Short Communication

Molecular Analysis of Isoniazid, Rifampin and Streptomycin Resistance in Mycobacterium tuberculosis Isolates from Patients with Tuberculosis in Düzce, Turkey

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SUMMARY: The aim of this study was to use DNA sequencing analysis to analyze the mutations in the most commonly targeted genes (katG, inhA, rpoB, rpsL) in isoniazid (INH)-, rifampin (RIF)- and streptomycin (SM)-resistant Mycobacterium tuberculosis strains obtained from subjects in Düzce, Turkey. Four isolates were found to be INH-resistant, 3 were RIF-resistant and 5 were SM-resistant, out of a total of 52 M. tuberculosis strains. In 3 of the 4 INH-resistant strains, a mutation in the katG gene in codon 315 appeared as AGC→ACC (Ser→Thr), and the other INH-resistant strain showed a mutation in the katG gene in codon 314 as ACC→CCC (Thr→Pro). There were no mutations in the inhA gene in INH-resistant isolates. Two of the 3 RIF-resistant strains were found to have mutations in the rpoB gene in codon 516 appearing as GAC→GTC (Asp→Val), and the other RIF-resistant strain had a mutation in the rpoB gene in codon 531 as TCG→TTG (Ser→Leu). These 3 RIF-resistant strains are also INH-resistant. All 5 SM-resistant strains have mutations in the rpsL gene in codon 43 appearing as AAG→AGG (Lys→Arg). Thus, we found common gene mutations that bring about the resistance of M. tuberculosis to antituberculosis drugs in all of our isolates from Düzce. To the best of our knowledge, the ACC→CCC (Thr→Pro) mutation in the katG gene in codon 314 has not been previously defined.

Mycobacterium tuberculosis is a slow-growing organism, and isolation, identification and drug susceptibility testing can therefore take several weeks. In recent years, many molecular methods have been developed for rapid detection, species identification and drug susceptibility testing of mycobacteria. Mutations in several genes and genomic regions of M. tuberculosis are involved in the occurrence of resistance to isoniazid (INH), rifampin (RIF), streptomycin (SM) and ethambutol (EMB), which are critical components of the first-line multidrug therapy for tuberculosis (1). The primary target of INH is mycolic acid synthesis, and mutations in the katG, inhA, kasA, and abpC genes result in resistance. INH resistance is apparently controlled by a more complex genetic system that involves several genes, as describe. Such mutations have been found in up to 90% of INH-resistant strains, and one particular substitution in codon 315 of the katG gene, AGC→ACC (Ser→Thr), is reported to be the most frequent (2-4). In general, mutation in the inhA gene is associated with a low level of INH resistance. Mutations in inhA not only cause INH resistance but also confer resistance to the structurally related second-line antituberculosis drug ethionamide (5). In previous studies, more than 97% of RIF-resistant M. tuberculosis strains showed a mutation in a 81-bp “core region” of the rpoB gene (6,7).

The target for SM resistance is the ribosomal proteins, Mutations in genes rpsL and rrs, which are involved in the synthesis of these proteins, have been shown to be responsible for 70% of SM-resistant strains (2,8). The drug resistance of M. tuberculosis to SM is related to rpsL gene mutation, with the mutation in codon 43 being the most common cause (9). EMB resistance is due primarily to emB gene mutations (47 to 65%) (10). In the present study, we analyzed mutations in the most commonly targeted genes (katG, inhA, rpoB, rpsL) in INH-, RIF- and SM-resistant M. tuberculosis strains obtained from clinical isolates from the Düzce School of Medicine Hospital.

M. tuberculosis cultures were grown on Löwenstein-Jensen (LJ) medium and drug susceptibility tests were performed according to the proportional method with susceptibility being defined as growth of less than 1% (10% for SM) in the presence of critical drug concentrations with LJ medium. Fresh colonies were collected from LJ medium and mixed with 200 UL distilled water to produce a suspension. Acid-fast bacteria were heated at 80°C for 60 min and mycobacterial DNA was then isolated from the sample using a NucleoSpin tissue isolation kit (Macherey-Nagel GmbH & Co., Düren, Germany). A total of 5 µl of the DNA extract was added to a reaction tube containing 45 µl of the PCR mixture (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1.5 mM MgCl2, 200 µM [each] deoxynucleoside triphosphate, 0.5 µM [each] primers) 5′-GAAACAGCGGCGCTGATCGT and 5′-GGCCGACAAACAGAACGT (14), 5′-CCTCGCTGCCCATTTCGTCGGGG for katG (12), 5′-CTCTGCTGCCCAGAGGGAG and 5′-ATCCCCGGTTTTCCTCCCGG for inhA (13), rpo95-5′-GTTTTCATGGAAGAGCGCCACAC and rpo397 5′-CTGGTCTGGAACCCCGAACGG GTTGAC for rpoB (14), 5′-GGCCGACAAACAGACGT and GTTCACCAACTGAGTGCAC for rpsL (11), and 1.25 U of Taq polymerase (Promega, Madison, Wis., USA). The

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amplification was carried out using a PCR System 9700 thermal cycler (Applied Biosystems, Foster City, Calif., USA). The product obtained after PCR was purified with a Microcon YM-100 spin column (Millipore-Amicon, Bedford, Mass., USA) and sequenced with a Big Dye terminator v3.0 cycle sequencing kit (ABI prism; Applied Biosystems). Next, the dye terminators were removed with a DyeEx 2.0 kit (Qiagen GmbH, Hilden, Germany). Finally, DNA sequencing analyses of the regions for katG, inhA, rpoB and rpsL were performed with an Automated 310 DNA sequencer (Applied Biosystems) to determine mutations in these regions.

The katG gene in 4 INH-resistant isolates was examined by sequencing, and mutations in codon 315 were detected in 3 of the 4 in the form of AGC→ACC, (Ser→Thr; the remaining INH-resistant strain mutation in the katG gene in codon 314 appeared as ACC→CCC (Thr→Pro), a mutation which we have not found in previous studies (2). There was no mutation in the inhA gene in INH-resistant isolates. Two of the 3 RIF-resistant strains were found to have mutations in the rpoB gene in codon 516 in the form of GAC→GTC (Asp→Val), while the remaining RIF-resistant strain had mutation TCG→TTG (Ser→Leu) in codon 531 of the rpoB gene. All 5 SM-resistant strains showed mutation AAG→AGG (Lys→Arg) in codon 43 of the rpsL gene. The mutations found in these resistant M. tuberculosis strains are shown in Table 1.

M. tuberculosis acquires drug resistance by antibiotic selection of mutations that occur randomly at chromosomal loci. Individual nucleotide changes (point mutations) confer resistance to single drugs, and the stepwise accumulation of these mutations leads to multidrug-resistant tuberculosis (1,12). In INH resistance, the most commonly observed alteration is a point mutation that results in an amino acid substitution at codon 315 of the katG gene. Typically, this substitution results in the replacement of the naturally occurring serine with threonine, AGC→ACC (Ser→Thr). It is estimated that this mutation occurs in 30 to 60% of all INH-resistant isolates (10,13-19,21); these data are consistent with the present results. In Russia, the katG AGC→ACC (Ser→Thr) mutation in M. tuberculosis isolates was screened by PCR collected from 1996 to 2001 and this mutation was found in 93.6% of 204 INH-resistant strains (20). The other INH-resistant strain mutation in codon 314 of the katG gene was ACC→CCC (Thr→Pro). Mutations in the promoter region of inhA occur in 20-34% of INH-resistant isolates, either alone or in combination with katG mutations (22). There was no mutation in the inhA gene in the 4 INH-resistant isolates.

The nucleotide sequences of the rpoB genes of M. tuberculosis strains isolated mostly from Asian countries were analyzed. In this gene, mutations in one of 3 codons (516, 526 and 531) account for the majority of RIF-resistant strains (70 to 95%), especially in areas with a high incidence of multiple drug-resistant tuberculosis (MDR-TB) (23-25). In a study by Karahan et al., 90% of RIF-resistant isolates from Turkish patients were found to carry a mutation in the rpoB gene, with 531 TCG→TTG (Ser→Leu) being the most frequent (26); similar results were also reported by Avkan et al. (27). Previously, it was shown that strains from the St. Petersburg area of Russia (24) and other Russian regions (28) show mutations in these three codons in the rpoB gene in 93.6% of RIF-resistant strains. Additionally, if an isolate is shown to be RIF-resistant, it often indicates multidrug resistance (29); in the present study, all RIF-resistant strains were also MDR-TB. Two of our 3 RIF-resistant strains showed mutation GAC→GTC (Asp→Val) in codon 516 and the third showed TCG→TTG (Ser→Leu) in codon 531. Mokrousov et al. have reported that the katG 315 and rpoB 531 mutations were found to be more prevalent among Beijing (96.8 and 77.3%, respectively), than among non-Beijing strains (85.7 and 28%) in St. Petersburg (30). We found similar mutation ratios to those obtained in previous studies on Turkey and Asian countries. The katG and rpoB mutations that were identified in previously reported MDR strains were also detected in our study. Ramaswamy et al. (23) found that the most common resistance-associated polymorphisms for the four drugs are the following: INH, AGC→ACC (Ser→Thr) (67.6%) in katG; RIF, TCG→TTG (Ser→Leu) (41.7%) in rpoB; and SM, AAG→AGG (Lys→Arg) (24%) in rpsL. In the present study, we also found similar mutations, in addition to an INH-resistant strain mutation in codon 314 of the katG gene, ACC→CCC (Thr→Pro), which has not been previously defined.

Table 1. The mutations found in resistant M. tuberculosis strains

<table>
<thead>
<tr>
<th>INH; katG and inhA</th>
<th>RIF; rpoB</th>
<th>STR; rpsL</th>
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<tbody>
<tr>
<td>Codon 314 ACC→CCC (Thr→Pro) Sample No. 1</td>
<td>Codon 531 TCG→TTG (Ser→Leu) Sample No. 1</td>
<td>Codon 43 AAG→AGG (Lys→Arg) Sample No. 2</td>
</tr>
<tr>
<td>Codon 315 AGC→ACC (Ser→Thr) Sample No. 2</td>
<td>Codon 516 GAC→GTC (Asp→Val) Sample No. 2</td>
<td>Codon 43 AAG→AGG (Lys→Arg) Sample No. 5</td>
</tr>
<tr>
<td>Codon 315 AGC→ACC (Ser→Thr) Sample No. 3</td>
<td>Codon 516 GAC→GTC (Asp→Val) Sample No. 3</td>
<td>Codon 43 AAG→AGG (Lys→Arg) Sample No. 6</td>
</tr>
<tr>
<td>Codon 315 AGC→ACC (Ser→Thr) Sample No. 4</td>
<td></td>
<td>Codon 43 AAG→AGG (Lys→Arg) Sample No. 7</td>
</tr>
</tbody>
</table>

1) There were no mutation at inhA gene in INH resistant strains.
The resistance of *M. tuberculosis* to SM is related to an *rpsL* gene mutation and mutations in codon 43 or 88 are the most common cause (2). Katsukawa et al. have reported that 18 t of 19 (94.7%) SM-highly resistant isolates carry a mutation in any *rpsL* gene at position 43 or 88 (31). Additionally, Huang et al. have found that 81.4% of mutations occur in codon 43 of TB-*rpsL* in the format of AAG→AGG, Lys→Arg (9). In the present study, we detected the same *rpsL* mutation on codon 43 in all 5 SM-resistant strains.

An understanding on the molecular level of the mechanisms of drug resistance in *M. tuberculosis* will enable us to develop improved tools for rapid diagnosis as well as to identify new targets to circumvent resistance and develop efficacious treatment regimens for patients with MDR-TB (29). In the present study, since we found common gene mutations that cause resistance of *M. tuberculosis* to antituberculosis drugs in all of our isolates. We also identified a new mutation, ACC→CCC (Thr→Pro) in codon 314 of the *katG* gene, that has not been previously defined.

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**REFERENCES**


