Rapid Detection of rpoB Gene Mutations in Rif-resistant M. tuberculosis Isolates by Oligonucleotide Microarray

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Objective To detect the specific mutations in rpoB gene of Mycobacterium tuberculosis by oligonucleotide microarray.

Methods Four wild-type and 8 mutant probes were used to detect rifampin resistant strains. Target DNA of M. tuberculosis was amplified by PCR, hybridized and scanned. Direct sequencing was performed to verify the results of oligonucleotide microarray.

Results Of the 102 rifampin-resistant strains 98 (96.1%) had mutations in the rpoB genes. Conclusion Oligonucleotide microarray with mutation-specific probes is a reliable and useful tool for the rapid and accurate diagnosis of rifampin resistance in M. tuberculosis isolates.

Key words: Mycobacterium tuberculosis; Rifampin resistance; rpoB gene / site mutation; Oligonucleotide microarray/detection

INTRODUCTION

Tuberculosis caused by M. tuberculosis is one of the most important re-emerging infectious diseases. The prevalence of drug resistant M. tuberculosis strains is the main cause for the increasingly serious infections[1-4].

Rifampin (rif), one of the principal first-line anti-tuberculosis drugs, inhibits DNA-directed RNA synthesis of M. tuberculosis by binding to the β-subunit of RNA polymerase. It has been reported that some site mutations in the β-subunit of M. tuberculosis RNA polymerase encoded by the rpoB gene result in drug resistance of the microbe. In general, rpoB mutations can be found in 96.1% of rif-resistant M. tuberculosis strains worldwide and these mutations usually locate in a region at the 507-533th amino acid residuals (81 bp) in the rpoB gene, which is often called rif-resistance-determining region (RRDR)[5-7]. Of the site mutations at RRDR, H526D/Y/L/R, S531L, D516V, and L533P are closely correlated with rif-resistance.

However, the biological features of bacteria, including drug resistance, usually differ in diverse geographical regions[8-12], and there may be diversities of site mutations of RRDR in the rpoB gene of M. tuberculosis isolates from different nations or various geographical areas. Tuberculosis is a common disease in China. Because of the vast areas of China, disparity in geographical environment, different sanitation levels and living habits in different regions, it is clinically important to determine the prevalent strains of rif-resistant M. tuberculosis and the dominant site mutation types at RRDR of rpoB gene in this country.

Therefore, in this study, we investigated the RIF-resistant frequency of M. tuberculosis strains isolated from sputum samples of active tuberculosis patients in Zhejiang province in southeast of China and the dominant site mutation types at RRDR in the rpoB gene of rif-resistant M. tuberculosis isolates. In addition, according to the investigation data, we developed an oligonucleotide microarray for the rapid detection of dominant site mutations at the 516, 526, 531, and 533 amino acid residuals at RRDR of M. tuberculosis rpoB gene extracted from sputum of the patients.

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MATERIALS AND METHODS

Source of Bacterial Strains

Rif-sensitive *M. tuberculosis* reference strain H37Rv (ATCC25618) was provided by the Chinese National Institute for the Control of Pharmaceutical and Biological Products. Sputum specimens were collected from active tuberculosis patients in nine hospitals in different areas of Zhejiang Province between January 2005 and December 2006. *M. tuberculosis* strains were isolated from the specimens according to the approved procedure of the Chinese Anti-tuberculosis Association. Briefly, the sputum samples were dealt with pollution for 15 to 20 minutes by 2% NaOH and cultured with Lowenstein-Jensen solid medium in an atmosphere containing 10% CO₂ at 37 °C for at least 7 days. Bacterial colonies were identified with Ziehl-Neelsen staining. A total of 235 clinical strains of *M. tuberculosis* were isolated and stored at -70 °C.

Drug Sensitivity Test

Drug sensitivity test for RIF was performed on Lowenstein-Jensen medium as previously described and MIC > 40 μg/mL was considered RIF resistant. In the drug sensitivity test, *M. tuberculosis* strain H37Rv (ATCC25618) was used as a control strain.

Detection of rpoB Gene

Genomic DNA from the 235 *M. tuberculosis* isolates and strain H37Rv (ATCC25618) was prepared with a bacterial genome DNA extraction and purification kit (BioColor). A loop of culture was suspended in 500 μL double distilled water and heated at 95 °C for 15 min. The DNA used for PCR amplification was obtained by heat shock extraction (1 min at 95 °C, 1 min on ice, repeated 5 times). The primer sequences for the rpoB gene amplification by PCR are 5'-TCG CCG CGA TCA AGG AGT -3' (forward) and 5'-TGC ACG TCG CGG ACC TCC A-3' (reverse). A high fidelity PCR kit (TaKaRa), in which Taq-Pfu mixture is used as DNA polymerase, was used to amplify the rpoB gene. The total PCR volume was 50 μL containing 200 nmol/L each primer and 5 μL bacterial lysate as a template. Amplification was initiated by incubation at 95 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, at 56 °C for 30 s, at 72 °C for 30 s, and a final extension at 72 °C for 5 min. The PCR products were routinely examined by electrophoresis on 1.5% agarose gel stained with ethidium bromide. The expected size of target amplification fragment was 157 bp.

Sequencing and Sequence Comparison

To obtain more stable and accurate sequencing results, a T-A cloning kit (TaKaRa) was used to clone the target amplification fragments. Sequencing of the inserted fragments in recombinant plasmids was performed by Invitrogen Company (Shanghai, China). Nucleotide sequences of the rpoB gene fragments were analyzed and compared using the BLAST software.

Preparation of RRDR Probes and Oligonucleotide Microarray

According to the sequencing results, over 90% of RIF resistant isolates harbored site mutations within an 81bp region (the codons from 507 to 533) in the rpoB gene. The most frequent mutation sites found in this study were H526D/Y/R/L (46.1%) and S531L.

<table>
<thead>
<tr>
<th>Probes Name</th>
<th>Target Sites</th>
<th>Sequences (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probe A</td>
<td>D516 (GAC)</td>
<td>5'-T15-TCATGGACCAGAACA-3'</td>
</tr>
<tr>
<td>Probe B</td>
<td>D516V (GAC→GTC)</td>
<td>5'-T15-TCATGTCAGAACA-3'</td>
</tr>
<tr>
<td>Probe C</td>
<td>D516Y (GAC→TAC)</td>
<td>5'-T15-ATTCTAGTACCAGAACA-3'</td>
</tr>
<tr>
<td>Probe D</td>
<td>H526 (CAC)</td>
<td>5'-T15-GTTGACCACACAGCCGA-3'</td>
</tr>
<tr>
<td>Probe E</td>
<td>H526D (CAC→GAC)</td>
<td>5'-T15-CTGTTGACCAGAACA-3'</td>
</tr>
<tr>
<td>Probe F</td>
<td>H526Y (CAC→TAC)</td>
<td>5'-T15-GTTGACCACACCAGC-3'</td>
</tr>
<tr>
<td>Probe G</td>
<td>H526R (CAC→CGC)</td>
<td>5'-T15-TTGACCACGCCGC-3'</td>
</tr>
<tr>
<td>Probe H</td>
<td>H526L (CAC→CTC)</td>
<td>5'-T15-TTGACCCTCAAAGC-3'</td>
</tr>
<tr>
<td>Probe I</td>
<td>S531 (TCG)</td>
<td>5'-T15-CGACGACTGCGCGTCG-3'</td>
</tr>
<tr>
<td>Probe J</td>
<td>S531L (TCG→TTG)</td>
<td>5'-T15-CGACTGTCGCGCGTCG-3'</td>
</tr>
<tr>
<td>Probe K</td>
<td>L533 (TCG)</td>
<td>5'-T15-GGCCGTCGGCCGGCGCG-3'</td>
</tr>
<tr>
<td>Probe L</td>
<td>L533P (TCG→TTG)</td>
<td>5'-T15-GTCGGCGCGCGCGCGCG-3'</td>
</tr>
</tbody>
</table>
(38.2%), and the rest were D516V/Y (6.9%) and L533P (2.9%), which are consistent with the reported findings\textsuperscript{[14-15]}. Therefore, we designed twelve labeled oligonucleotide probes, eight of which were designed to detect the different site mutations at D516, H526, S531, and L533, and the other four were designed to detect the same four non-mutated sites (Table 1). An oligonucleotide microarray including the twelve probes was prepared by TaKaRa Company and named as rpoB-RRDR8.

Detection of rpoB Gene in Sputum Specimens by Oligonucleotide Microarray

Each sputum specimen collected from 235 patients with positive \textit{M. tuberculosis} isolates was mixed with an equal volume of freshly prepared 4% NaOH -0.5% NaCl solution, and incubated at room temperature for 15-30 minutes to lyse the sputum. Then, 10 mL of 0.5 mol/L PBS (pH 7.4) was added, mixed and centrifuged at 3000 \( r/min \) for 15 min. The precipitate was suspended in 50 \( \mu \)L bacterial lysis solution and the suspension was transferred into a fresh 1.5 mL tube which was water-bathed at 100 °C for 5 min and centrifuged for collecting the supernatant. A high fidelity PCR kit (TaKaRa) was used to amplify the segment from the rpoB gene. The total PCR volume was 50 \( \mu \)L containing forward primer (200 nmol/L) and Cy3 fluorescein-labelled reverse primer with a proportion of 1:5 (forward:reverse) and 2 \( \mu \)L of the supernatant was used as a template. The PCR conditions were the same as described in the rpoB gene detection. The microarray (rpoB-RRDR8) was washed twice with 0.2% SDS and double distilled water, respectively, and dried at room temperature. The fluorescein-labelled PCR products were mixed with hybridization solution (10×SSC - 5% formamide - 0.2% SDS) with a proportion of 1:5, and water-bathed at 100 °C for 5 min to denature DNA and immediately cooled on ice for 5 min after heating. Ten \( \mu \)L of the mixture was added into hybridization area of the microarray water-bathed at 42 °C for 1 h and washed with 1×SSC-0.2%SDS, 0.2×SSC, and 0.1×SSC for 1 min, respectively. The dried microarray was scanned with a GenePix 4000B array scanner at a wave length of 532 nm for detecting Cy3 fluorescein (10 \( \mu \)m resolving power, 550V voltage and 10% PMT). The scanned images were analyzed using GenePix Pro4.0 software\textsuperscript{[16]} Hybirdization result was considered a non-mutant if the mean value of fluorescence signal using wild type probe divided by mutant type probe was larger than 3, while it was considered a mutant when the mean value of fluorescence signal using mutant type probe divided by wild type probe was larger than 3.

RESULTS

Rif-resistant Rate of \textit{M. Tuberculosis} Isolates

\textit{M. tuberculosis} strain H37Rv (ATCC25618) was sensitive to RIF with a MIC value of 0.016 \( \mu \)g/mL. Among the 235 \textit{M. tuberculosis} isolates, 102 (43.4%) were resistant to RIF and 133 (56.6%) were sensitive to RIF.

rpoB Gene Amplification Results

PCR confirmed that the 235 isolates of \textit{M. tuberculosis} were all rpoB positive (Fig. 1).

![Fig. 1. rpoB gene amplification fragments of \textit{M. tuberculosis} isolates.](image)

1: DNA marker; 2: blank control; 3: positive control using strain H37Rv (ATCC25618) DNA as atemplate; 4-7: rpoB gene amplification fragments of the isolates with No. 15 (S), 25 (S), 28 (R), and 43 (R), respectively.

rpoB Gene Sequencing of Isolates

In the sequences of RRDR in rpoB gene of RIF-sensitive \textit{M. tuberculosis} strain H37Rv (ATCC25618) (GenBank accession No.: NC_000962), the codons 516, 526, 531 and 533 encode Asp (D), His (H), Ser (S) and Leu (L), respectively\textsuperscript{[17]}. From our sequencing results, 96.1% (98/102) of the rif-resistant \textit{M. tuberculosis} isolates were found to have site mutations at the RRDR of rpoB gene (Table 2). Among the 98 rif-resistant variants, the most frequent site mutation was H526D/Y/R/L (46.1%, 47/102) followed S531L (38.2%, 39/102). Furthermore, two types of double site mutations were found in 98 resistant variants, namely D516V+H526D and H526L+S531L. Of the 133 rif-sensitive isolates, three showed site mutations at the RRDR of rpoB gene (Table 2). Among the 98 rif-resistant variants, the most frequent site mutation was H526D/Y/R/L (46.1%, 47/102) followed S531L (38.2%, 39/102). The distribution of probes and oligonucleotide microarray of rpoB gene are shown in Fig. 2. Rmix
was a mixture containing the probes for all the rif-resistant-related site mutations selected in this assay, and DMSO served as the negative control. The oligonucleotide microarray showed that the site mutations at RRDR in rpoB gene of sputum specimens were identical to those from the sequence analysis of PCR products. All the site mutation types found in this study are shown in Fig. 3.

### TABLE 2

<table>
<thead>
<tr>
<th>Isolates (n)</th>
<th>Site Mutation at the RRDR in rpoB Gene of M. Tuberculosis Isolates</th>
<th>Cases (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistant (102)</td>
<td>rpoB Codon No.</td>
<td>rpoB Base Change</td>
</tr>
<tr>
<td>516</td>
<td>GAC→GTC</td>
<td>D→V</td>
</tr>
<tr>
<td>516</td>
<td>GAC→TAC</td>
<td>D→Y</td>
</tr>
<tr>
<td>526</td>
<td>CAC→GAC</td>
<td>H→D</td>
</tr>
<tr>
<td>526</td>
<td>CAC→TAC</td>
<td>H→Y</td>
</tr>
<tr>
<td>526</td>
<td>CAC→CGC</td>
<td>H→R</td>
</tr>
<tr>
<td>526</td>
<td>CAC→CTC</td>
<td>H→L</td>
</tr>
<tr>
<td>531</td>
<td>TCG→TTG</td>
<td>S→L</td>
</tr>
<tr>
<td>533</td>
<td>CTG→CCG</td>
<td>L→P</td>
</tr>
<tr>
<td>516 and 526</td>
<td>GAC→GTC and CAC→GAC</td>
<td>D→V and H→D</td>
</tr>
<tr>
<td>526 and 531</td>
<td>CAC→CTC and TCG→TTG</td>
<td>H→L and S→L</td>
</tr>
<tr>
<td></td>
<td>Wild Type</td>
<td>Wild Type</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sensitive (133)</th>
<th>rpoB Codon No.</th>
<th>rpoB Base Change</th>
<th>Amino Acid Change</th>
<th>Cases (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>511</td>
<td>CTG→ATG</td>
<td>L→M</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>516</td>
<td>GAC→GAG</td>
<td>D→E</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>533</td>
<td>CTG→ATG</td>
<td>L→M</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wild Type</td>
<td>Wild Type</td>
<td>130</td>
<td></td>
</tr>
</tbody>
</table>

*Note.* D, V, Y, H, L, R, S, P, M, and E indicate aspartic acid, valine, tyrosine, histidine, leucine, arginine, serine, praline, methionine, and glutamic acid, respectively.

![Fig. 2. Spot matrix distribution of probes and oligonucleotide microarray of rpoB gene.](image)

DISCUSSION

Tuberculosis remains a major global public health problem[^3]. It is generally perceived that drug resistance is the major factor for its high prevalence[^1-4,12]. The reported epidemiological data indicate that *M. tuberculosis* RIF-resistant strains are
rpoB GENE MUTATIONS IN RIF-RESISTANCE M.TUBERCULOSIS

![Image](image.jpg)

Fig. 3. Oligonucleotide microarray showing different site mutations at RRDR of rpoB gene.


widespread in many countries and areas of the world, and its drug resistance is closely related to some special site mutations in the rpoB gene, especially at RRDR\[5-7,17-18\]. Although Zhejiang province is one of the most developed areas in aspects of economy, environmental sanitation and medical care in China, the morbidity of tuberculosis is high and the number of tuberculosis patients reach to approximate 100/100 000. Therefore, it is quite necessary to investigate the RIF-resistant rate of M. tuberculosis isolates and the correlation between RIF-resistance and site mutations at RRDR of rpoB gene, and to develop a convenient and fast oligonucleotide microarray for detecting the associated site mutations.

Of the 235 M. tuberculosis isolates from Zhejiang province, 102 (43.4%) were found to be RIF-resistant, and 133 (56.6%) were found to be RIF-sensitive. Although nearly half of the isolates were RIF-resistant, their resistance rate is still lower than that in many other areas, such as Europe, North America, and Africa (51.9%-68.9%), and Beijing Municipality of China\[12,18-23\]. However, it is similar to that in Korea and Shandong province of China\[13,24\].

We used Taq-Pfu mix as DNA polymerase. The expected target products were obtained from the M. tuberculosis isolates after PCR amplification. The sequencing results of PCR products showed that, among the 102 RIF-resistant isolates, the most common mutations at RRDR of rpoB gene appeared at H526 (46.1%) and S531 (38.2%) with a total rate of 84.3% (86/102). It was reported that S531 displayed the highest mutation frequency (54.5%-63.3%), followed by H526\[13,22,25-27\]. The above inconsistency with our findings suggests that there may be different mutation sites at RRDR in rpoB gene of RIF-resistant M. tuberculosis isolates in different countries or areas. It is interesting to note that two types of the three isolates found in this study displayed double site mutations (D516V+H526D and H526L+S531L), and the three isolates were RIF resistant with their MIC $\geq$ 256 μg/mL. Pontus and his colleagues (2006) reported a high RIF-resistant strain of M. tuberculosis with double mutations of L511P+D516G\[18\], which were also found in our study.

Moreover, our sequencing results demonstrate that multiple amino acid residuals (D, Y, R, and L) could substitute histidine at the site 526 with a percentage of 19.1%-29.8%, while leucine at the site 531 could only substitutes the original serine. Besides, the lower frequencies of D516V/Y (6.9%, 7/102) and L533P (2.9%, 3/102) indicate that D516V/Y and L533P are not the major mutation sites or types associated with RIF-resistance at RRDR in rpoB gene of the M. tuberculosis isolates.

Our sequencing results indicate that H526D/Y/R/L,
SS31L, D516V/Y, and L533P could cover almost the major mutation sites and patterns of RRDR in rpoB gene of the M. tuberculosis RIF-resistant isolates. M. tuberculosis needs a long culture cycle and its positive cultivation rate from clinical samples is relatively low. For a convenient and fast detection of site mutations, we developed an oligonucleotide microarray rpoB-RRDR8, by which spumt from tuberculosis patients could be directly used as a specimen for assay of mutation sites at RRDR in rpoB gene. The data about the associated site mutations could be accurately obtained by comparing hybridization signal levels detected using both the wild and mutant type probes.²⁶ According to these results, microarray rpoB-RRDR8 has a high sensitivity (fg DNA) and needs a shorter span of time.

In conclusion, microarray rpoB-RRDR8 can be used to detect RRDR-associated site mutations of RIF-resistant M. tuberculosis isolates from tuberculosis patients.

REFERENCES


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