-Beijing family in St. Petersburg, Russia. Eur J Clin Microbiol Infect Dis 2002; 21:596-602.
- 10- Kim SY, Park YJ, Kim WI, Lee SH, Chang CL, Kang SJ, *et al*. Molecular analysis of isoniazid resistance in *Mycobacterium tuberculosis* isolates recovered from South Korea. Diagn Microbiol Infect Dis 2003; 47:497-502.
11. Guo H, Seet Q, Denkin S, Parsons L, Zhang Y. Molecular characterization of isoniazid-resistant isolates of *Mycobacterium tuberculosis* from the USA. J Medical Microbiol 2006; 55:1527-1531.
12. Goodwin A. *Mycobacterium tuberculosis* and other Nontuberculosis mycobacteria. In: Mahon CR, Lehman DC, Manuselis G. Textbook of Diagnostic Microbiology, 3rd ed. New York: Saunders Elsevier; 2007.p.673-716.
13. Marttila HJ, Soini H, Huovinen P, Viljanen MK. *katG* mutations in isoniazid-resistant *Mycobacterium tuberculosis* isolates recovered from Finnish patients. Antimicrob Agents Chemother 1996; 40:2187-2189.
14. Poojary A, Nataraj G, Kanade S, Metha P, Baveja S. Rapid antibiotic susceptibility testing of *Mycobacterium tuberculosis*: it's utility in resource poor settings. Indian J Med Microbiol 2006; 24:268-272.
15. Murray PR, Rosenthal KS, Pfaller MA. Medical Microbiology. 5th ed. USA: Elsevier Mosby; 2005.p.297-310.
16. Leung ET, Kam KM, Chiu A, Ho PL, Seto WH, Yuen KY, *et al*. Detection of *KatG* Ser315Thr substitution in respiratory specimens from patients with isoniazid-resistant *Mycobacterium tuberculosis* using PCR-RFLP. J Med Microbiol 2003; 52, 999- 1003.
17. Marttila HJ, Soini H, Huovinen P, Viljanen MK. *KatG* mutations in isoniazid-resistant *Mycobacterium tuberculosis* isolates recovered from Finnish patients. Antimicrob Agents Chemother 1996; 40:2187-2189.
18. Velayati AA, Masjedi MR, Farnia P, Tabarsi P, Ghanavi J, Ziazarifi AH, *et al*. Emergence of new forms of totally drug-resistant tuberculosis bacilli: super extensively drug-resistant tuberculosis or totally drug-resistant strains in Iran. Chest 2009; 136:420-425.
19. Zaker Bostanabad S, Titov LP, Bahremand R. Frequency and molecular characterization of isoniazid resistance in the *KatG* region of MDR isolates from tuberculosis patients in southern endemic border of Iran. Infect Genet Evol 2008; 8:15-29.

20. Mohajeri P, Tavakoli A, Moghim S. Detection of mutation at codon 315 *KatG* gene as a gene marker associated with isoniazid resistance, in *Mycobacterium tuberculosis* strains isolated from patients in Isfahan and Tehran by PCR-RFLP method. *J Zanjan Unive Med Sci Health Services* 2009; 17:29-40.
21. Cavusoglu C, Turhan A, Akinci P, Soyler I. Evaluation of the Genotype MTBDR assay for rapid detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* isolates. *J Clin Microbiol* 2006; 44:2338-2342.
22. Aktas E, Durmaz R, Yang D, Yang Z. Molecular characterization of isoniazid and rifampin resistance of *Mycobacterium tuberculosis* clinical isolates from Malatya, Turkey. *Microb Drug Resist* 2005; 11:94-99.
23. Ahmad S, Mokaddas E. Contribution of AGC to ACC and other mutations at codon 315 of the *katG* gene in isoniazid-resistant *Mycobacterium tuberculosis* isolates from the Middle East. *Int J Antimicrob Agents* 2004; 23:473-479.
24. Ahmad S, Fares E, Araj GF, Chaugh TD, Mustafa AS. Prevalence of S315T mutation within the *KatG* gene in isoniazid-resistant clinical *Mycobacterium tuberculosis* isolates from Dubai and Beirut. *Int J Tuberc Lung Dis* 2002; 6:920-926.
25. Valvatne H, Syre H, Kross M, Ruth Stavrum R, Phyu S, Grewal M. Isoniazid and rifampicin resistance-associated mutations in *Mycobacterium tuberculosis* isolates from Yangon, Myanmar: implications for rapid molecular testing. *J Antimicrob Chemother* 2009; 64:694-701.
26. Musser JM. Antimicrobial agent resistance in mycobacteria: molecular genetic insights. *Clin Microbiol Rev* 1995; 8:496-514.
27. Zhang M, Yue J, Yang Y, Zhang H, Lei J, Jin R, Zhang X, *et al.* Detection of mutation associated with Isoniazid resistance in *Mycobacterium tuberculosis* isolates from China. *J Clin Microbiol* 2005; 43:5477-82.
28. Haas WH, Schilke K, Brand J, Amthor B, Weyer K, Fourie PB, *et al.* Molecular analysis of *KatG* gene mutations in strains of *Mycobacterium tuberculosis* complex from Africa. *J Antimicrob Chemother* 1997; 41:1601-1603.
29. Telenti A, Honoré N, Bernasconi C, March J, Ortega A, Heym B, *et al.* Genotypic assessment of isoniazid and rifampin resistance in *Mycobacterium tuberculosis*: a blind study at reference laboratory level. *J Clin Microbiol* 1997; 35: 719-23.
30. Dalla Costa ER, Ribeiro MO, Silva MS, Arnold LS, Rostirolla DC, Cafrune PI, *et al.* Correlations of mutations in the *KatG*, *oxyR-ahpC* and *inhA* genes and in vitro susceptibility in *Mycobacterium tuberculosis* clinical strains segregated by spoligotype families from tuberculosis prevalent countries in South America. *BMC Microbiol* 2009; 9:39.
31. van Soolingen D, de Haas PE, van Doorn HR, Kuijper E, Rinder H, Borgdorff MW. Mutations at amino acid position 315 of the *KatG* gene are associated with high-level resistance to isoniazid, other drug resistance, and successful transmission of *Mycobacterium tuberculosis* in the Netherlands. *J Infect Dis* 2000; 182:1788-1790.