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Mutations in rpoB Gene and Genotypes of Rifampin Resistant Mycobacterium Tuberculosis Isolates in Iran

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ABSTRACT

Background: *Prevention and treatment of drug-resistant clones is important in guiding TB control strategies. The simultaneous rapid detection of the type of mutation conferring resistance and the genotype reflect the extent of drug resistant TB transmission within the communities.Mutations conferring resistance to rifampin in rifampin-resistant clinical Mycobacterium tuberculosis isolates occur mostly in the 81 bp rifampin-resistance-determining region (RRDR) of the rpoB gene.*

Materials and Methods: *Spoligotyping, IS6110- restriction fragment length polymorphism (RFLP) typing and sequencing of the rpoB gene were performed for 30 rifampin resistant M. tuberculosis isolates from patients referred to "Iranian National TB Laboratory" from 2006 to 2007.*

Results: *Mutations in the RRDR of the rpoB gene were identified in 96.6% of rifampin-resistant isolates. The spoligotyping analysis identified one (3.3%) East African-Indian (EAI) family, 7 (23.3%) Haarlem family, 9 (30.0%) Beijing family and 12 (40.0%) Central Asia (CAS) family isolates. Sixty- six percent of CAS isolates carried a mutation in codon 516, 37% of Beijing isolates carried a mutation in codon 531 and 33% of Haarlem isolates carried a mutation in codon 526.*

Conclusion: *Overall, there appeared to be a correlation between the genotype and specific mutations conferring resistance to rifampin in the Beijing and Haarlem families.* ***(Tanaffos 2008; 7(2): 11-17)***

Key words: *Mycobacterium tuberculosis, Rifampin resistance, rpoB gene, Spoligotyping*

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INTRODUCTION

Mycobacterium tuberculosis remains one of the most significant causes of death from an infectious agent, leading to 2 million deaths annually worldwide. As the incidence of tuberculosis has increased, there has been a corresponding rise in the incidence of drug- resistant strains of *M. tuberculosis* (1).Rifampin has proven to be an effective antituberculosis agent and its use has greatly shortened the duration of chemotherapy for the treatment of TB (2). The action of rifampin is believed to interfere with transcription in bacteria by binding to the β-subunit of RNA polymerase (the product of the rpoB gene) (3).There are newly identified areas with a high prevalence of drug-resistant tuberculosis in heavily populated countries such as China and Iran. Among new cases of tuberculosis in Iran, the prevalence of resistance to at least one drug is 5.0 percent and the prevalence of multi-drug-resistant tuberculosis among previously treated cases reaches 48.2 percent (4).In several regions of the world, the spread of drug-resistant TB has been associated with the expansion of strains belonging to the Beijing family (8).Genotyping of *M. tuberculosis* isolates has proven to be a powerful approach for investigating transmission dynamics, circulating strains, and the natural history of TB. The commonly used genotyping methods are spoligotyping and IS6110 restriction fragment length polymorphism (RFLP) typing. Families of *M. tuberculosis* isolates are defined on the basis of shared spoligotypes or closely related IS6110 RFLP patterns (9, 10). Because genotyping techniques measure variations in the *M. tuberculosis* genome unrelated to the acquisition of drug resistance, it was somewhat surprising to find that certain mutations appear to predominate in drug-resistant members of some families (9, 10). Mutations in the *rpoB* gene, encoding the β subunit of RNA polymerase, have been shown to be strongly associated with rifampin-resistant phenotypes in multiple study populations. The *rpoB* mutations are more likely segregated in an 81-bp region called the rifampin resistance-determining region (RRDR) (11, 12). The objective of the present study was to assess the presence of large families of strains in Iran to investigate correlations between specific rifampin resistance mutations and the genotypes of rifampin-resistant strains of *M. tuberculosis*.

MATERIALS AND METHODS

Strains and resistance testing: A set of 30 rifampin resistant *M. tuberculosis* complex strains obtained from sputum samples of patients referred to Iranian National TB laboratory from 2006 to 2007, were used. They were identified as *M. tuberculosis* using conventional methods including routine microscopy, culture and positive nitrate and niacin tests (13). The standard *M. tuberculosis* strains H37Rv was employed as control.

Drug susceptibility of all strains was determined by proportional method (13, 14). The rifampin concentration was 40 μg/1 ml. If the number of colonies grown on the rifampin-containing tube was less than 1% of the number of colonies grown on a drug-free medium, the isolate was defined as susceptible to rifampin. The isolate was resistant if the number was≥ 1% (13, 14).

DNA extraction: Genomic DNA was extracted as described by Van Soolingen et al. with modifications (15). Bacteria were harvested from the Lowenstein–Jensen slopes, heat-killed and incubated with lysozyme (1 h, 37°C) followed by digestion with 50 μg proteinase K in 10% SDS for 30 min at 65 °C. A further incubation with CTAB/NaCl for 10 min at 65°C was followed by sedimentation using chloroform/isoamyl alcohol (24: 1, v/v). Genomic DNA was extracted with phenol/chloroform and precipitated with 70% ethanol.

PCR amplification of mycobacterial strains: Aliquots of purified mycobacterial DNA (10–20 ng) were added to PCR reagents. The 250 bp rpoB fragment was amplified by using the primers rpob F (5-GGTCGGCATGTCGCGGATGG-3) and rpob R (5-GTAGTGCGACGGGTGCACGTC-3) as described previously (16); an initial step of 95°C for 5 min, 30 cycles of 95°C for 15 s, 62°C for 15 s, 72°C for 15 s, and a final step of 72°C for 5 min. Each 50µl reaction mixture contained 1 mM MgCL2, 200 nM concentrations of each primer, 100 µM concentrations of each deoxynucleoside triphosphate(dNTP), 10 ng template, 5 µl of 10XPCR buffer (Qiagen , Germany), and 1 U AmpliTaq Gold (Qiagen , Germany). The presence of PCR products was confirmed by agarose gel electrophoresis.

Purification of PCR products and DNA sequencing: PCR products were purified by using PCR purification kit (Roche, Germany). The PCR primers were also used for the direct sequencing of both strands of the amplification products with an automated ABI Prism 373 DNA sequencer and corresponding kits from the same manufacturer (Applied Biosystems, Foster City, Calif.). The Blast 2 sequences computer program was used for DNA sequence comparisons (http:// www. ncbi. nlm. nih. gov/BLAST/).

DNA fingerprinting: Mycobacterial genomic DNA from various isolates was digested with PvuII and then run on a Tris-borate-EDTA-0.8% agarose gel. The IS6110 probe used in southern hybridization was a 245-bp PCR DNA fragment amplified by PCR using INS-1 and INS-2 primers as described previously (17). The 245-bp PCR product was labeled with the DIG DNA labeling kit (Roche Diagnostics, Inc., Germany) before southern hybridization. Southern blotting was performed as described previously (18) and the hybridization membrane was examined photometrically.

Spoligotyping was performed with a commercially available kit to detect 43 known spacers in the DR locus according to the instructions supplied by the manufacturer (Isogen Bioscience B.V., Maarsen, The Netherlands). To avoid contamination on the commercial membranes, appropriate controls i.e, DNA from M. bovis P3, *M. tuberculosis* H37Rv and autoclaved purified water (adequate number of negative controls) were included in each experiment. The results obtained were entered in a binary format as excel spreadsheets, and compared to the updated international spoligotype database of the Pasteur Institute, Guadeloupe (19).

RESULTS

In addition to being resistant to rifampin, 5 isolates were resistant to isoniazid (MDR isolates).The spoligotyping analysis identified 1 (3.3%) East African-Indian (EAI) family, 7 (23.3%) Haarlem family, 9 (30.0%) Beijing family and 12 (40.0%) Central Asia (CAS) family isolates. The spoligotype of one isolate did not belong to a known family(orphan).The IS6110 RFLP assay of the rifampin-resistant and sensitive isolates revealed that the number of hybridizing bands ranged from 5 to 17. All isolates had unique fingerprints.

Mutations in the RRDR of the rpoB gene were identified in 29 (96.6%) of the 30 rifampin resistant isolates. The most frequent mutations were substitutions in codons 516, 526 or 531 of the rpoB gene, which were found in 25 (83.3%) of isolates (Table 1). Mutations in codon 531 of the rpoB gene were detected in 16 (53.3%) of 30 rifampin resistant isolates. Six isolates from these isolates belonged to the Beijing, 5 isolates belonged to the Central Asia (CAS) and 4 belonged to the Haarlem family (Figure 1).

Table 1. Description of spoligotypes and associated rpoB mutations identified in Iranian rifampin resistant *M. tuberculosis* isolates

Mutated Codon(s)	Amino acid substitution(s)	Genotype family				
		CAS	Beijing	Haarlem	EAI	Orphan
490,526 CAG3CAT; CAC3TAC	Gln3 His ; His3Tyr	1				
490,531 CAG3CAT;TCG3TTG	Gln3His; Ser3Leu		1			
513 CAA3CCA	Gln3Pro	1				
516 GAC3GTC	Asp3Val	1	1			
516 GAC3TAC	Asp3Tyr	1				
522 TCG3TTG	Ser3Leu	1				
526 CAC3TAC	His3Tyr	1	1	1	1	
526 CAC3GAC	His3Asp			1		
531 TCG3TTG	Ser3Leu	3	5	4		
531 TCG3TGG	Ser3Trp	2	1			
533 CTG3CCG	Leu3Pro			1		1
No mutation		1				
Total (30)		12	9	7	1	1

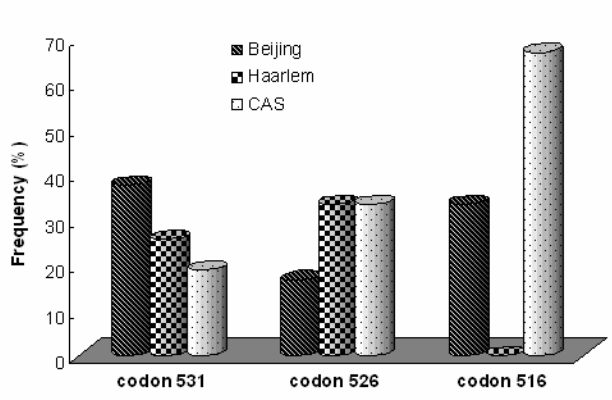

Figure1. Mutations in the rpoB gene in rifampicin-resistant *M. tuberculosis* isolates belonging to different families.

Mutations in codon 526 of the rpoB gene were detected in 6 (20.0%) of rifampin-resistant isolates. Two of these isolates belonged to the CAS, 2 isolates belonged to the Haarlem and the other 2 isolates belonged to the Beijing and EAI family.Mutations in codon 516 of the rpoB gene were detected in 3 (10.0%) of rifampin-resistant isolates. Two of these isolates belonged to the CAS and one isolate belonged to the Beijing family.Mutations in codon 533 of the rpoB gene were detected in 2 (6.6%) of rifampin resistant isolates. One of these isolates belonged to the Haarlem and the other did not belong to a known family (Orphan).

A mutation at codon 513 and a mutation at codon 522 were identified in two isolates belonging to the CAS family.

Two rifampin-resistant strains of *M. tuberculosis* contained missense mutations in two separate codons, including codons 490 and 526, codons 490 and 531. These two isolates were MDR isolates and belonged to CAS and Beijing families. The three other MDR isolates contained mutations at codon 531. Two of these isolates belonged to the Beijing and one belonged to the Haarlem family (Table 1).

DISCUSSION

Ten different missense mutations involving codons 490, 513, 516, 522, 526, 531 and 533 were identified in 33 (94.3 %) rifampin-resistant strains. It was observed that the majority (83.3%) of rifampin-resistant *M. tuberculosis* isolates contained missense mutations that led to substitutions of amino acids at

Ser-531 (53.3%), His-526 (20.0%) or Asp-516 (10.0%) in the core region of rpoB. Relative frequencies of the rpoB gene mutations in this study were similar to the world data (20,22). Various workers worldwide have shown different frequencies of mutation at codon 531 (29–74%) , 526 (0–43%) and 516 (0–38%) (20, 22), respectively.One (3.3%) rifampin resistant isolate did not show any mutation on sequencing. Similar results have been reported in earlier studies (23).

According to the spoligotypes, among clustered isolates, CAS, Beijing and Haarlem families accounted for 40.0%, 30.0% and 23.3% of the strains studied, respectively (Table 1). A previous study has shown that CAS super family is one of the major super families in Iran (24). CAS super family has been reported from 34 countries in varying numbers and is essentially limited to the Middle-East and Central Asia (Iran, Pakistan, Bangladesh, Sri-Lanka, Mauritius, and India) (25).

The frequencies of mutations in rpoB codons 531 (37.5%), 526 (16.6%) and 516 (33.3%) were detected in the Beijing family isolates. Previous studies have reported a significant correlation between Beijing genotype and multi-drug resistance. In earlier studies, 78.3% of MDR isolates belonging to this genotype from the Archangel Oblast had 531TTG mutation while 87.5% isolates from Estonia belonging to this genotype were MDR (26, 27). In Iran also the Beijing and Haarlem genotypes are overrepresented among MDR tuberculosis cases according to a previous study (28). But as the most of our isolates are mono resistant isolates, the percent of the Beijing super family containing mutations in this report**,** is less than previous studies which highlights the importance of this family in multi-drug resistance compared to mono-drug resistance.The frequencies of mutations in rpoB codons 531 (25.0%), 526 (33.3%) and 516 (0%) were detected in the Haarlem family isolates.

A previous study showed that the nature and frequency of occurrence of mutations in the rpoB gene were independent of spoligopatterns (29). But as it is shown in Figure 1 the Beijing and Haarlem families are important in rifampin resistance as well as multi-drug resistance in Iran. The figure shows that codon 531 and codon 526 mutations are over expressed in the Beijing and Haarlem isolates respectively. The predominance of mutations in rpoB codon 531 and codon 526 of the rifampin-resistant isolates belonging to the Beijing and Haarlem families is in good agreement with frequencies of mutations in codons of the rpoB gene and rates of growth of spontaneous rifampicin-resistant mutants of M. tuberculosis strain H37Rv (30).As different mutations in the rpoB gene were detected in the same IS6110 RFLP cluster, the rpoB gene mutations appear to have been acquired independently in most isolates during the course of chemotherapy.

Overall, the data indicated that the frequencies of individual mutations in the genes associated with rifampicin and streptomycin resistance vary dramatically among isolates belonging to different genotype families. Such variation may influence the performance of molecular diagnostic tests designed to detect mutations associated with drug resistance in *M. tuberculosis* isolates. This emphasizes the importance of validating the performance of a diagnostic test in the population being tested.

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