Isoniazid Resistance and the Point Mutation of Codon 463 of *katG* Gene of *Mycobacterium tuberculosis*

It has long been known that almost all isoniazid (INH) resistant mycobacteria lose the catalase and peroxidase activities along with reduced or no virulence for guinea pigs. Recently resistance to INH has become known to be associated with mutations of *katG* gene encoding the HPI (Hydroperoxidase I) type catalase and peroxidase. Among these mutations, the point mutation of codon 463 of *katG* gene is found frequently, and is suggested as being associated with INH resistance. Therefore we performed this study in order to confirm the correlation between the point mutation of codon 463 of the *katG* gene and INH resistance of *M. tuberculosis* in Korea. Fifty isolates, 32 of which were resistant to INH, and 18 of which were sensitive to INH, were selected for this study. We used PCR-SSCP and RFLP analysis to detect the point mutation of the codon 463 of *katG* gene and confirmed the CGG (arginine) to CTG (leucine) mutation by direct sequencing analysis. Among 32 resistant isolates, 7 isolates (22%) had the same restriction pattern compared with that of the reference strain (H37Rv), and 25 isolates (78%) showed a different restriction pattern. Among 18 sensitive isolates, 7 isolates (39%) had the same restriction pattern compared with that of H37Rv, and 11 isolates (61%) showed a different restriction pattern. These results suggest that the CGG to CTG change of codon 463 of *katG* gene of *M. tuberculosis* may be a polymorphism not related with INH resistance.
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Key Words : *Mycobacterium, Tuberculosis, katG gene, Drug resistance, Isoniazid*

Tae Sun Shim, M.D.*, Chul-Gyu Yoo, M.D., Sung Koo Han, M.D., Young-Soo Shim, M.D., Young Whan Kim, M.D.

Department of Internal Medicine and Tuberculosis Research Institute, Seoul National University College of Medicine, Seoul, Korea

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Address for correspondence
Young Whan Kim, M.D., Department of Internal Medicine, Seoul National University Hospital, 28 Yongon-dong, Chongno-ku, Seoul 110-744, Korea. Tel : (02) 760-2856

* present address : Tae Sun Shim, M.D., Department of Internal Medicine, University of Ulsan-College of Medicine, Asan Medical Center, 388-1 Poongnap-dong, Songpa-ku, Seoul 138-040, Korea. Tel : (02) 224-3130, Fax : (02) 224-6968

INTRODUCTION

Intractable pulmonary tuberculosis is the leading cause of death due to pulmonary diseases in Korea. One of the most important factors is drug resistance, especially multi-drug resistance (MDR). The prevalence of radiologically diagnosed pulmonary tuberculosis was 5.1% in 1965 in Korea. The prevalence had been decreased continuously by the national anti-tuberculous program to become 1.0% in 1995. Now it is estimated that more than 429,000 people still have pulmonary tuberculosis in Korea. Recently, the incidence of tuberculosis has begun to increase in many countries due to HIV infection, and the control of this disease is further threatened by the emergence of drug resistance. Korea is one of the countries with a high prevalence of multi-drug resistant tuberculosis not related with AIDS. Today, INH resistance of *M. tuberculosis* accounts for 25.5% (1990) in Korea. Because the prevalence of AIDS is increasing in Korea, it is possible that the prevalence of MDR tuberculosis will be higher in the future. However, the development of new drugs for the treatment of slowly growing mycobacteria is difficult because of the long treatment periods, the use of multidrug therapy, and the difficulty of evaluation of relapse rate.

INH and rifampicin (RFP) are the backbone of the current antituberculous chemotherapy regimen and the resistance to one of these bactericidal drugs may result in therapeutic failure. Recently, the molecular basis of mycobacterial resistance to the antituberculous agents has been elucidated. INH resistance is associated with *katG* or *inhA* gene mutations (1, 2). RFP resistance is associated with *rpoB* gene mutation (3). SM resistance is associated with *rspL* or *rrS* gene mutations (4, 5). And fluoroquinolone resistance is associated with *gyrA* gene mutation (6). Among these, RFP resistance has been studied more extensively. The main mechanism of mutaion of the *rpoB* gene is point mutations in a confined region of 69 bp sequence. So RFP resistant strains can be rapidly detected by single-strand conformational polymorphism (SSCP) and sequencing analysis instead of the time consuming drug sensitivity test. SSCP analysis is based on the fact that separated strands of DNA adopt a folded conformation as a result of self

complementary and intramolecular interactions. A single nucleotide mutation usually leads to an altered conformation that can be identified as a change in DNA strand mobility by nondenaturating polyacrylamide gel electrophoresis. Rapid detection of RFP resistant *M. tuberculosis* by PCR-SSCP analysis shows a high level of sensitivity and specificity, near 100% (7~9). Also, PCR-SSCP analysis is being tried in detecting INH resistance, but the situation in INH resistance is quite different from that of RFP resistance. The mutation of *katG* gene is distributed evenly throughout the *katG* gene over 2,000 bp and the frequency of *katG* gene mutation in INH resistant *M. tuberculosis* is low compared with that of *rpoB* gene mutations in RFP resistant *M. tuberculosis*. So, our efforts to detect INH resistance by observing *katG* gene mutations has been disappointing. But recently there was a very interesting observation that the point mutation of codon 463 of *katG* gene is found frequently in INH resistant strains, and may be associated with INH resistance (10, 11). Also, the mutation can be detected easily by RFLP analysis because codon 463 is the restriction site of *Nci*I and *Msp*I enzyme. Here we present our experience using PCR-SSCP, RFLP, and direct sequencing analysis for the evaluation of *katG* gene, especially the point mutation of codon 463 of *katG* gene.

MATERIALS AND METHODS

M. tuberculosis isolates

Randomly selected 50 clinical culture isolates of *M. tuberculosis* in Seoul National University Hospital between 1994 and 1995 were evaluated. Susceptibility tests of all *M. tuberculosis* isolates were done by the absolute concentration method at the Korean Tuberculosis Institute. The critical concentration of INH by which probably resistant strains have been separated from sensitive strains was 0.2 ug/ml. The tests were done in Lowenstein-Jensen medium. Resistance to INH was found in 32 isolates and the remaining 18 isolates were INH-susceptible. For control purpose, a full-drug susceptible reference strain (H37Rv) was used.

DNA extraction of *M. tuberculosis*

We extracted DNA by modifying the Hurley et al's method (12). The cultured colony were suspended in 200ul of TEN buffer solution. The sample was mixed with 200ul of PCI (phenol/chloroform/isoamyl alcohol), 200ul of Zirconium bead (0.1mm), and 100ul of TEN solution, and was disintegrated in a mini-bead beater for 3 minutes. Then the sample was centrifuged for 5 minutes at 3000rpm. Then the 200ul of transparent supernatant was moved to another Eppendorf tube, mixed with 200ul of CI (chloroform/isoamyl alcohol) solution, and centrifuged for 5 minutes at 3000rpm. The 100ul of supernatant was mixed with 10ul of 3M sodium acetate and 220ul of cooled 100% ethanol, and incubated for 15 minutes at -70℃. Then it was centrifuged for 10 minutes at 10,000rpm, the supernatant was removed by vacuum-suction, and the pellet was dried in the air. The extracted DNA was resuspended in 100ul of distilled water and used for PCR analysis.

RFLP analysis by *Nci*I and *Msp*I restriction

For RFLP (Restriction fragment length polymorphism) analysis, we amplified 1364 bp amplicon representing the 3'-half of the *katG* gene with primers B1 and B2 and the amplicon was digested with restriction endonuclease *Nci*I or *Msp*I. We used a thermal cycler (GeneAmp PCR System 9600 ; Perkin Elmer Cetus, Norwalk, CT, USA) and 50ul of lyophyllized, PCR Pre-Mix kit (Korea Biotech. Inc., Seoul, Korea) for PCR reaction. We mixed 2.5 ul of each primer (10 pmole/ul), 2ul of template DNA, and 43ul of distilled water into the Pre-Mix kit microtube. The PCR reaction mixture was subjected to 5 minutes at 95℃ followed by 50 cycles of 1 minute at 95℃ and 2 minutes at 72℃. PCR products were analyzed on 2% agarose gels for confirmation of 1364 bp amplicon. 1364 bp DNA fragments were purified with the Wizard PCR Preps DNA purification kit (Promega, Madison, Wis., USA). We mixed 2 units of *Nci*I or *Msp*I (1.2 unit/0.1ul) with 1 ug of DNA and incubated it for 2 hours at 37℃. The fragments were electrophoresed using 2% Nusieve gel.

In one case, 1364 bp sequence was not amplified by PCR , so we amplified 189 bp sequence including codon 463 of *katG* gene with primers 463a and 463b and performed RFLP analysis.

Identification of mutations by PCR-SSCP analysis

We amplified the 237 bp sequence (3rd to 239th) of *katG* gene by PCR using primers katg1 and katg2 and the PCR prodct was subjected to SSCP analysis. Also we amplified the 189 bp sequence of *katG* gene including codon 463 and performed SSCP analysis.

1. PCR

PCR was done as described above. PCR reaction mixture (20 ul) contained 1 ul of 10 pmole/ul primers, 0.1ul of ^{32P}-dCTP, 1ul of DNA preparation, and 16.9ul of distilled water. The thermocycler reactions were 5

minutes at 95℃ followed by 35 cycles of 1.5 minutes at 95℃, 1 minute of 59℃, and 1 minute at 72℃. In cases of PCR with primers 463a and 463b, the thermocycler reactions were 5 minutes at 95℃ followed by 40 cycles of 1 minute at 95℃, 1 minute of 60℃, and 1 minute at 72℃. Then the final elongation step was done for 5 minutes at 72℃.

2. SSCP analysis

After amplification, 1ul of PCR product was mixed with 2ul of SSCP loading buffer (95% formamide, 0.05% bromphenol blue, 0.05% xylene cyanol, 10% NaOH), heated for 5 minutes at 95℃, cooled on ice, and loaded onto a non-denaturating 0.75×MDE gel (Hydrolink, AT Biochem, Malvern, Pennsylvania, USA) (composed of 28ml of MDE solution, 4.5 ml of 10×TBE, and 42.5ml of H_2O) polymerized with 30ul of 10% TEMED and 300ul of 10% ammonium persulfate. Electrophoresis was performed at room temperature for 12 hours at a constant power of 6W. Gels were dried for 2 hours at 80℃ gel drier and exposed overnight for autoradiography.

Direct DNA sequencing

All 9 isolates which showed different SSCP pattern compared with that of H37Rv showed the same band pattern each other. So we selected one of them and did direct sequencing analysis by Sanger's dideoxy sequencing method using Sequenase PCR Product Sequencing Kit (USB, Sequenase version 2.0, Illinois, USA). We amplified 189 bp sequence by PCR with primers 463a and 463b, treated the PCR products with a combination of Exonuclease I and Shrimp alkaline phosphatase, and inactivated Exonuclease I and Shrimp alkaline phosphatase by heating to 80℃ for 15 minutes. Then we used 0.5 pmole of the template for sequencing reaction. The sequencing reaction was stopped by adding 4 ul of stop solution. Then we heated samples to 75℃, 2 minutes immediately before loading onto sequencing gel and loaded 3ul in each lane of urea containing 6% polyacrylamide gel. We electrophoresed for about 90 minutes at 55W and soaked the gel in 5% acetic acid and 15% methanol to remove urea. After transfer to Whatmann 3M paper, drying was done for 2 hours at 80℃. After removing the wrap, we did overnight autoradiography.

Primers

We used these oligonucleotide primer sets to amplify *katG* gene of *M. tuberculosis*.

katg1 : 5'- GCCCGAGCAACACCC -3' (15 mer)
katg2 : 5'- ATGTCCCGCGTCAGG -3' (15 mer)
B1 : 5'- CACCCCGACGAAATGGGACAACAGTTTCCT -3' (30 mer)
B2 : 5'- GGGTCTGACAAATCGCGCCGGGCAAACACC -3' (30 mer)
463a : 5'- TGGCAGGATCCGGTCCCTGCG -3' (21 mer)
463b : 5'- CTGCAGGCGGATGCGACCACC -3' (21 mer)

RESULTS

*Nci*I and *Msp*I restriction of 1364bp *KatG* gene PCR product

We amplified 1364 bp sequence including codon 463 of *katG* gene by PCR in 50 clinical isolates. And we did RFLP analysis using *Nci*I (Fig. 1) or *Msp*I restriction endonuclease. In one case that was not amplified 1364 bp band by PCR, we amplified 189 bp band containing codon 463 with primers 463a and 463b, digested the amplicon with *Nci*I enzyme, and evaluated the RFLP pattern. Seven out of 18 (39%) INH susceptible isolates and 7 out of 32 (22%) INH resistant isolates showed the same RFLP pattern compared with that of H37Rv (Table 1). Eleven out of 18 (61%) INH sensitive isolates and 25 out of 32 (78%) INH resistant isolates showed differnet RFLP pattern. Statistically INH susceptibility and RFLP pattern by *Nci*I or *Msp*I of *katG* gene did not show any correlation ($p>0.05$).

PCR-SSCP analysis of 189bp *KatG* gene PCR product

We evaluated 4 INH susceptible isolates containing H37Rv and 11 INH resistant isolates. Three INH sensitive and 3 INH resistant isolates showed the same band pattern and 1 INH sensitive strain and 8 INH

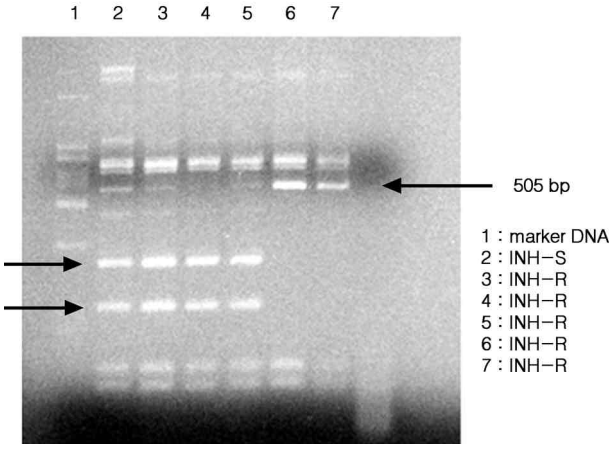

Fig. 1. RFLP analysis of 1364 bp amplicon of *katG* gene of *M. tuberculosis* by *Nci*I enzyme. Lane 6 and 7 show 505 bp band (right arrow), indicating point mutation of codon 463. Lane 2 to 5 show 294 and 211 bp band (left arrows), identical with that of H37Rv. INH-S: INH sensitive isolates. INH-R: INH resistant isolates.

Table 1. INH susceptibility and RFLP results of 1364 bp amplicon including codon 463

Restriction	INH-sensitive	INH-resistant	Total
Yes[a]	7	7	14
No[b]	11	25	36
Total	18	32	50

a. Yes : codon 463 of *KatG* gene is restricted by *NciI* or *MspI* enzyme
b. No : codon 463 of *KatG* gene is not restricted by *NciI* or *MspI* enzyme

resistant isolates showed the different band pattern compared with that of H37Rv, but they showed the same band pattern each another (Fig. 2). These results are completely identical with that of RFLP analysis.

Direct sequencing of 189 bp amplicon including codon 463 of *katG* gene

We amplified 189 bp sequence containing codon 463 of *katG* gene with primers 463a and 463b by PCR in case of strains with different RFLP and SSCP pattern. And we analysed the sequence using primer 463a and compared it with that of H37Rv. In case of H37Rv, the sequence of codon 463 of *katG* gene is CGG (arginine), whereas in the strain with different RFLP or SSCP pattern, the sequence is CTG (leucine) (Fig. 3).

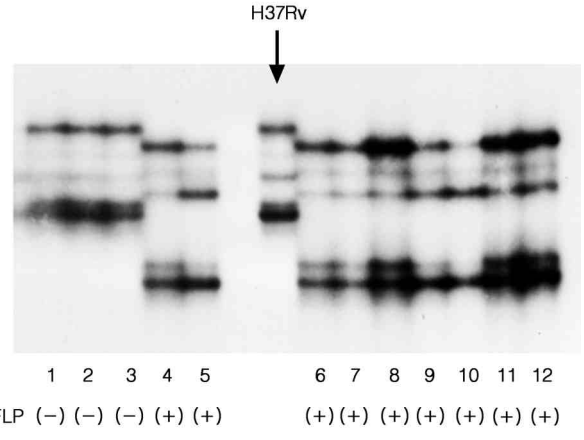

Fig. 2. PCR-SSCP analysis of 189 bp *katG* gene PCR product including codon 463. All *M. tuberculosis* strains with different RFLP pattern (+) compared with that of H37Rv showed different PCR-SSCP band pattern, whereas all the other *M. tuberculosis* strains with the same RFLP pattern (−) compared with that of H37Rv showed the same PCR-SSCP band pattern. Namely they showed completely identical results between RFLP and SSCP analysis.

Other evaluations (PCR-SSCP analysis of *KatG* gene 237 bp product)

We amplified the 237bp sequence (3rd to 239th) of *katG* gene by PCR using primer katg1 and katg2 and performed SSCP analysis. Two INH-susceptible isolates including H37Rv and 20 INH-resistant isolates were evaluated. They all showed the same band pattern (data not shown).

DISCUSSION

INH (Isoniazid) is a key component of the antituberculous, short-course chemotherapy. INH was first chemically synthesized in 1912 and has been used in the treatment of tuberculosis since 1952. But until now, its mode of action and the mechanism of resistance are not completely understood. The recent outbreaks of multiple-drug-resistant (MDR) tuberculosis in western countries are primarily associated with AIDS, but in Korea the high incidence of MDR tuberculosis is not related with AIDS yet.

It has long been known that some INH-resistant isolates are associated with the loss of catalase-peroxidase activity and show reduced or no virulence in guinea pigs (13). Recently the molecular basis of resistance of mycobacteria to the anti-tuberculous agents has been elucidated. INH resistance in *M. tuberculosis* was iden-

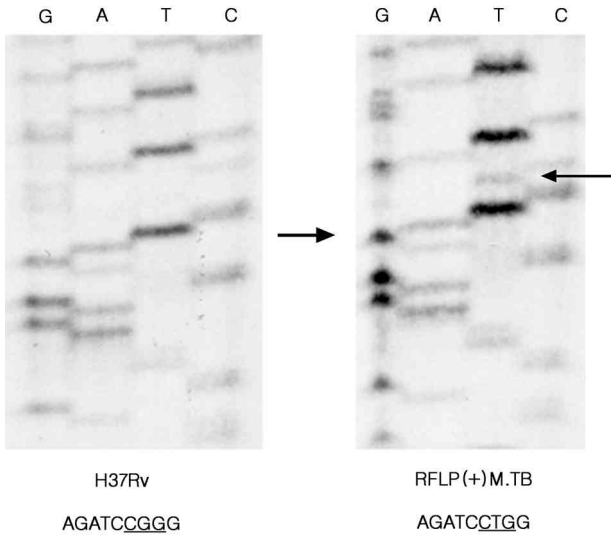

Fig. 3. Direct sequencing analysis of 187 bp amplicon including codon 463 of *katG* gene. The mutant *M. tuberculosis* isolate shows point mutation from CGG (arginine) to CTG (leucine).

tified for the first time at the molecular level by Zhang et al. (2). INH susceptibility could be restored by re-introducing the cloned *M. tuberculosis katG* gene (14). Furthermore, they showed that deletion of *katG* occured in 2 out of 3 clinical isolates of highly resistant isolates of *M. tuberculosis* (2). They concluded that in a subset of INH-resistant isolates, the lack of catalase activity is due to the complete loss of the catalase-peroxidase gene (2). But the more recent reports provided evidence that the main mechanism of mutation of *katG* gene is missense mutation rather than total deletion (15, 16). Furthermore INH resistant isolates of *M. tuberculosis* show various rates of *katG* gene mutations. Altamirano et al. (15) reported that all 9 INH ressistant *M. tuberculosis* showed *katG* gene mutations using the same primers katg1 and katg2 decribed in our study. By contrast, the other reports demonstated that *katG* gene mutations were detected only in 48~66% of INH resistant *M. tuberculosis* using 11 primers covering whole *katG* gene (8, 9, 11, 17). Altamirano et al. (15) detected *katG* mutations only in 237bp amplicon, but they detected mutations in 100%, so the result is doubtful for its confidence. We performed PCR-SSCP analysis using the same primer set and could not find any mutations. Our results are consistent with those of Heym et al. (11). Like these results, the *katG* gene mutation is thought to be less frequent in INH resistant isolates than we had thought, and the site of mutation is distributed evenly through the *katG* gene. So in order to detect the *katG* gene mutations, we must use several primer sets. But one recent report showed that the mutation at codon 463 of *katG* gene is so frequent in INH resistant isolates that it might be indictive of INH resistance. Codon 463 is a *Nci*I and *Msp*I restriction site (10). So when the wild type sequence CGG is mutated to CTG, it is no longer recognized by either enzyme. Thus, if the mutation of codon 463 of *katG* gene is the index of INH resistance, we can find the INH resistance easily by RFLP analysis. 1364 bp amplicon of H37Rv have 6 restriction sites by *Nci*I enzyme, wherease in codon 463 mutant (CGG to CTG), the number of restriction sites is reduced to 5. So in RFLP analysis by *Nci*I enzyme, 505 bp band appears with the disappearance of 294 bp and 211 bp band (Fig. 4).

It should be noted that all the other bacterial HPI proteins have a hydrophobic residue (Leu, Ala) at the same position (18). In particular, a Leu residue is present in the catalase-peroxidase from *Mycobacterium intracellulare*, an organism naturally resistant to INH (19) and in BCG strains that are less susceptible to INH (11, 20). Cockerill et al. (10) showed that 19 (44.2%) of the 43 isolates with INH MICs>1.0 ug/ml had lost the *Nci*I-*Msp*I restriction site at the locus of codon 463 while only 1 of 32 isolates with INH MICs<1.0 ug/ml had this restriction polymorphism. These results suggest that the mutation arginine to leucine in codon 463 of the *katG* gene might be a indicative of INH resistance. And Morris et al. (9) reported that 20 out of 42 INH resistant *M. tuberculosis* had a *katG* gene mutations and 8 out of 20 *katG* gene mutants had a codon 463 mutations. Musser et al. (21) demonstrated that most (84%) of INH resistant isolates had mutations in *katG* or *inhA* locus or lacked *katG*. Together, approximately 75% of INH-resistant isolates had replacement at amino acid 315 or 463 in *katG* or nucleotide substitution upstream of *inhA* (21). Heym et al. (11) studied INH resistance more extensively. All 3 INH susceptible isolates had no mutations in *katG* gene. In 36 INH resistant isolates, 21 isolates showed 7 distinct *katG* gene mutations. The majority of the mutations were due to single nucleotide substitution. Among these, the most frequent mutation was codon 463 CGG (arginine) to CTG (leucine) substitution which occured in 7 isolates. The interesting point is that 5 distinct mutations of the N-terminal domain result in decreased or abscent catalase activity, while the C-terminal mutations including codon 463 mutation conserved the catalase activity.

But all the other reports do not show that the mutation of codon 463 is associated with INH resistance. In view of our results, the G to T point mutation of codon 463 of *katG* gene may be a polymorphism not associated with INH resistance. So we can suggest that only the muations of N-terminal of *katG* gene influence the catalase activity and INH resistance. Morris et al. (9) didn't mention whether INH sensitive isolates also have codon 463 mutatioon or not. So we don't know whether the codon 463 mutation is the cause of INH resistance or merely a plymorphism not related with INH resistance in that study. Rouse and Morris (17) showed that catalase positive and INH sensitive isolates of *M. bovis* normally

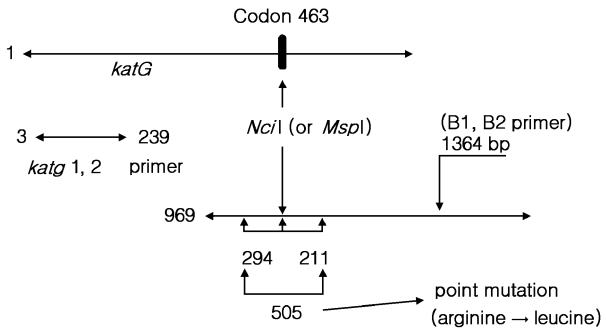

Fig. 4. Creation of 505 bp band by losing the restriction site of codon 463 by *Nci*I enzyme. The arrow (between 3 and 239) represents the PCR product of primers katg1 and katg2.

had a codon 463 mutation. In the present study, we found that 11 out of 18 INH-sensitive isolates and 25 out of 32 INH-resistant isolates had the point mutation of codon 463 of *katG* gene. This data mean that INH resistance *M. tuberculosis* has nothing to do with codon 463 mutation of *katG* gene. These results are consistent with those of Park et al. (22) which showed that 21 (75%) out of 28 INH sensitive *M. tuberculosis* had a leucine substitution at codon 463 of *katG* gene, even though they did not investigated the resistant isolates.

We performed both RFLP analysis by both *Nci*I and *Msp*I enzyme and PCR-SSCP analysis in order to reduce the error rate and the results were completely identical. We also compared our susceptibility test with others'. But we could not find any big difference.

Our results suggest that the G to T point mutation of codon 463 of *katG* gene of *M. tuberculosis* may be a polymorphism not related with INH resistance. Therefore it is not reasonable to think that different SSCP pattern of *katG* gene compared with reference strain (H37Rv) is the marker of INH resistance. So the cause and effect relationship between susceptibility and each mutation should be confirmed. And it seems to be insufficient to diagnose INH resistance only by detecting *katG* gene mutations. Recent reports show that the *katG* or *inhA* gene mutations are found ony in 60% to 75% of INH resistant isolates (9, 11). And the evidence that all the mutations are the cause of the INH resistance is lacking. On the other hand, reduced or abscent catalase activity is observed in INH resistant strain without *katG* or *inhA* gene mutations. Heym et al. (11) shows that 27 out of 36 INH resistant isolates had *katG* or *inhA* gene mutations, and in 9 INH resistant isolates without *katG* or *inhA* gene mutaions, all 5 isolates studied were catalase negative. So it is suggested that the other genetic mechanism of drug resistance should be associated with catalase activity. The other possible alternative drug targets have been suggested. For example, *oxyR* regulon mutations can influence the sensitivity of *E. coli* to INH (23, 24). Recently another gene, *ahpC* gene, was reported to be associated with INH-resistance of the *M. tuberculosis* complex (25). So it seems that INH-resistance of *M. tuberculosis* is associated with mutations of multiple genes rather than a mutation of single gene. Further studies are needed for complete insight into the mechanisms of INH resistance.

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