

A Mutation at Codon 516 in the *rpoB* Gene of *Mycobacterium leprae* Confers Resistance to Rifampin¹

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ABSTRACT

A missense mutation at codon 516 in the *rpoB* gene of *Mycobacterium leprae* conferring rifampin resistance was confirmed by the correlation between sequencing results and mouse footpad assay. The isolate was obtained from a relapsed lepromatous leprosy patient. This is the first report on the complete concordance between the mutation located at codon 516 in the *rpoB* gene and the corresponding resistance to rifampin in leprosy. The novel profile of mutation in the *rpoB* gene will contribute to the comprehensive understanding of rifampin resistant patterns and offer a useful tool for developing simple and rapid drug susceptibility testing approaches, which would promise more effective and successful control of leprosy.

RÉSUMÉ

Une mutation faux-sens localisée au codon 516 du gène *rpoB* de *Mycobacterium leprae* a permis l'expression d'une résistance à la rifampicine, qui a été confirmée par une corrélation entre les résultats du séquençage et le test d'inoculation à la patte de souris. L'isolat a été obtenu à partir d'un patient souffrant de lèpre lépromateuse et qui a rechuté. Ceci est le premier article rapportant une concordance complète entre la mutation localisée au codon 516 du gène *rpoB* et une résistance à la rifampicine dans le contexte de la lèpre. Ce nouvel éventail de mutation du gène *rpoB* va contribuer à une compréhension plus complète des alternatives de résistance à la rifampicine. Il devrait offrir un outil utile au développement d'approches pour le test simple et rapide de la résistance à la rifampicine, qui devrait résulter en un contrôle plus efficace et réussi de la lèpre.

RESUMEN

Una mutación sin sentido en el codón 516 del gene *rpo B* de *Mycobacterium leprae*, que le confiere resistencia a la rifampina, fue confirmada por correlación de los resultados de la secuenciación y del ensayo en la almohadilla plantar del ratón. La cepa fue obtenida de un caso de recaída de lepra lepromatosa. Este es el primer reporte sobre la concordancia perfecta entre la mutación localizada en el codón 516 del gene *rpo B* y la resistencia a la rifampina en la lepra. El nuevo perfil de mutación en el gene *rpo B*, aparte de que ayudará a entender los patrones de resistencia a la rifampina, constituye una nueva herramienta para el desarrollo de métodos simples y rápidos para probar la susceptibilidad de la bacteria a la droga, lo cual seguramente contribuirá al control exitoso y efectivo de la lepra.

Rifampin is a key component of multi-drug therapy (MDT) suggested by the World Health Organization (WHO) in the

treatment of leprosy (19). Resistance to rifampin often gives rise to treatment failure, which subsequently threatens the effective control of leprosy (1, 8). However, resistance to rifampin has been constantly documented since 1976, by using standard mouse footpad assay (2, 4, 12, 13, 15, 18). For this reason, it is suggested that clinically suspected relapsed leprosy cases and those who exhibit an unsatisfactory response to antileprosy therapy should be subjected to drug susceptibility testing. Understanding of drug resistance is essential for the effective

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tive treatment and control of leprosy. Therefore, simple and rapid methods for drug resistance testing are necessary. To establish such methods, solid basic data for the correlations between mutation and phenotype of drug resistance are required. In the present study, an isolate obtained from a relapsed Japanese leprosy patient was investigated for drug susceptibility testing by both genetic analysis and the standard mouse footpad method. It has been assumed that mutations which cause rifampin resistance in *Mycobacterium tuberculosis* are almost the same as in *M. leprae*. Although it has been confirmed that mutations at codon 513, 526, 531, and 533 in the *rpoB* gene of *Mycobacterium leprae* confer rifampin resistance, no substantial evidence shows whether a mutation at codon 516 relates to rifampin resistance or not. The goal of this report was to confirm the missense mutation at codon 516 in the *rpoB* gene conferring rifampin resistance.

MATERIALS AND METHODS

***M. leprae* isolate.** The isolate, named as Kusatsu-6, was detected in a skin biopsy sample obtained from a 75-yr-old Japanese lepromatous leprosy male patient. The patient had been treated with dapsone monotherapy for 18 yrs, and then with rifampin alone for 10 yrs before another 14 yrs monotherapy of dapsone. The patient relapsed and, because he was considered likely to have taken his medicine irregularly and had had long-term monotherapy, he was suspected of harboring drug-resistant *M. leprae*.

Drug susceptibility testing in the mouse footpad. The biopsy specimen was processed to recover *M. leprae* in the same manner as previously described (12). The initial bacillary suspension containing 1.0×10^6 in 0.05 ml of Hank's balanced salt solution (HBSS) was inoculated into the hind footpads of BALB/c-*nu/nu* mice since the viability of the bacilli in the material treated with antileprosy drugs was unknown. Approximately 12 months after inoculation, bacterial suspension for drug susceptibility testing was prepared from the nude mice footpads, which had shown bacillary multiplication. Drug susceptibility testing, for dapsone, rifampin, ofloxacin, sparfloxacin, clofazimine, and clarithromycin was per-

formed in the same manner as previously presented (11, 12, 13). Additionally, 0.08% minocycline (3) was added to the drug group for susceptibility testing in the present study. Bacillary growth in the mice footpads was examined after treatment with each drug for 25 weeks (17).

Genetic analysis for mutation. Sequencing was conducted as previously reported (11). Briefly, the initial biopsy suspension of Kusatsu-6 was partially purified by differential centrifugation and the pellet was resuspended in 50 μ l of lysis buffer consisting of Proteinase K and Tween 20, then incubated at 60°C for 18 hr, followed by snap freeze-heating (-84°C for 30 min and then 98°C for 10 min) to extract the genomic DNA and inactivate proteinase K.

Primers with the following sequences were used: folP F 5'GCT TCT CGT GCC GAA GCG CTC3' and folP R 5'GCC ATC GCG GGA TCT GCT CGC CCA3'; rpoB F0 5'CAG GAC GTC GAG GCG ATC AC3' and rpoB R0 5'CAG CGG TCA AGT ATT CGA TC3'; gyrA FN 5'CAG GTG ACG GTT CTA TAC AG3' and gyrA RN 5'TAC CCG GCG AAC CGA AAT TG3'. These amplimers target a 388-bp fragment of the *folP* gene, a 382-bp fragment of the *rpoB* gene and a 342-bp of the *gyrA* gene in *M. leprae*, which contain mutations corresponding to dapsone-, rifampin-, and quinolone-resistance, respectively. DNA was amplified by G mixture of FailSafe PCR System (EPICENTRE, Madison, WI, U.S.A.), the amplified product was verified by electrophoresis and recovered by using MinElute Gel Extraction Kit (QIAGEN, GmbH, Germany). The sequencing reaction was performed by the BigDye Terminator Cycle Sequencing FS Ready Reaction kit (Perkin-Elmer Applied Biosystems, Norwalk, CT, U.S.A.). Direct sequencing of the PCR products was performed with the ABI Prism 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems, Norwalk). Sequencing data was analyzed by the DNASIS program (Hitachi Software Engineering, Yokohama, Japan), as presented elsewhere (9, 11, 14). The DNA sequence was compared with that in the GenBank database.

RESULTS

Drug susceptibility in the mouse. Bacillary growth in mouse footpads administered

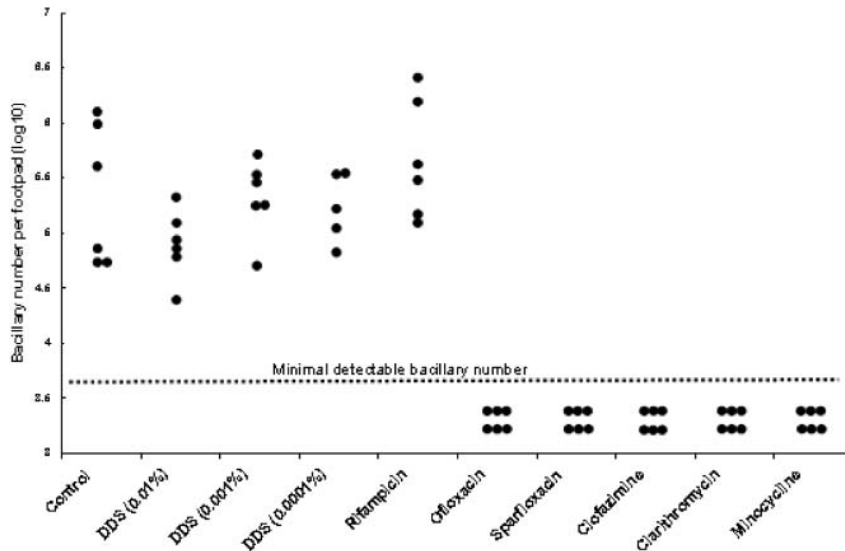


FIG. 1. Bacillary growth in footpads of mice administered anti-leprosy drugs.

0.01% rifampin, 0.01%, 0.001% and 0.0001% dapson showed almost the same level of growth as observed in the control mice. No bacillary growth was noticed in footpads of mice treated with ofloxacin, sparflloxacin, clofazimine, clarithromycin, and minocycline (Fig. 1). According to the results of this mouse footpad assay Kusatsu-6 was concluded to be resistant to rifampin and dapson at high concentration, but susceptible to the other drugs mentioned above.

Genetic analysis. The expected PCR products of the *folP*, *rpoB* and *gyrA* gene was successfully obtained from Kusatsu-6. The sequencing results displayed a missense mutation in the *rpoB* gene, affecting the codon at position 516 (numbering system applied for *E.coli*), GAT → TAT, leading to an amino acid substitution, Asp → Tyr, simplified as Asp-516-Tyr (Fig. 2). Similarly, a missense mutation at codon 55 (CCC→CTC, Pro-55-Leu) in the *folP* gene was revealed. No mutation was found at codon 89 or 91 in the *gyrA* gene.

DISCUSSION

Single point mutations within an 81-bp region in the *rpoB* gene involving 5 codons, Gly-513, Asp-516, His-526, Ser -531 and Leu-533 have been proved to lead to rifampin resistance in *Mycobacterium tuber-*

culosis (16, 20). The deduced amino acid sequence of this region presented 100% identity to that in *M. leprae* (22). According to the highly conserved nature of this region in the *rpoB* gene, six distinct mutations affecting 4 codons (Gly-513, His-526, Ser -531 and Leu-533) within this region carrying resistance to rifampin have been already clarified in *M. leprae* (1, 5, 11, 13, 22). Nevertheless, until now there has been direct evidence to explain whether the mutation at codon Asp-516 in the *rpoB* in *M. leprae* is linked to rifampin resistance, even if in *Mycobacterium tuber culosis* mutation at this codon Asp-516 confers rifampin resistance. To our knowledge, the present study is the first report to elucidate that this mutation at codon 516 is responsible for rifampin resistance in *M. leprae*.

As we know, the standard mouse footpad assay, which has been employed for drug resistance testing in leprosy for 40 yrs, requires not only long periods of at least 12 months to get results, but also requires considerable facilities, expertise and rigorous restrictions on the conditions of the *M. leprae* examined (7, 8). Therefore, it is necessary and urgent to establish rapid and routinely applicable approaches for the detection of drug resistance in leprosy. Recently, the characterization of the mutations at codons Gly-513, His-526, Ser -531 and Leu-533

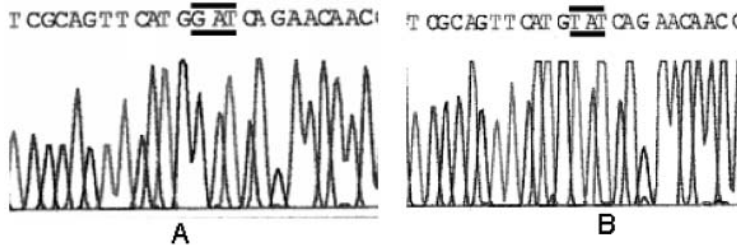


FIG. 2. rpoB sequencing result. (A) codon 516 GAT for amino acid Asp in wild type *M. leprae*. (B) codon 516 TAT for the substituted amino acid Tyr in Kusatsu-6.

have been used to set up simple and feasible alternative protocols. PCR-single strand conformation polymorphism (PCR-SSCP) (6), PCR-heteroduplex formation assay (PCR-HDF) (22), solid-phase hybridization to oligonucleotide capture probes (7) and Touch-Down PCR (10) have yielded satisfactory preliminary evaluations in leprosy. On the other hand, a comprehensive understanding of mutations in the *rpoB* gene correlated with rifampin resistance enhances the reliability and integrity of promising methods for drug susceptibility testing. This novel profile of the mutation at codon Asp-516 contributes to data on mutation patterns of rifampin resistant *M. leprae*, and is certainly worthwhile in the determination of drug susceptibility testing in leprosy. The multiplication of Kusatsu-6 in mouse footpads treated by rifampin indicated the full concordance with the missense mutation of Asp-516-Tyr in the *rpoB* gene.

In addition, the molecular mechanisms of dapsone- and quinolone-resistant *M. leprae* have been adequately described so far. Dapsone-resistant relevant mutations are limited at condons 53 and 55 in the *folP* gene (9, 21) whereas mutations reflecting resistance to quinolone affect codon 89 and 91 in the *gyrA* gene (11). Our results clearly demonstrated that the dapsone-resistant *M. leprae* harbored a missense mutation at codon 55 (CCC→CTC, Pro-55-Leu) in the *folP* gene. Mutations in the *folP* gene are commonly detected among Japanese relapsed leprosy cases because MDT was not applied to them until 2000, as we discussed previously (13). In spite of this, the lack of bacillary growth in the mice footpads administered ofloxacin and sparfoxacin was identical to the result of the *gyrA* sequencing. All the results presented complete

agreement between *in vivo* susceptibility and genetic tests.

In conclusion, the verification of the mutation at condon Asp-516 in the *rpoB* gene is involved in rifampin resistance in *M. leprae*. This finding offers valuable information for molecular drug susceptibility testing in leprosy and hopefully will help to provide a useful tool for the further successful control of leprosy all over the world.

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