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Association among the Rv2629 gene, rpoB gene, RFP resistance and Beijing genotype in *M. tuberculosis* clinical isolates

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Rifampin (RFP) is a major first-line anti-tuberculosis drug. The molecular assay currently used is the detection of rpoB gene mutations in *M. tuberculosis*. Recently, the Rv2629 191C allele was found to have a correlation with RFP resistance, and might become a valuable marker for the detection of RFP resistance or the Beijing genotype. We studied the association among the Rv2629 gene, rpoB gene, RFP resistance and Beijing genotype in 69 *M. tuberculosis* clinical isolates using DNA sequencing, conventional drug susceptibility and spoligotyping. The 191C allele was present in 92.8% (64/69) isolates. Of 29 RFP-sensitive strains, none exhibited any mutations in rpoB genes, only one strain (3.4%) was found to carry the 191A allele and 28 strains (96.6%) had the 191C alleles. Of 40 RFP-resistant strains, 30 (75%) strains had *rpoB* gene mutations, only 4 strains (10%) carried 191A alleles, and 36 strains (90%) exhibited 191C alleles. The 191C allele was also present in INH-sensitive, SM-sensitive or EMB-sensitive isolates. Spoligotyping analysis showed 8 distinct spoligotyping patterns. 81.1% (30/37) strains were divided into one big cluster, which had a characteristic of the Beijing genotype. Rv2629 191C allele was present in 93.3% (28/30) Beijing genotype strains, but also in non-Beijing genotype strains. These results indicate that high association is present between the ropB gene and RFP resistance. No association is present between the Rv2629 191C allele and RFP resistance, or between the Beijing genotype and RFP resistance.

Key words: *M. tuberculosis,* Rv2629, rpoB, rifampin resistance, spoligotyping.

INTRODUCTION

The combination of a tuberculosis (TB) epidemic and its drug resistance are quite severe in China. There were 4.5 million patients with active pulmonary TB and 2 million sputum smear-positive or culture-positive patients in 2000. About 27.8% of *M. tuberculosis* isolates were resistant to at least one primary anti-tuberculosis drug, and 10.7% were multidrug-resistant tuberculosis (MDR-TB) that is at least resistant to RFP and isoniazid (INH) (Ministry of Health of the People's Republic of China, 2000). Rapid identification of drug-resistant *M. tuberculosis* is very helpful for early effective treatment of TB and control of the spread of strains. The conventional drug susceptibility test requires more than 4 weeks and sometimes cannot meet the need in clinics. Rifampin (RFP), a rapid bactericide, is a major first-line anti-tuberculosis drug. It is documented that the rates of primary resistance and acquired resistance to RFP were 10.3 and 29.5%, respectively (Ministry of Health of the People's Republic of China, 2000). Previous studies have shown that it acts by binding to the β subunit of RNA polymerase and interferes with transcription and RNA elongation. The acquired resistance to RFP in 89 - 96% RFP-resistant *M. tuberculosis* clinical isolates resulted from mutations in the gene encoding the

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RNA polymerase β subunit (rpoB). Most of the mutations were situated from codon 509 to codon 533, especially in codon 531 and 526 (Lee Ann et al., 2003; Hirano et al., 1999; Varma-Basil et al., 2004). Recently, Wang et al. (2007) found a new 191A/C mutation in the Rv2629 gene that was significantly associated with RFP resistance in *M. tuberculosis*. However, Chakravorty et al. (2008) reported that Rv2629 191C allele was not associated with RFP resistance and that the allele could not be used as a molecular target to detect RFP resistance. The allele appears to be an excellent marker for the Beijing-W clade/SCG-2 phylogenetic group. Homolka et al. (2008) also confirmed that variations in Rv2629 (191 A/C) were specific for genotypes Beijing and Ghana, respectively, but not involved in the development of RFP resistance. Therefore, we studied the association among the Rv2629 gene, rpoB gene, RFP resistance and Beijing genotype in *M. tuberculosis*. Sixty-nine *M. tuberculosis* clinical isolates was analyzed using DNA sequencing, conventional drug susceptibility and spoligotyping.

MATERIALS AND METHODS

Strains and clinical isolates

The *M. tuberculosis* (H37Rv) reference strain was obtained from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. Sixty-nine *M. tuberculosis* clinical isolates were selected from the strain bank of the Institute for Tuberculosis Research of PLA, which were isolated from patients with tuberculosis at the Tuberculosis Departments of the Institute for Tuberculosis Research of PLA, Thorax Disease Hospital of Hebei province, China, between 2002 and 2005.

Conventional identification

The mycobacterial culture, conventional species identification and drug susceptibility testing performed in our laboratory were performed in accordance with the Chinese Laboratory Science Procedure of Diagnostic Bacteriology in Tuberculosis (Chinese Antituberculosis Association, 1995). Drug susceptibility testing was performed using the absolute concentration method on Lowenstein–Jensen medium. The presence of bacterial growth at an RFP concentration of 50 µg/ml was considered as an index of RFP resistance. Resistance to other antituberculosis drugs was also determined at the following drug concentrations: INH 1 µg/ml, streptomycin (SM) 10 µg/ml and ethambutol (EMB) 5 µg/ml.

DNA extraction

Cultured *M. tuberculosis* complex (about 5 mg) were transferred to microcentrifuge tubes containing 500 µl of TE buffer (10 mM Tris–1 mM EDTA, pH 8.0), heat killed at 80°C for 30 min, and harvested by centrifugation at 2,000 × g for 30 min. After the supernatant was discarded, cells were resuspended in 1 ml TE buffer containing 2 mg lysozyme and incubated at 37°C for 2 h. Sodium dodecyl sulfate (SDS) and proteinase K were added to final concentrations of 1% and 50 µg/ml, respectively, and the mixture was incubated at 55°C for 2 h. Cell lysates were extracted with phenol and chloroform. DNA was precipitated with 2 volumes of ethanol and a 1/10 volume of 3 M sodium acetate (pH 5.2), dissolved in TE buffer, and stored at -20°C.

PCR Polymerase chain reaction amplification and DNA sequencing

DNA was amplified at a final volume of 25 µl polymerase chain reaction (PCR) mixture containing 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM MgCl2, 200 µM dNTP (Saibaisheng, Beijing, China), 0.4 µM (each) primer, 10 to 100 µg of genomic DNA extracted from *M. tuberculosis* isolate as a template and 1 unit of *Taq* polymerase (Saibaisheng, Beijing, China). Specific primers for rpoB (forward primer, 5'-GGT GGT CGC CGC GAT CAA G -3', reverse primer, 5'-CGA GCC GAT CAG ACC GAT GT -3') were used to amplify a 240 bp fragments, and specific primers for Rv2629 (forward primer, 5'-GAC GAC TCG CAC GAC ACT CTT-3', reverse primer, 5'-CAG CCG AAT CAC CGT AG-3') were used to amplify a 238 bp fragments. The PCR reaction included 35 cycles of denaturation at 94°C for 1 min, primer annealing at 58 or 65°C for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. The PCR products were analyzed on ethidium bromide-stained 2% agarose gels. Each rpoB and Rv2629 gene fragments were sequenced by Canada Sangon Ltd., Beijing, China, with the primers used for PCR amplification. Obtained sequences were compared with those registered in the GenBank database by BLAST analysis.

Spoligotyping

Spoligotyping was performed on genomic DNA by the standard method described by (Kamerbeek et al., 1997). The oligo-nucleotides DRa (GGTTTTGGGTCTGACGAC, 5' digoxin-labeled) and DRb (CCGAGAGGGGACGGAAAC) were used as primers to amplify the whole DR region by PCR. The amplified PCR products were hybridized with spoligotyping Biodyne C membrane immobilized 43 oligonucleotide probes (Isogen Life Science, Holland). Detection of hybridizing DNA was done using digoxin label and chemiluminescent detection kit (Roche, Germany), followed by exposure to X-ray film (China) in accordance with the instructions of the manufacturer.

RESULTS

Phenotype identification of *M. tuberculosis* clinical isolates

An overview of the isolates investigated is given in Table 1. Of 69 clinical isolates of *M. tuberculosis*, 26 isolates were fully susceptible to RFP, INH, SM and EMB. Forty-three isolates were resistant to at least one anti-tuberculosis drug. Among these, RFP resistance was detected in 40 isolates (93.0%, 40/43), INH resistance in 32 isolates (74.4%, 32/43), SM resistance in 32 isolates (74.4%, 32/43) and EMB resistance in 21 isolates (48.8%, 21/43). 27 isolates were multidrug-resistant being at least resistant to INH and RFP.

Detection of the rpoB gene and Rv2629 gene by DNA sequencing

Sixty-nine *M. tuberculosis* clinical isolates was analyzed for the association among phenotypic drug resistance, rpoB genotype and Rv2629 191 allele type, by DNA sequencing (Tables 1 and 2). The 191C allele was

Table 1. Detection of 69 *M. tuberculosis* clinical isolates.

	No. of strains	Detection result			
		Conventional methods	rpoB sequencing	Rv2629 sequencing	Spoligotyping
	Standard strain H37Rv	Pansusceptible	Wild type	Site 191 A	
	1	Pansusceptible	Wild type	Site 191 A	403777707760771
	14	Pansusceptible	Wild type	Site 191 C	000000000003771
RFP-sensitive strains (n = 29)	1	Pansusceptible	Wild type	Site 191 C	400000000003771
	1	Pansusceptible	Wild type	Site 191 C	000000000003371
	1	Pansusceptible	Wild type	Site 191 C	777777777760771
	1	S	Wild type	Site 191 C	000000000003771
	1	E	Wild type	Site 191 C	000000000003771
	8	Pansusceptible	Wild type	Site 191 C	Not done
	1	HE	Wild type	Site 191 C	Not done
	1	RHE	Wild type	Site 191 A	000000000003771
	1	RSE	Codon 531 TTG	Site 191 A	000000000003771
	1	RHE	Codon 531 TTG	Site 191 A	774777777720311
	1	RS	Codon 531 TTG	Site 191 A	677777777700771
	2	RHSE	Codon 531 TTG	Site 191 C	000000000003771
	5	RHSE	Codon 531 TTG	Site 191 C	Not done
	1	RHSE	Codon 526 TAC	Site 191 C	000000000003771
	1	RHSE	Codon 533 CCG, Codon 515 ATT	Site 191 C	000000000003771
	1	RHSE	Codon 533 CCG, Codon 517 CAC	Site 191 C	Not done
	2	RHS	Codon 531 TTG	Site 191 C	000000000003771
	1	RHS	Codon 531 TTG	Site 191 C	000000000003761
	3	RHS	Codon 531 TTG	Site 191 C	Not done
RFP-resistant strains (n = 40)	1	RHS	Codon 531 TGG	Site 191 C	000000000003771
	1	RHE	Codon 531 TTG	Site 191 C	Not done
	1	RHE	Codon 526 CTC	Site 191 C	000000000003771
	1	RHE	Codon 517 CAC	Site 191 C	Not done
	2	RSE	Codon 531 TTG	Site 191 C	000000000003771
	1	RSE	Codon 531 TTG	Site 191 C	Not done
	1	RSE	Codon 511 CCG, Codon 516 GGC	Site 191 C	000000000003771
	1	RS	Codon 526 GAC	Site 191 C	Not done
	1	R	Codon 531 TGG	Site 191 C	Not done
	1	R	Codon 526 GAC	Site 191 C	Not done
	1	RHSE	Wild type	Site 191 C	Not done
	3	RHS	Wild type	Site 191 C	Not done
	1	RSE	Wild type	Site 191 C	Not done
	1	RH	Wild type	Site 191 C	000000000003771
	2	RS	Wild type	Site 191 C	Not done
	1	RE	Wild type	Site 191 C	000000000003771

R = rifampin-resistant, H = isoniazid-resistant, S = streptomycin-resistant and E = ethambutol-resistant.

present in 92.8% (64/69) of isolates. Of 29 RFP-sensitive strains, none exhibited any mutations in the rpoB genes, only one strain (3.4%) was found to carry the 191A allele and 28 strains (96.6%) had the 191C alleles in theRv2629 genes.

Of 40 RFP-resistant strains, 30 strains had a single-point mutation or two-point mutation involving codon 531, 526, 533, 517, 515 511 and 516 in the *rpoB* gene, only 4 strains (10%) carried 191A alleles and 36 strains (90%) exhibited 191C alleles in Rv2629 genes. The

Table 2. Rv2629 191A/C allele distribution.

Characteristic	Rv2629 191C allele no. (%)	Rv2629 191A allele no. (%)
Sensitive to all 4 drugs	25 (96.2)	1 (3.8)
Resistant to RFP	36 (90.0)	4 (10.0)
Sensitive to RFP	28 (96.6)	1 (3.4)
Resistant to INH	30 (93.8)	2 (6.2)
Sensitive to INH	34 (91.9)	3 (8.1)
Resistant to SM	30 (93.8)	2 (6.2)
Sensitive to SM	34 (91.9)	3 (8.1)
Resistant to EMB	18 (85.7)	3 (14.3)
Sensitive to EMB	46 (95.8)	2 (4.2)
rpoB mutation	27 (90.0)	3 (10.0)
rpoB wild type	37 (94.9)	2 (5.1)
Spoligotyping		
000000000003771	28 (93.3)	2 (6.7)
400000000003771	1 (100.0)	0 (0.0)
000000000003761	1 (100.0)	0 (0.0)
000000000003371	1 (100.0)	0 (0.0)
777777777760771	1 (100.0)	0 (0.0)
403777707760771	0 (0.0)	1 (100.0)
774777777720311	0 (0.0)	1 (100.0)
677777777700771	0 (0.0)	1 (100.0)

191C allele was also present in INH-sensitive, SM-sensitive or EMB-sensitive isolates (Table 2).

Genotyping by spoligotyping and Rv2629 191 alleles

Thirty-seven of 69 isolates were analyzed by spoligotyping. A total of 8 distinct spoligotyping patterns were observed. 30 strains (81.1%) were divided into one big cluster, which exhibited 9 dots of hybridization signals with oligonucleotide probe No. 35 to No. 43. In this biggest cluster (containing 16 drug-resistant strains and 14 drug-sensitive strains), 93.3% (28/30) strains had Rv2629 191C alleles. 7 isolates had unique spoligotypes (Figure 1, Tables 1 and 2).

DISCUSSION

The drug-resistant TB has been a serious public health problem in China. RFP is one of the major antituberculous drugs. RFP-resistant TB has a poor outcome of treatment. Rapid detection of RFP resistance would impel the early, effective therapy of TB. At present, available molecular markers for RFP resistance are rpoB gene mutations. It has been accepted as the “gold standard” for bacterial molecular identification (Mäkinen et al., 2006). However, approximately 5% of RFP-resistant M. *tuberculosis* isolates were found to have no mutations in the rpoB gene. New markers are needed to remedy the defect.

We studied the association among Rv2629 191 alleles, rpoB gene and RFP resistance in 69 *M. tuberculosis* clinical isolates. Compared with the conventional testing, rpoB gene mutations occurred in 30/40 (75%) RFP-resistant isolates and none in the RFP-sensitive isolates, which is in accordance with previous reports (Lee Ann et al., 2003; Hirano et al., 1999; Varma-Basil et al., 2004). The 191C allele was present in 28/29 (96.6%) RFP-resistant isolates and 36/40 (90%) RFP-sensitive isolates. The finding that 191C allele was present in RFP-susceptible isolates is similar to the data published by Chakravorty et al. (2008) and Homolka et al. (2008), but contrary to the result of Wang Q et al., who found 191C allele in 99.1% RFP-resistant isolates and not in any RFP-susceptible isolates (Wang et al., 2007). Chakravorty et al. found that 191C allele in 30.6% RFP-resistant isolates and 16.9% RFP-susceptible isolates. Homolka et al. found the 191C allele in 62.1% of MDR isolates. They also found that over expression of either Rv2629 191 allele in *M. smegmatis* did not produce an increase in RFP resistance. Furthermore, the Rv2629 191C allele was also present in the isolates with other first-line drug resistance. Therefore, Rv2629 191C allele is not correlative with RFP resistance and other drug resistances and cannot be used as a molecular marker to detect *M. tuberculosis* RFP resistance or any drug resistances.

In this study, the 191C allele was present in 64/69 (92.8%) isolates and the 191A allele was only present in 5/69 (7.2%)

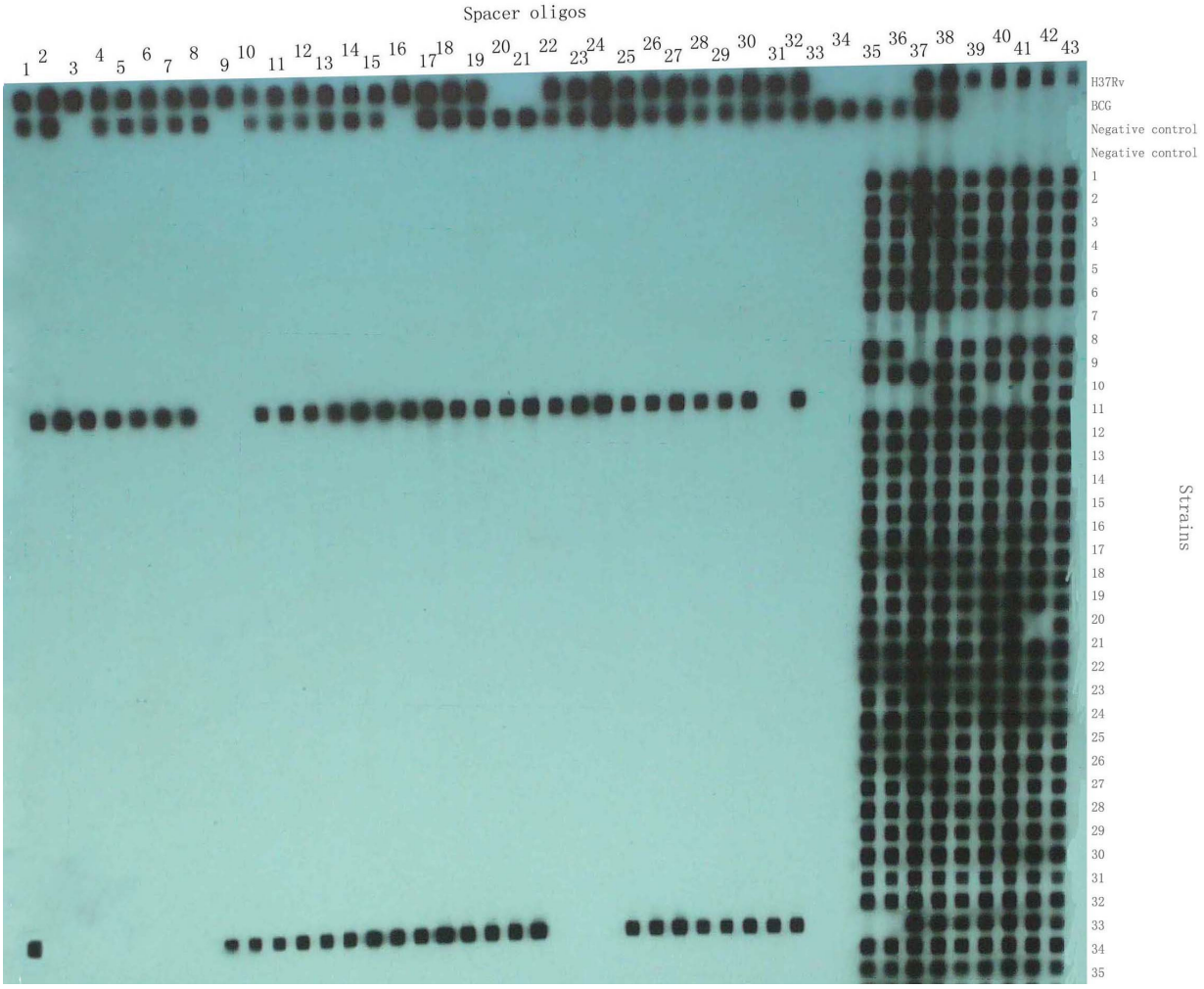

Figure 1. A representative spoligotyping result of *M. tuberculosis* H37Rv, *M. bovis* BCG and 37 different clinical isolates analyzed in this study. The PCR products of different isolates were hybridized with a membrane immobilized by 43 spacer oligonucleotide probes (vertical lines). The black dot represents the presence of the spacer and the blank space indicates the absence of the particular spacer within the amplified DNA from the isolates. The numbers in the right indicate the isolate number.

isolates. This suggests that the 191C allele is dominant in isolates from China. Chakravorty et al. found that the 191C allele was present in 46.5% isolates from Asia, while the 191A allele was present in 83.3 to 98.0% isolates from Africa, Europe, North American and South American. It is obvious that the 191C allele was more common in the isolates from Asia.

M. tuberculosis Beijing genotype strains exhibited the characteristic of 9 spacer hybridization among the probes No. 35 to No. 43 by spoligotyping analysis. They were extensively distributed in Asia, Europe, North American, and South American. They came from a same ancestor and were easy to spread in the population. In this study, the biggest cluster was accordance with the characteristic of Beijing genotype. In China, the distribution of Beijing genotype strains varies among the different provinces, higher (79 to 91%) in the Beijing region, 67% in Ningxia Hui Autonomous Region, 59% in Henan province, 55% in Guangxi province and lower (25%) in Guangdong province (Cao et al., 2008; Liu et al., 2007; Li et al., 2003; Li et al., 2003). All of these strains were collected from TB patients in local hospitals and therefore may have suffered from a selection bias. However, the results suggest that Beijing genotype strains were absolutely dominant in the northern area of China and do not correlate with drug resistance. Although, the 191C allele and Beijing genotype strain are common in Asia, Chakravorty et al. (2008) and Homolka et al. (2008) also found that the 191C allele appears to be a specific marker for the Beijing genotype strains, the 191C allele was present in Beijing genotype strains and non-Beijing genotype strains in this study. This study included fewer non-Beijing genotype strains, therefore the association between 191C allele and the Beijing genotype must be further investigated.

In conclusion, we confirmed that high association is present between the ropB gene and RFP resistance, but Rv2629 191C allele was not correlative with RFP resistance and any other drug resistances in *M. tuberculosis*. This suggests that 191C allele cannot be used as a molecular marker of any drug resistance. However, we found that the Rv2629 191C allele and the Beijing genotype strains were dominant in China. The Rv2629 191C allele was also present in non-Beijing genotype strains and their association should be further studied.

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