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Mutations in the *rpoB* Gene of *Mycobacterium leprae* from Korean Leprosy Patients

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Abstract A fast and easy PCR-SSCP method was developed and assessed for the early detection of rifampin-resistant *Mycobacterium leprae* in skin biopsy samples from Korean leprosy patients. The 190 bp of the *rpoB* gene, in which mutation is known to cause resistance to rifampin, was amplified by PCR and then analyzed by SSCP and DNA sequencing. All PCR products showing mobility shift on PCR-SSCP contained mutations, demonstrating that this method can be used for an early diagnostic method to detect a putative rifampin-resistant *M. leprae* strain. DNA sequence analysis revealed that 19 of 34 patient samples contained *M. leprae* strains with missense mutations in the *rpoB* gene: five were the same mutations previously reported to cause rifampin resistance and eight were the new type of mutations that likely cause rifampin resistance. These newly identified mutations, whose all five cytosine bases of four amino acids were substituted with thymine, were found at different sites from those reported in *Mycobacterium tuberculosis* or *M. leprae*. Therefore, they may provide additional clues to understand the molecular biological basis on the rifampin resistance of *M. leprae*.

Key words: *Mycobacterium leprae*, leprosy, rifampin resistance, PCR-SSCP, *rpoB*

Rifampin is known to be the most effective and important chemotherapeutic agent in the multidrug therapy (MDT) for tuberculosis and leprosy. In Korea, rifampin has been used in MDT since 1974 under the standard prescription guideline of the World Health Organization [7]. The purpose of MDT is to prevent the reservoir formation of leprosy by effectively eradicating the causative bacteria as fast as possible, thereby stopping the spreading of leprosy and decreasing the expression of drug resistant strains. A possibility of a drug resistant strain to appear is high when dapsone, clofazamine, or rifampin alone is used, and in a patient who receives drug treatment intermittently over a long time. A rifampin-resistant strain can appear in a patient who discontinued the drug therapy. For example, leprosy was reported to relapse 9 years after stopping the drug treatment, due to rifampin resistance [4]. Jacobson and Hastings reported rifampin-resistant strains for the first time in 1976 [6]. Grosset *et al.* isolated 39 strains of *M. leprae* from recurring leprosy patients in 1989, of which 22 were rifampin-resistant [4]. Even with MDT, many patients face difficulties clinically, due to recurrence.

A molecular biological study on rifampin resistance was first carried out in *Escherichia coli* [8]. It has been reported that the cause of resistance is due to the mutation in the *rpoB* gene. The *rpoB* gene encodes a RNA polymerase β-subunit which is the target of rifampin. Alternation of an amino acid in a RNA polymerase β-subunit due to a point mutation, an insertion, or a deletion of a nucleotide base weakens rifampin binding to a β-subunit of RNA polymerase, resulting in rifampin resistance. Another possible resistance mechanism is alternation of the membrane permeability of bacteria that leads to a reduced drug uptake [1, 2, 8, 15].

Although there are numerous reports on the isolation of rifampin-resistant strains in tuberculosis, only a few reports on the isolation of resistant strains in leprosy has been documented worldwide. This is due to the facts that 1) the *in vitro* culture of a strain is currently impossible, 2) the proliferation time *in vivo* is long, and 3) the evaluation of

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resistance by an *in vivo* test using nude mice takes long so that cost can be prohibitive.

The traditional method of investigating resistance includes the isolation of a putative resistant strain, its inoculation and growth in nude mice, and investigation of resistance to rifampin treatment after nodules development. However, it is not clinically easy to practice. In addition, mutations are difficult to detect with the restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNAs (RAPD) methods. Since its development by Orita *et al.* in 1989, the single strand conformation polymorphism (SSCP) method has been frequently used in detecting gene mutations [13]. The PCR-SSCP method that employs amplification of DNA fragments by PCR became useful for confirming a mutation in the *M. leprae rpoB* gene in biopsy tissue of leprosy patients.

In Korea, many patients have been suffering from relapse in the course of MDT since introduction of rifampin 25 years ago. Among 19,517 leprosy patients who underwent MDT from 1989 to 1997, 347 (1.78%) and 201 (1.03%) patients experienced recurrence and risk of relapse, respectively [10]. In spite of these statistical figures, any experimental attempt to detect a rifampin-resistant strain from Korean leprosy patients has not been reported. The present study was undertaken to detect infection of rifampin-resistant *M. leprae* early. All PCR products showing mobility shift on SSCP gels contained mutations. Therefore, our PCR-SSCP method that is very simple and fast to perform can be used for early detection of a putative rifampin-resistant *M. leprae* strain. In addition, the types and sites of mutations identified through this study will be informative for understanding the molecular biological basis of the rifampin resistance of *M. leprae*.

MATERIALS AND METHODS

Bacteriological Index (BI), Morphological Index (MI), and Anti-Leprosy Regimens of Leprosy Patients

We examined leprosy patients who were being treated in the outpatient clinics at the Institute of Hansen's Disease, College of Medicine, The Catholic University of Korea (Seoul, Korea), Korean Leprosy Control Association (Euiwang, Korea), and Jesus Clinic (Taegu, Korea). Four-mm punch biopsies were performed on skin of patients who had recurrent leprosy and who did not show definitive clinical improvement with a MDT (DDS; rifampin, clofazimine or ROM; rifampin, ofloxacin, minocyclin). The bacterial index (BI) was calculated by counting smears stained by the Ziehl-Neelsen method and decolorized with 1% acid alcohol under the 100× oil immersion lens: 1+, 2+, and 3+ indicate at least 1 rod in every 100, 10, and 1 field; 4+, 5+, and 6+ at least 10, 100, 1,000 in every field, respectively. The morphological index (MI) was calculated by counting the numbers of solid-staining acid-fast rods. DNA was purified from the fresh or frozen punch biopsy samples in liquid nitrogen. Recurrent patients were defined as those who had been released from MDT for 24 months because of decreased clinical symptoms and BI, but showed positive BI again. Also included were those patients who did not show recurrence but did not respond well to MDT. Table 1 shows the BI and MI of biopsies, relapse, and types of used drugs.

Extraction of DNA from *M. leprae*

After cutting a biopsy sample into small pieces with a knife (No. 15) on a class II clean bench (Nuair Co, Plymouth, MN, U.S.A.), a sample was homogenized in 1 ml of PBS (phosphate-buffered saline) with a Mickle

Table 1. Characteristics of bacteriological status and MDT regimens for leprosy patients.

Patient	Sex/ Age	Relapse	BI/MI (Year. Month)			Treatment (Tx) (Year. Month)
4-7	F/39		5+10% (96.04)	2+/1% (98.03)		No. Tx (94-96); DDS+RFP+B663 (96.4); DDS+OFLX+B663 (98.3)
6-3	M/49		5+20% (97.03)	3+/5% (97.10)	2-/0% (98.07)	DDS, RFP, B663 (97.03); ROM (98.01)
4-3	M/52		4+/0% (96)	4+/0% (97.04)	4+/0% (98.2)	DDS (58-61); irregular (62-79); No. Tx (79-86); irregular (87-96)
8-2	M/47		6+/5% (98.01)	6+/0% (98.08)	5+/1% (99.05)	DDS, RFP, B663 (98.01); ROM (99.8)
5-11			2+/0% (95)			defaulter
1-4	M/59		4+/1% (95.06)	4+/0% (96.09)	4+/0% (97.06)	MDT (95.6); OFLX+MINO+B663 (96.3)
1-5	F/64	relapse	2+/+ (95.05)	2+/+ (95.09)	4+/0% (96.04)	DDS+RFP=B663 (96.3)
1-6	M/52	relapse	4+/8% (95.04)	3+/0% (96.03)		DDS+RFP+B663 (95.4); DDS+OFLX+B663 (96.3)
1-7	M/52	relapse	4+/1% (95.06)	5+/10% (96.06)		DDS+RFP+B663 (95.06)
1-8	F/72		4+/1% (95.06)			DDS (84-84); No. Tx (88-95); deaulter
5-10	M/63		2+/1% (95.10)			defaulter
6-2	M/55		3+/10% (93.06)	2+/2% (94.11)	1+/0% (97.06)	DDS+RFP+B663 (95); RMP+DDS+OFLX+CAM (96)
8-1	M/52		4+/10% (95.03)	3+/4% (97.04)	3+/5% (97.08)	DDS+RFP (95.03); DDS+B663=RFP (97.04); ROM (97.08)

RFP, rifampin; OFLX, ofloxacin; MINO, minocycline; CAM, clarithromycin.

homogenizer (Mickle Laboratory Engineering Co., Gomshall, Surrey, U.K.). After precipitating tissues at 1,000 ×g for 5 min, the supernatant was obtained. By alternately freezing and thawing 100 μl of bacterial solution in liquid nitrogen and in an 100°C water bath 5 times for 1 min each, total DNA was extracted and used as a PCR template to amplify the *rpoB* gene from *M. leprae*. *M. leprae* Thai 53 strain, which has been a seed culture for infection in nude mice at Institute of Hansen's Disease, was used as the control group for DNA extraction, PCR, SSCP, and sequencing.

Polymerase Chain Reaction

Because rifampin resistance has been suggested to be due to a point mutation in the *rpoB* gene that encodes an RNA polymerase β-subunit [1, 3, 8, 14], we synthesized the following PCR primers. The forward primer, rpo35 (5'-GCCGCAGACGCTGATCAATA-3'), was designed according to the study of Horone and Cole [1]. It was a 20mer starting from the 1,239th base on the *M. leprae rpoB* gene. The reverse primer, rpo38 (5-CACGTCACGGACCTCT-AGCC-3), was designed to yield 190 bp DNA fragments.

The reaction mixture for PCR contained 100 pmole of each primer, 0.25 mM of dNTP mixture, 48–49 μl of master reaction mixture containing 5 μl of 10× Ex Taq DNA polymerase buffer and 0.5 unit of Ex Taq DNA polymerase (Takara Shuzo Co, Otsu, Japan), and 1–2 μl of biopsy tissue extract. One or two drops of mineral oil (Sigma, St. Louis, MO, U.S.A.) were placed on the upper layer, and PCR was performed with a DNA thermal cycler 480 (Perkin Elmer Cetus, Norwalk, CT, U.S.A.). After denaturing at 94°C for 3 min, the mixture was put under 30 cycles of reaction at 98°C for 20 s and at 66–70°C for 1 min, and followed by incubation at 72°C for 10 min.

SSCP Analysis

Using 0.5× MDE (Mutation Detection Enhancement) gel solution (2× concentrate, FMC Co., Troy, NY, U.S.A.) and TBE buffer, 10 μl of PCR reaction was mixed with an equal amount of 2× loading solution (95% formamide, 10 mM NaOH, 20 mM EDTA, 0.02% bromophenol blue, and 0.02% xylene cyanol). The mixture was denatured at 95°C for 3 min and cooled fast, immediately loaded onto a 0.5× MDE gel [18 cm×18 cm×1.5 mm (H×W×D), Hoeffer Scientific Instruments, San Francisco, CA, U.S.A.], and followed by electrophoresis in a cold room at 150 V and 20 mA for 6–9 h. The mobility shift of a DNA band was examined after silver staining [11].

PCR-Directed DNA Sequencing

The nucleotide sequences were determined with a Taq Cycle Sequencing Kit (Takara Shuzo Co.) that combines a PCR technique and Sanger's dideoxy sequence determination method. DNA to be sequenced was first amplified by PCR with the same primer used for PCR-SSCP. The primer (540 pmole) was labeled at the 5'-end with ^{32}P by incubating for 30 min at 37°C in a buffer containing 67.5 mM Tris-HCl, pH 8.8, 67.5 mM KCl, 3.4 mM $MgCl_2$, [γ-^{32}P]dATP (50 pmole, 6000 Ci/mmol, Amersham, Piscatway, NJ, U.S.A.), and T_4 polynucleotide kinase (10 U, Takara Shuzo Co.) An 18 μl of reaction solution containing 1 pmole of the labeled primer, 10–100 fmole of template DNA, 10× cycle buffer, and Taq polymerase was divided into 4 μl aliquots in 4 PCR tubes, each containing 2 μl of 4 types of dNTP-ddNTP mixture. After overlaying mineral oil onto each tube, 1 cycle at 94°C for 30 s and 15 cycles of repetitive reaction at 94°C for 30 s, at 60°C for 30 s, and at 72°C for 1 min were performed, followed by 15 cycles of PCR at 94°C for 30 s and at 72°C for 1 min. After adding 4 μl stop solution into each of 4 tubes, 2 μl of PCR product was loaded into a 6% sequencing gel, and a gel was run at constant power of 50 W for 2 h. After electrophoresis, a gel was transblotted to a Whatman 3MM chromatography paper, and the paper was exposed to a X-ray film at -70°C for 12 h.

When a mutation was confirmed by the PCR-directed sequencing, the DNA fragment amplified with PCR was cloned into a PCR vector in order to further confirm the mutation. The nucleotide sequence was analyzed with an automatic sequencer (Pharmacia Biotech Co., Piscatway, NJ, U.S.A.). The PCR and nucleotide sequence analysis was repeated at least 3 times to eliminate mistakes from DNA sequencing.

RESULTS

The nucleotide sequence for the *rpoB* gene of *M. leprae* has already been reported [3], and the analysis of deduced amino acid sequence of RpoB protein shows 6 highly conserved regions in several bacteria [19]. Many mutations, which are known to induce rifampin resistance, are found to be strictly clustered in the two regions of the 3,540 bp of the *M. leprae rpoB* gene, the 320 bp nucleotide stretch spanning from 448 to 767th base and the 710 bp from 1,219 to 1,929th from the start codon [1, 3, 15, 17]. For this study, we designed the primers so that a shorter *rpoB* region than the one in other studies could be amplified (see Materials and Methods; [1, 3, 5, 15, 17]). When the annealing temperature was raised up to 68°C, nonspecific amplification was eliminated, resulting in more sharp bands (data not shown; [9]). The GC content of *M. leprae* genes is slightly high at 54–58%. Therefore, the high annealing temperature might decrease nonspecific amplification from *M. leprae* genomic DNA. However, we assumed that it decreased the amplification from contaminants in the DNA solution that was coextracted from the patients' biopsy tissues.

SSCP analysis is a technique widely used to examine gene mutations of a single nucleotide change. Since the

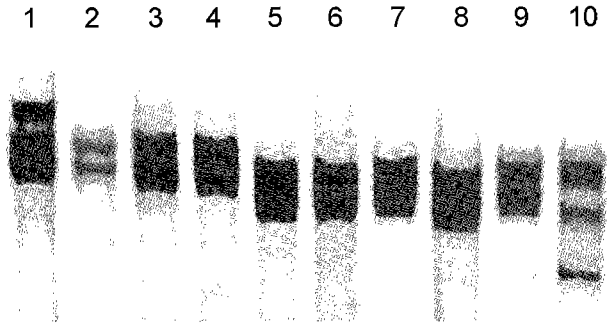

Fig. 1. PCR-SSCP patterns of the 190 bp *rpoB* region of *M. leprae*.
The 190 bp *rpoB* region amplified from biopsy samples of leprosy patients was analyzed by SSCP: lane 1, isolates from patients 1-4; lane 2, 1-5; 3, 1-6; 4, 1-7; 5, *M. leprae* Thai 53; 6, 4-1; 7, 4-3; 8, 4-7; 9, 5-11; 10, 8-2. Different mobility shift was observed among samples. Sequence analysis confirmed that the 190 bp fragments showing the same mobility shift (lane 5) as those from wild-type *M. leprae* Thai 53 (lane 6) did not contain a mutation, suggesting that SSCP is a reliable screening technique to detect mutations in the *rpoB* gene.

formation of single-stranded DNA is very sensitive to temperature and denaturants such as formamide and sodium hydroxide, changes of these conditions can cause drastic changes in the conformation, which can be determined with SSCP analysis. A small 0.5× MDE gel (18×18 cm) was prepared without adding glycerol, electrophoresed, and silver stained. As shown in Fig. 1, the SSCP patterns were quite variable among the samples obtained from several patients. Sequence analysis confirmed that all 190 bp fragments showing the different mobility shift from wild-type *M. leprae* Thai 53 contained at least one mutation, demonstrating that this SSCP is a reliable screening technique to detect mutations in the *rpoB* gene.

The nucleotide sequence analysis of PCR product from a total of 34 study subjects revealed that 19 subjects contained point mutations of missense mutations. In some cases, a missense mutation occurred only at one site, but in most cases occurred at several sites. The insertion or deletion mutations were not observed (Table 2, Fig. 2). Among 19 isolates, 5 strains contained the same mutations as already reported to cause rifampin resistance [1, 3, 5, 15, 17]. A ser-leu substitution in position 456, which was previously reported by Honore *et al.* [1, 2], was discovered in the samples from 3 patients, 4-7, 5-11, and 6-3. Interestingly, in the sample from the patient 5-11, an additional his-arg substitution in position 451 occurred, which has not been reported in a resistant strain of *M. leprae* but most frequently in a resistant strain of *M. tuberculosis*. In the sample from the patient 4-3, leu was substituted by pro in position 436, as in a resistant strain of *M. tuberculosis*, and arg in position 465 was additionally changed to his. Unlike as in a resistant strain of *M. tuberculosis*, where ser was substituted by leu in position 447, it was substituted by gly in the sample from patient 8-2. Because these mutations were discovered at the same sites in resistant strains of both *M. tuberculosis* and *M. leprae*, we suggest that the identified changes resulted from infection of a rifampin-resistant strain.

We also found the new type of mutations that occurred at the same sites in isolates from 8 Korean leprosy patients

Table 2. Sequence analysis of the Rif region from isolates of leprosy patients.

Isolates	Amino acid residue affected	Sequence change	Amino acid change
4-7	456	TCG→TTG	Ser→Leu
5-11	451, 456	CAC→CGC, TCG→TTG	His→Arg, Ser→Leu
6-3	456	TCG→TTG	Ser→Leu
4-3	436, 465	CTG→CCG, CGT→CAT	Leu→Pro, Arg→His
8-2	447	TCG→GGG	Ser→Gly
1-4	453, 464, 465, 467, 468	CGC→CGT,TCG→TTG,CGT→TGT, CGT→TGT, GCC→GTT	Arg→Arg, Ser→Leu, Arg→Cys, Arg→Cys, Ala→Val
1-5	454, 461, 464, 465, 467, 468	CGG→CGA, GGT→TGT, TCG→TTG, CGT→TGT, CGT→TGT, GCC→GTT	Arg→Arg, Gly→Cys, Ser→Leu, Arg→Cys, Arg→Cys, Ala→Val
1-6	464, 465, 467, 468	TCG→TTA, CGT→TGT, CGT→TGT, GCC→GTT	Ser→Leu, Arg→Cys, Arg→Cys, Ala→Val
1-7	420, 428, 464, 465, 467, 468	ATC→ATT, AAG→GAG, TCG→TTG, CGT→TGT, CGT→TGT, GCC→GTT	Ile→Ile, Lys→Glu, Ser→Leu, Arg→Cys, Arg→Cys, Ala→Val
1-8	464, 465, 467, 468	TCG→TTG, CGT→TGT, CGT→TGT, GCC→GTT	Ser→Leu, Arg→Cys, Arg→Cys, Ala→Val
5-10	453, 463, 464, 465, 467, 468	CGC→CGT, TTG→TCG, TCG→TTG, CGT→TGT, CGT→TGT, GCC→GTT	Arg→Arg, Leu→Ser, Ser→Leu, Arg→Cys, Arg→Cys, Ala→Val
6-2	449, 461, 464, 465, 467, 468	CTG→CCG, GGT→GGC, TCG→TTG, CGT→TGT, CGT→TGT, GCC→GTT	Leu→Pro, Gly→Gly, Ser→Leu, Arg→Cys, Arg→Cys, Ala→Val
8-1	420, 464, 465, 467, 468	ATC→ATT, TCG→TTG, CGT→TGT, CGT→TGT, GCC→GTT	Ile→Ile, Ser→Leu, Arg→Cys, Arg→Cys, Ala→Val

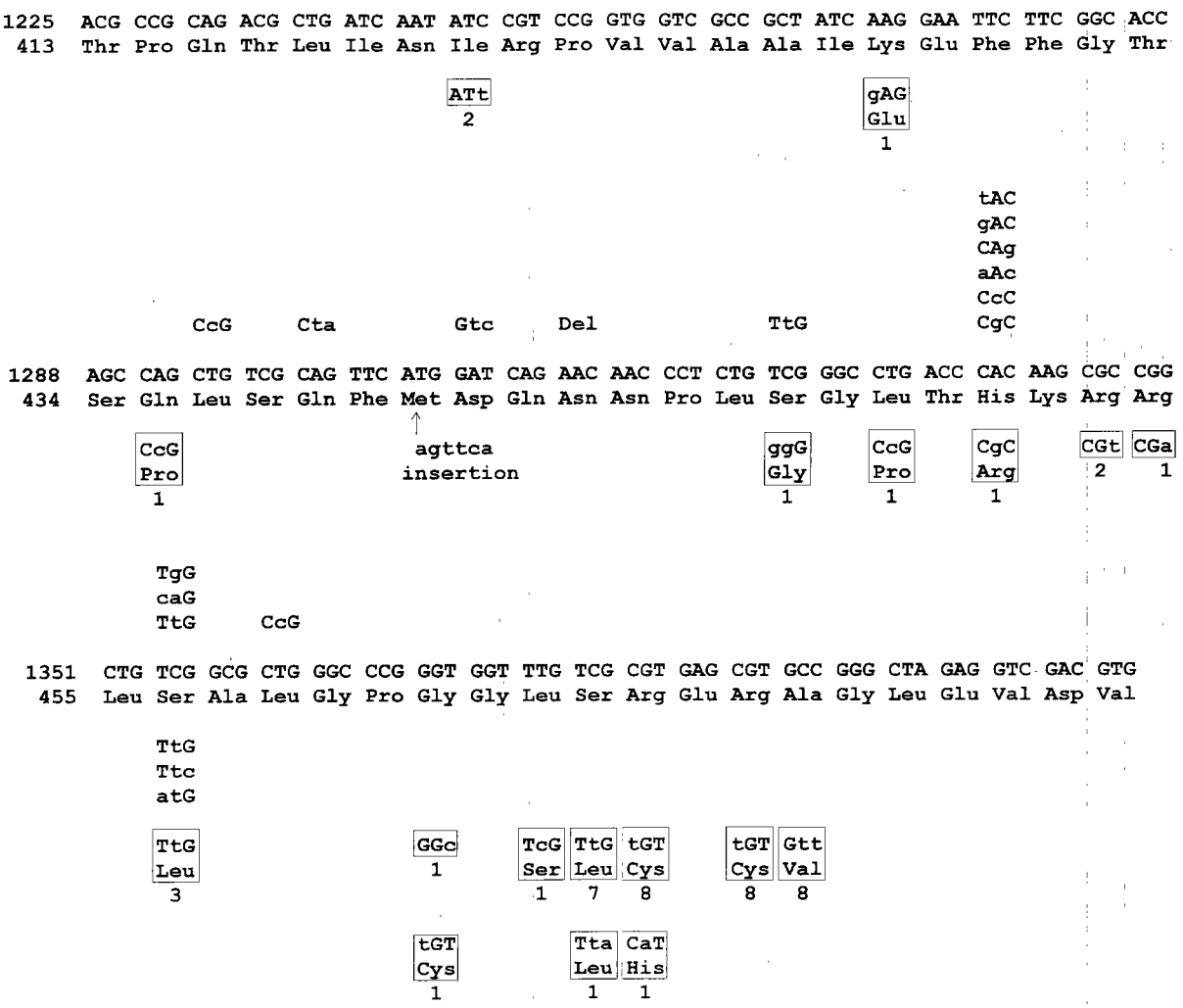

Fig. 2. Nucleotide sequence of the 190 bp region of the *rpoB* harboring mutations.
A nucleotide sequence and codon numbering system of *M. leprae* is adopted. The previously reported nucleotide changes in the *rpoB* genes of *M. tuberculosis* and *M. leprae* are aligned to the wild-type *rpoB* gene and indicated at above and below of the wild-type sequence, respectively. The mutations found in this study are indicated by a square, and the frequency of isolated mutation is indicated below the square. The nucleotide sequences reported in this study have been deposited in GenBank (Accession Nos are from AF200631 to AF200648 and AF200924).

(Table 2, Fig. 2). It was the substitution mutation of all cytosine to thymine from the 464th to 468th amino acids resulting in converting ser (464th) -arg (465th) -glu (466th) -arg (467th) -ala (468th) to leu-cys-glu-cys-val. Among these, a ser-leu substitution in position 464 resulted from a codon change of TCG to TTG, except for the sample from patient 1-6 in which it was changed to TTA (Table 2, Fig. 2). In addition to these mutations, samples from patients 1-4 and 8-1 showed two more base changes that were silent mutations. In samples from four patients, an additional amino acid change was observed. Gly was substituted by cys in position 461 in the sample from patient 1-5, lys by glu in position 428 from patient 1-7, leu by ser in position 463 from patient 5-10, and leu by pro in position 449 from patient 6-2. This type of mutation was frequently found (24%; 8 out of 34 patients) among the subjects under the current investigation. To confirm if mutations at this area induce resistance to rifampin, the strain have to be tested with an *in vivo* experiment. However, the mutations at this region can induce changes in the rifampin recognition site by altering the primary and secondary structure of the protein. The amino acid hydrophobic analysis revealed that hydrophilic amino acids were mutated to hydrophobic amino acids (Ser→Leu, Arg→Cys, Arg→Cys, and Ala→Val) (Fig. 3), and the prediction of secondary structure by a Chou and Fasman system showed that the α-helix structure of this region was also changed to a β-sheet form (Fig. 4). Therefore, these mutations may weaken the interaction of rifampin to RpoB protein, resulting in rifampin resistance.

The other mutations found in the samples from 6 patients may be responsible for rifampin resistance; however, they

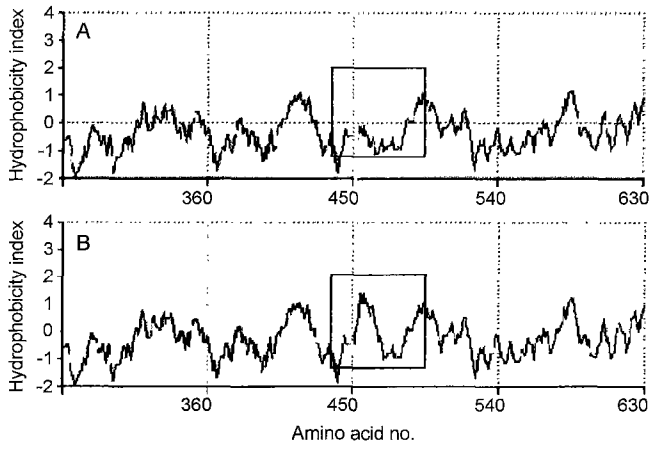

Fig. 3. Comparison of the hydrophobic profile of the mutated region in a RNA polymerase β-subunit with that of a corresponding region in wild-type *M. leprae*.
Among 1,179 amino acids of RpoB, only mutated regions are represented in the Kyte-Doolittle plots using window of 14. They show a remarkably different profile in the wild-type (A) and a mutant from the patient 1-8 (B) as indicated by boxes, demonstrating that the hydrophobicity of the mutated region is substantially changed.

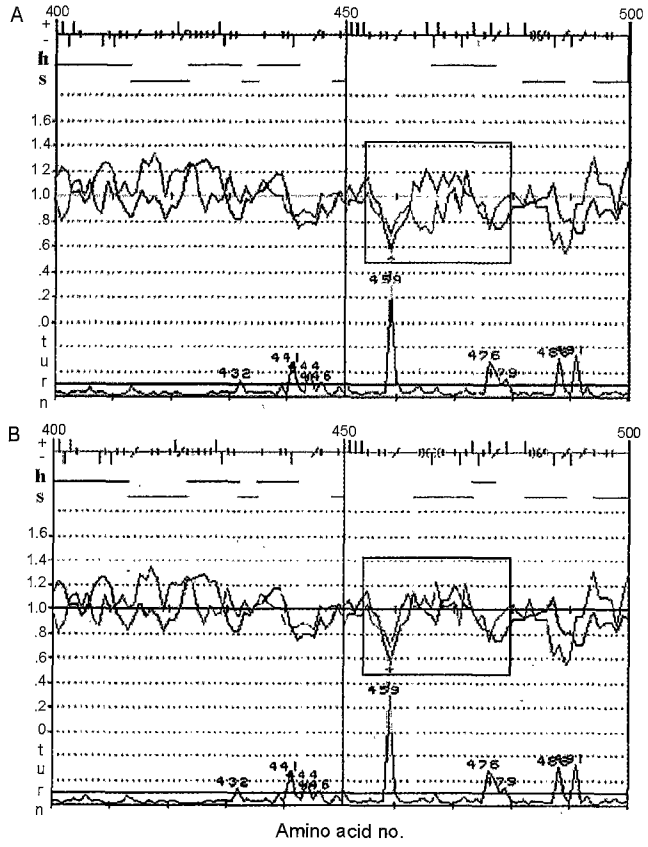

Fig. 4. Prediction of the secondary structure of the mutated region of RNA polymerase β-subunit in a mutant *M. leprae* from the patient 1-8 (B) and of the corresponding region in wild-type (A).
The change of α-helix (blue line) to β-sheet (red line) was shown in the boxes. Systems of Chou and Fasman were used in a Prosis program.

are not described in detail because there is no clue linking them to resistance.

DISCUSSION

In the context of increasing incidence of relapse in leprosy patients and the very slow growth rate of *M. leprae*, the rapid diagnosis of infection of rifampin-resistant *M. leprae* is in great need for efficient treatment. We developed a simple and easy PCR-SSCP method and applied it to detect mutations in the 190 bp of the *rpoB* gene. This SSCP method was far more efficient and faster than a previous PCR-SSCP method [15, 17, 18] that uses a large gel and nucleotides labeled with a radioisotope. In addition, the samples showing mobility shift contained mutations that are likely to cause resistance to rifampin. Therefore, this method would be very useful for early detection of rifampin-resistant *M. leprae* from biopsy samples of leprosy patients and thus help greatly in the treatment of leprosy with fast diagnosis of rifampin resistance.

Sequence analysis of PCR products showed mutations on the 190 bp of the *rpoB* gene in biopsy samples from 19 of 34 Korean leprosy patients who were being treated with MDT using rifampin, and who showed rifampin resistance during the course of treatment with rifampin or after a long period of treatment. This frequency of mutation was comparable to that reported by Grosset *et al.* in 1989, in which 22 out of 39 strains, separated from patients with recurrent leprosy, showed rifampin resistance [4]. Therefore, all of these 19 patients are likely infected with rifampin-resistant *M. leprae*. However, because only 190 bp sequences, which cover the most frequent mutation sites in the *rpoB* gene of a rifampin-resistant strain, were investigated in this study, the samples that did not show mutation at this region may have mutations at other regions.

As expected, the similar mutations known to cause resistance to rifampin were observed in samples from several patients. The substitution mutation of ser to leu in position 456, which was previously reported in rifampin-resistant *M. leprae,* was observed in the *M. leprae* isolated from 3 patients. The mutation at the same site as in *M. tuberculosis* but not reported previously in *M. leprae* was also detected in the samples from 2 patients. Therefore, the above 5 patients can be judged to have rifampin-resistant bacteria. On the other hand, in *M. leprae* separated from 8 patients, all cytosine bases were mutated to thymine at the 464, 465, 466, 467th amino acids and these sites were located at a different region from the most frequent mutation sites of *M. leprae* reported by Honore *et al.* [1, 2]. Although these mutations need to be evaluated with an *in vivo* test using nude mice, they are expected to be very important for resistance to rifampin. First, some of the *M. leprae* strains containing this type of mutations were isolated from patients

who were unresponsive or relapsed even on MDT (patients 1-7 and 8-1). Second, the mutations would change the primary and secondary structures of the RpoB protein. This may weaken the interaction of rifampin with RpoB.

Specific mutations have been identified in isolates of *Mycobacteria* of different geographical origin, which supported the concept of spontaneous mutations leading to rifampin resistance [12, 14, 16]. However, our finding of a new type of mutation in samples from Korean patients suggests the possible presence of clustering of rifampin-resistant mutants within a community.

The occurrence of multiple mutations in a single strain was unexpected. Most rifampin-resistant strains are known to harbor only one, or exceptionally two, mutations. It is unlikely that these mutations resulted from sequencing error because we confirmed it by three independent sequencings. Moreover, the PCR products were sequenced directly.

In summary, we established a fast and easy PCR-SSCP method that can be applied for early diagnostic detection of infection with a rifampin-resistant strain of *M. leprae*. In addition, we identified not only the mutations known to cause resistance but also the multiple mutations never before reported elsewhere. Our future study will be focused to confirm if the newly identified mutations are responsible for resistance to rifampin.

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