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Detection of Rifampin Resistance Mutation and Its Altered Nucleotide Sequences in *Mycobacterium leprae* Isolated from Korean Patients with Leprosy

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Rifampin is the most powerful drug for treating leprosy and tuberculosis today. It inhibits initiation and elongation of RNA transcription by binding to β-subunit of RNA polymerase, leading to kill mycobacteria. We isolated one variant strain of *Mycobacterium leprae* from 24 Korean leprosy patients who are less susceptible to rifampin or have suffered from relapse by polymerase chain reaction and single strand conformation polymorphism (PCR-SSCP) of the *rpoB* gene. Direct sequencing of the *rpoB* region of *M. leprae* variant revealed missense mutations which altered the amino acids sequence of RpoB to Ser-464, Arg-465, Arg-467 and Ala-468. This is the first finding on *rpoB* gene mutation of *M. leprae* from Korean patients; moreover the mutant type was found to be different from the previously reported cases in other countries.

Key words: ***Mycobacterium leprae*, PCR-SSCP, direct sequencing, multibacillary leprosy, missense mutation**

Rifampin is the most powerful and important chemotherapeutic agent of the multidrug therapy(MDT) for treating leprosy and tuberculosis because of its powerful antibiotic activity and low frequency of resistant strain. Rifampin inhibits initiation and elongation of RNA transcription by binding β-subunit of RNA polymerase in *Mycobacterium leprae*.

Resistance to rifampin has not been reported as well as it is to dapsone. Rifampin resistant strain of *M. leprae* was first reported by Jacobson and Hastings (7). Grosset *et al.* (1) reported a study of 39 documented relapse of leprosy after treatment with rifampin. Therefore, a rapid and efficient detection method is needed to find the rifampin resistant strains.

The molecular analysis of rifampin resistance has been studied in *E. coli* (8), and it has been demonstrated that rifampin resistance is due to a missense mutation in the highly conserved regions of the *rpoB* gene, which encodes the β-subunit of RNA polymerase in several bacteria (17). This missense mutation is caused by a single nucleotide substitution, which leads to a change in the amino acid of the RNA polymerase; more specifically, polar hydrophillic amino acids are substituted with nonpolar hydrophobic amino acids, resulting in the physical obstruction for rifampin to bind to RNA polymerase (3, 14).

In order to detect these single base changes by clinical diagnostic test, Orita *et al.* (10, 11) had devised the SSCP technique which uses PCR and mobility shifts in nondenaturing polyacrylamide gel electrophoresis. Simple and rapid PCR-SSCP and magnetic bead based direct sequencing techniques are suggested for PCR-amplified *rpoB* fragment in *M. leprae* and in *M. tuberculosis* (3, 4, 6, 12).

Rifampin has been used for the last 20 years in Korea and many of the leprosy patients are suffering from relapse during MDT. Actually, 73.6% of multibacillary leprosy patients are prescribed rifampin for more than 10 months, and of them 1.37% have a relapse. Nevertheless, the reports for rifampin resistant strain of *M. leprae* are very rare because mutation of *M. leprae* has not been detected by RFLP or RAPD method comparing with *M. tuberculosis*. And also, any experimental results for detecting rifampin resistant strain have not been reported in Korea.

We here report an isolation of one variant of *M. leprae*

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that is resistant against rifampin after an investigation of 14 Korean patients with leprosy along with the determination of its altered nucleotide sequence using our established PCR based SSCP analysis and direct sequencing. This finding will be very helpful for leprosy control program and for studying the molecular basis of rifampin resistance in *M. leprae*.

Materials and Methods

Preparation of genomic DNA of *M. leprae* for PCR

M. leprae was obtained from skin biopsies which were taken from lesions of 24 Korean leprosy patients and held in liquid nitrogen and shipped to the chronic diseases laboratory. *M. leprae* DNA was prepared from tissue homogenates of biopsies by freeze-thawing procedure and then used directly for PCR (15, 16).

Polymerase chain reaction

The region of *rpoB* known to harbour mutations conferring rifampin resistance (Rif^r) was amplified by PCR. Primers were synthesized by Pharmacia DNA synthesizer and the nucleotide sequence of the primers used in this study are listed on Table 1.

One microliter of biopsy extract was mixed with 5 μl of 10× Taq DNA polymerase buffer (Poscochem.), and boiled for 2 min. After then, this mix was added to 44 μl of master reaction mixture containing 2 μM of each primer, 0.125 μM of dNTP mixture, 2.5 mM of $MgCl_2$, and 0.1 U of Taq DNA polymerase (Poscochem.). The reaction mixture in 0.5 ml microcentrifuge tube were overlaid with mineral oil (Sigma) and cycled for 2 min at 94°C, 30 sec at 70°C, and 30 sec at 72°C for 35 cycles, and then for 10 min at 72°C for final elongation in a DNA thermal cycler 480 (Perkin Elmer Cetus). PCR products of 390 bp fragment were amplified by rpo22 and rpo32, and 190 bp fragment was amplified by rpo35 and rpo38 (Fig. 1).

SSCP analysis

To detect mutations in the *rpoB* fragment, SSCP analysis was employed using 0.5× MDE (Mutation Detection Enhancement, FMC) gel and silver staining.

Twenty microliters of PCR amplified fragment was mixed with 20 μl of 2× loading solution containing 95% formamide, 10 mM NaOH, 20 mM EDTA, 0.02% bromophenol blue, and 0.02% xylene cyanol, and heated at 95°C for 3 min to denature the PCR fragment. Samples were quick-chilled on ice and immediately loaded onto a 0.5× MDE gel (2× concentrate: 18×18 cm×1.5 mm (H×W×D), Hoeffer Co. SE600) made with 0.6× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA), 400 μl of 10% ammonium persulfate, and 40 μl of TEMED. Electrophoresis was carried out for 4-6 hrs at 150 V, 20 mA using a thermal circulator which was adjusted at 16°C. After electrophoresis, the gel was silver stained (9).

Table 1. Nucleotide sequences of the designed primers

Primer	Sequence
rpo22	CAGGACGTCGAGGCGATCAC
rpo32	TCCTCGTCAGCGGTCAAGTA
rpo35	GCCGCAGACGCTGATCAATA
rpo38	CACGTCACGGACCTCTAGCC

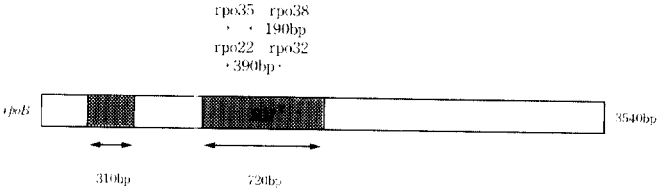

Fig. 1. Schematic representation of the *rpoB* gene in *M. leprae* and PCR strategy for amplifying the Rif^r regions. Various mutations have been reported in the shaded regions of *rpoB*.

PCR-directed DNA sequencing

Nucleotide sequences were determined using the Taq Cycle Sequencing Kit (Takara Shuzo Co.) which is a combination of PCR technique and Sanger's dideoxy sequence determination method. DNA for sequencing was prepared by PCR the same as it was for SSCP analysis, and the same primers were also used. The primer (540 pmol) was 5'-end labeled with [γ-^{32}P]dATP (50 pmole, 6000 Ci/mmol, Amersham) with T_4 polynucleotide kinase (10 U, Takara Shuzo Co.) in 10 μl of 67.5 mM Tris-HCl, pH 8.8, 67.5 mM KCl, and 3.4 mM $MgCl_2$ at 37°C for 30 min. And then thermal cycle reaction was performed at 94°C for 30 sec for 1 cycle, and at 94°C for 30 sec, at 60°C for 30 sec, at 72°C for 1 min for 15 cycles, and at 94°C for 30 sec, and at 72°C for 1 min for 15 cycles. After thermal cycling, 4 μl of stop solution was added into each of the four tubes. Two microliters of each of the four PCR reactants were run on a 6% sequencing gel. For 40 cm gel, the gel was run at constant power (50 W) for 2 hrs. After electrophoresis, the gel was transferred onto a chromatography paper (Whatman 3MM) and exposed to an X-ray film for 12 hrs at −70°C.

Results and Discussion

PCR-SSCP analysis for detection of rifampin resistance

The nucleotide sequence of the *rpoB* gene in *M. leprae* was determined recently (5, EMBL databank Z14314)

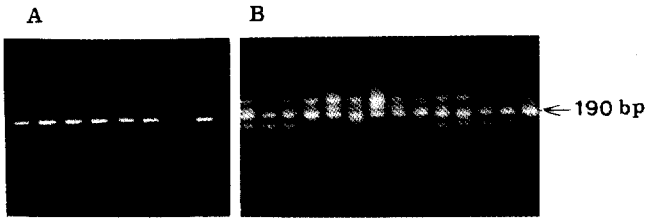

Fig. 2. Electrophoretic patterns of amplified *rpoB* region. PCR products showed sharper DNA bands on agarose gel electrophoresis when annealing temperature was increased to 70℃ (A) from 62℃ (B).

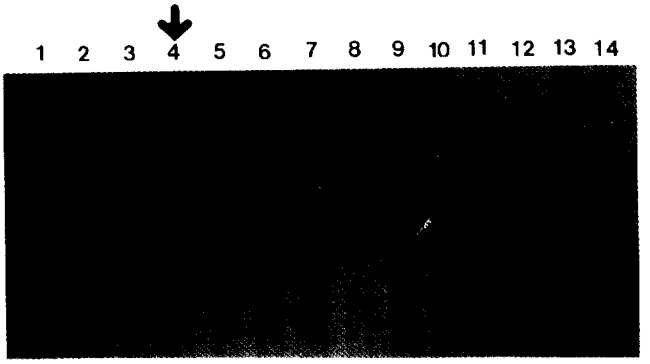

Fig. 3. PCR-SSCP analysis of *rpoB* region of *M. leprae*. One variant (lane 4) among 24 independent isolates of *M. leprae* had different mobility shift on a gel. Only 14 isolates of *M. leprae* was represented in photograph. Electrophoresis was carried out in 0.5× MDE gel without glycerol at 16℃. After electrophoresis, the gel was subjected to silver staining.

and has been deduced that the amino acid sequence of RpoB proteins have six regions of highly conserved sequence in several bacteria (17). From the size of *rpoB* gene in *M. leprae* of 3540 bp (5), the mutations were strictly clustered in the 310 bp and 720 bp regions as described in Fig. 1, leading to rifampin resistance. Primers were designed to amplify the shorter region than that was used in other studies (3, 14). PCR products showed sharper bands on the gel electrophoresis when annealing temperature was increased to 70℃ from 62℃, eliminating amplification of miscellaneous fragments (Fig. 2). The GC content of *M. leprae* is little high, as high as 54-58%; thus, it was speculated that such high annealing temperature would increase the amplification specificity while decreasing amplification of any contaminants which might exist in the biopsy extracts.

SSCP analysis is widely used to detect a genetic variation that is often the result of a difference at a single nucleotide. The conformation of single-stranded DNA is very sensitive to environmental conditions such as temperature and denaturant, formamide and sodium hydroxide. Therefore, changes of these conditions cause a

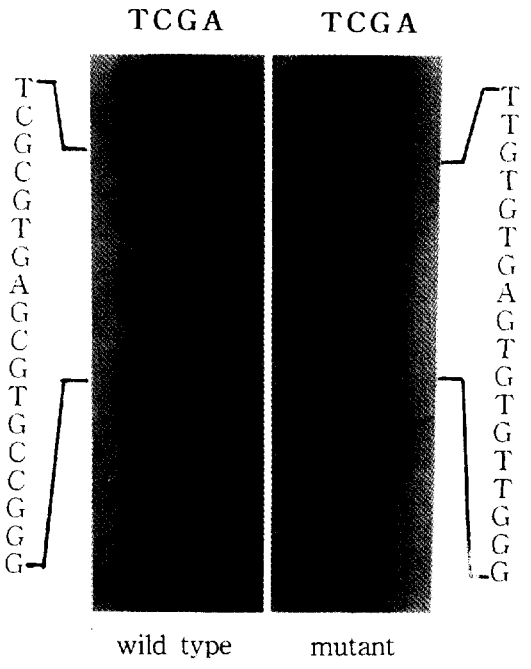

Fig. 4. Mutations were identified by direct sequencing of PCR products. The region analyzed in PCR-SSCP was directly sequenced by Taq cycle sequencing kit without subcloning. DNA sequencing was performed by using a reverse primer rpo3. The arrows indicate the band corresponding to a nucleotide substitution: C→T.

change in conformation, which can be detected in SSCP analysis. We performed SSCP analysis with 0.5× MDE gel electrophoresis without glycerol, using small size glass plates (18×18 cm) and silver stained instead of the large sequencing plates (34×40 cm), with radioactive labeled nucleotides. It is a more convenient and rapid method than other methods developed (2, 5, 13). In spite of the fact that no rifampin susceptible or resistant strain of *M. leprae* had been reported in Korea in stringent *in vivo* growth, we found one variant among 24 independent isolates of *M. leprae*, which had a different mobility shift (Fig. 3). With the establishment of this method for a rapid diagnosis of rifampin resistance cases, it might be helpful for the effective treatment.

Nucleotide sequence analysis of *rpoB* from SSCP variant

In order to detect the altered nucleotide sequence, we applied the PCR-directed sequencing method since the DNA sequencing is relatively time-consuming and labour-intensive. One of the PCR primers was end labeled with [γ-^{32}P] radioactive nucleotide, thus allowing the PCR products to be amplified in a thermal cycle reaction.

After nucleotide sequence analysis, sequence differences were found in the PCR-amplified region, which was a single base-change point mutation of C→T. It is

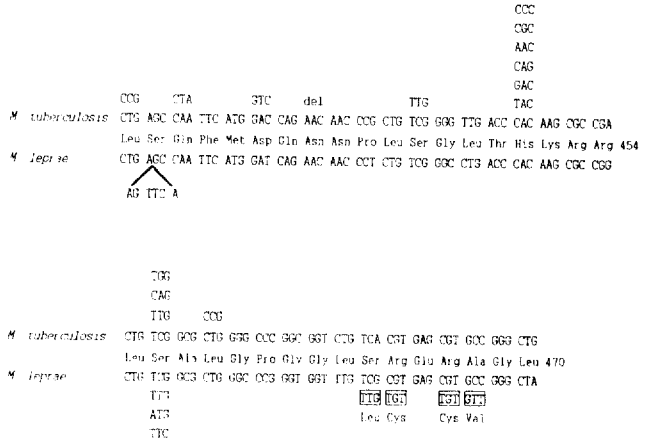

Fig. 5. Alignment of *M. tuberculosis* and *M. leprae rpoB*. Point mutated nucleotide sequences of *M. tuberculosis* and *M. leprae* are lined against the upper and the downstream of the conserved amino acid sequences. The mutations found in this study are in the box, and the sites are different from the frequent mutation sites, which has been reported in other articles (4, 14).

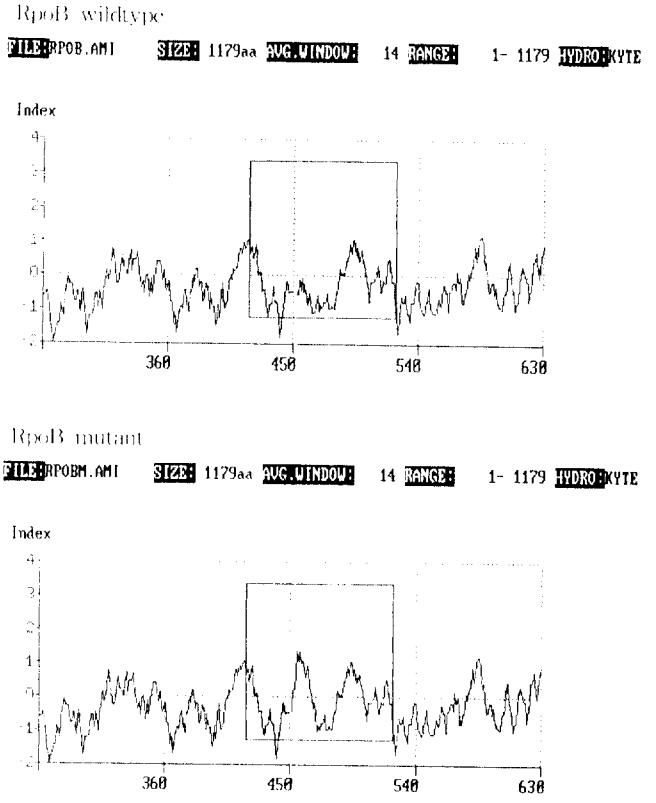

Fig. 6. Comparison of the hydrophobic profile of the region of mutations in the RNA polymerase β-subunit in *M. leprae* wildtype with mutant one. Among 1179 amino acids of RpoB, only mutated region has been represented in the Kyte-Doolittle plots using window of 14. It showed remarkably different profile in the wildtype and mutant at the shaded boxes, which is changed the hydrophobicity.

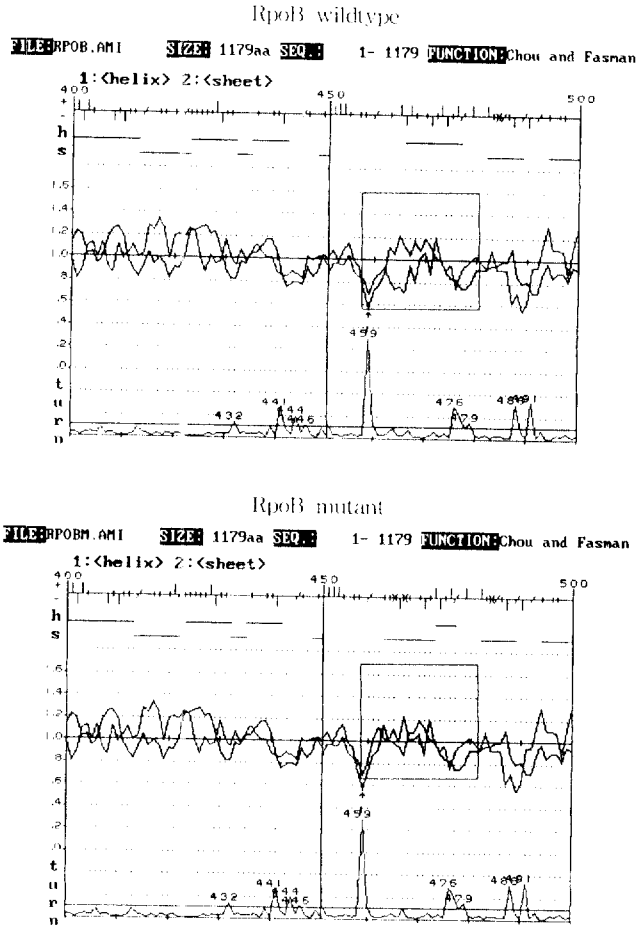

Fig. 7. Prediction of secondary structure of the mutated region of RNA polymerase β-subunit in *M. leprae* wildtype and mutant. The change of α-helix to β-sheet was shown in the shaded boxes. System of Chou and Fasman was used in Prosis program.

involved in 464, 465, 467 and 468 codon of amino acids in RpoB protein (Fig. 4, 5). With hydrophobicity analysis and predicted secondary structure of RpoB in *M. leprae* wildtype and mutant (Fig. 6, 7), these mutations caused the change of the amino acids from hydrophillic amino acids to hydrophobic amino acids: Ser→Leu, Arg→Cys, Arg→Cys, and Ala→Val. Although other studies are required for understanding the interaction of RpoB with rifampin, mutations in the RNA polymerase β-subunit may reduce the binding affinity for rifampin that is lead to physical obstruction, resulting in rifampin resistance.

In this study, we found that the single base substitution was repeated four times continuously within a well documented conserved region.

It was a different type of mutant compared to other geographical isolates that was substituted with one base in each mutant strain (3, 4, 14). Moreover, the mutation sites were slightly different from that of published cases (Fig. 5).

The main goal of this study was to elucidate the molecular mechanism of rifampin resistance in *M. leprae* and to

establish an early diagnostic method of rifampin resistant *M. leprae*. Our findings in this study were the first report in Korea on rifampin resistance at the molecular level. Therefore, these results will be very helpful for leprosy control program and for understanding of molecular mechanism of rifampin resistance in *M. leprae*.

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