Identification of mutations in the *rpoB* encoding the RNA polymerase beta subunit in rifampicine-resistant *Mycobacterium tuberculosis* strains from Iran

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ÖZET

İran'da rifampisine dirençli Mycobacterium tuberculosis suşlarında rpoB kodlayan RNA polymerase beta subunit mutasyonlarının tanımlanması

Bu çalışmanın amacı; İranlı hastalardan izole edilen Mycobacterium tuberculosis suşlarında rpoB mutasyonlarının sıklığı, lokalizasyonu ve tiplerini belirlemekti. Tüberküloz şüphesi olan 91 hastadan balgam örnekleri alındı. M. tuberculosis olarak tanımlananların 34 (%87)'ünde Rif-r izole edildi. Polimeraz zincir reaksiyonu (PCR) amplifikasyonu ve DNA sekanslama metotları kullanıldı. rpoB geni 411 bp fragmanları sekanslandı ve 81 bp bölgelerinin mutasyonları incelendi. Yirmi dokuz RIF-r MBT'de (%85) 60 mutasyon ve 13 mikrodelesyon saptandı. Altmış mutasyon içinde altı sessiz ve 54 missens mutasyon belirlendi. Missens mutasyonlar 23 tip aminoasit değişikliğini ortaya çıkardı. Beş RIF-r MBT izolatında (%15) rpoB geni core bölgesinde hiçbir mutasyon saptanmadı. Tüm sessiz mutasyonların 507 numaralı kodonda yerleştiği belirlendi. İranlı suşlarda saptanan mutasyonlar en sık 523 ve 526 numaralı kodonlardaydı. Beş yüz yirmi altı numaralı kodonda 5 allel ve 507, 508 ve 513 numaralı kodonların her birinde üç allelde tripletler bulundu. Altı (%19) suşta 526, 510 numaralı kodonlarda tekli mutasyonlar varken, geri kalan 23 (%69) izolatta multipl mutasyonlar: ikili 11 (%34), üçlü 7 (%22), dörtlü 1 (%3) ve beşli 4 (%12) olduğu belirlendi.

Anahtar Kelimeler: *İran'da tüberküloz rpoB geni mutasyonu.*

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SUMMARY

Identification of mutations in the rpoB encoding the RNA polymerase beta subunit in rifampicine-resistant Mycobacterium tuberculosis strains from Iran

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The aim of this study was to investigate the frequency, location and type of rpoB mutations in Mycobacterium tuberculosis isolated from patients in Iran. 91 sputum were collected from suspected tuberculosis patients, 34 Rif-r isolates (87%) were identified as M. tuberculosis. Polymerase chain reaction (PCR) amplification and DNA sequencing methods were performed. 411 bp fragments of rpoB gene were sequenced and mutations in 81 bp regions were analyzed. 60 mutations and 13 micro deletions were identified in 29 RIF-r MBT (85%). Among 60 mutations, 6 silent and 54 missense were identified. Missense mutations produced 23 types of amino acid substitutions. In 5 RIF-r MBT isolates (15%) no mutations were found in the core region of the rpoB gene. All silent mutations were localized in codon 507. Most frequent mutations detected from Iranian strains were in codons 523 and 526. Five alleles in codon 526 and 3 alleles in triplets in each codons 507, 508, 513 were found. 6 (19%) strains harboured single mutations 6 (18%) placed in codons 526, 510 while the rest of isolates 23 (69%) had multiple mutations: Double 11 (34%), triple 7 (22%), and quartile mutations 1 (3%) and 4 (12%) of strains harboured 5 mutations respectively.

Key Words: *rpoB gene mutation tuberculosis in Iran.*

The World Health Organization estimates one-third of the world's population or approximately 2 million persons are or have been infected with *Mycobacterium tuberculosis*. In bacterial populations the generation of antibiotic resistance depends on the rate of emergence of resistant mutants (1-4). A correlation between high mutation rate, antibiotic resistance and virulence in bacteria has been reported in several studies (5-8). The detection of resistant *M. tuberculosis* strains is generally performed by conventional susceptibility method which requires to culture the bacilli in presence of the different drugs. The rapid detection of RMP resistance is of particular importance, since it also represents a valuable surrogate marker for MDR resistance, which is a tremendous obstacle to TB therapy (5,9,10). Collectively, DNA sequencing studies have demonstrated that > 95% of RMP-resistant (Rmp) *M. tuberculosis* strains have a mutation within the 81bp hot-spot region (codon 507 to 533) of the RNA polymerase beta-subunit (*rpoB*) gene (11,12). The prevalence of the mutations determined so far varies for *M. tuberculosis* strains obtained from different countries. Multidrug-resistant *M. tuberculosis* is an emerging problem of great importance to public health of Iran. Recently, the molecular basis of rifampicine resistance in *M. tuberculosis* was identified by Telenti, et al. (13). Thus, it is important to determine the distribution of resistance mutations at the level of each country prior to molecular tests being introduced for routine diagnostics (14-21). The key to control the spread of tuberculosis include proper case finding, rapid diagnosis of tuberculosis and prompt initiation of effective chemotherapy. Advances in molecular biology have provided powerful epidemiological tools for typing and detecting *M. tuberculosis* deoxyribonucleic acid (DNA). Drug resistance has been known since the discovery of the first anti-TB drug, streptomycin, in 1954 and the presence of resistant mutants in wild populations of mycobacteria has been well documented (9,22-29). Sur-

veillance of the primary and secondary resistance patterns are important in assessing the quality of chemotherapy programs over several years and detecting errors in past treatments respectively (30,31). The aim of this study was to determine resistance-associated mutations in the 81 bp region of the *rpoB* gene in 34 rifampicine-resistance *M. tuberculosis* among Iranian strains.

MATERIALS and METHODS

M. tuberculosis Isolates

From July to September 2005, 91 patients suspected of tuberculosis were referred to Mycobacteriology Department of Pasteur Institute of Iran with their clinical and radiological data and PPD skin tests. 34 isolates were recovered from sputum culture samples of patients who had been resistance to rifampicine.

Drug Susceptibility Testing

The anti-microbial drug susceptibility tests (AMST) of the isolated organisms were performed with conventional anti-tubercle drugs such as rifampicine (RMP) - 40 mg/L, isoniazid (INH) - 0.2 mg/L, etambutol (EMB) - 2 mg/L, ethionamide (ETH) - 20 mg/L, streptomycin (SM) - 4 mg/L, and kanamycin (K) - 20 mg/L, using proportional method (32).

PCR Amplification

DNA extraction were purified using Fermentas kit's (K512). A 411-bp fragment of the *rpoB* gene was amplified by PCR with primers *rpoB*-F (5-TACGGTCGGCGAGCTGATCC-3) and *rpoB*-R (5-TACGGCGTTTCGATGAACC-3). PCR was carried out in 50 µL of a reaction mixture containing 50 mM KCl, 10 mM Tris (pH 8.0), 1.5 mM $MgCl_2$, 5 µM of deoxynucleoside threephosphates (dNTPs), 1U *Taq* polymerase, 20 pmoles of each set of primers, and 6 µM of chromosomal DNA. Samples were then subjected to one cycle at 94°C for 5 min, followed by 36 cycles at 94°C for 1 min, 57°C for 1 min, 72°C for 1 min, and a final cycle at 72°C for 10 min to complete the elongation of the PCR intermediate products. PCR products were then run on 2% agarose gels and examined for the presence of the 411-bp band after ethidium bromide staining (Figure 1).

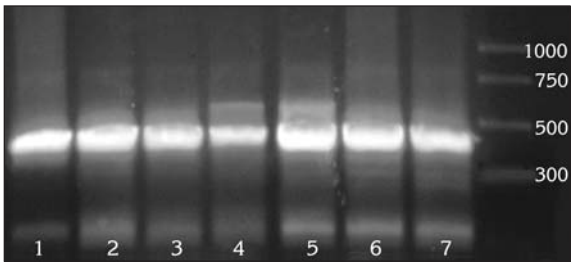

Figure 1. PCR amplification genome *rpoB* fragment 411bp.

34 cultures of Rif-r *Mycobacterium* spp. were isolated from 91 sputum specimens collected in Iran, 34 Rif-r isolates (87%) were identified as *M. tuberculosis*. The agarose DNA Extraction were performed by using Sigma Kit (124K6083). Sequencing reactions were performed with the DNA polymerase terminator cycle sequencing kit (Amersham) with 8 µL of PCR-amplified DNA as the template and 2.5 pmol of either the forward or the reverse primer.

DNA Sequencing

A 411-bp fragment of the *rpoB* gene, containing 81-bp *rpoB* fragment, was amplified by PCR using two primer: *rpoB*-F (5-TACGGTCGGCGAGCTGATCC-3) or *rpoB*-R (5-TACGGCGTTTCGATGAACC-3). PCR was carried out in 8 µL containing (0.25 µL polymerase, 0.9 µL Buffer for DNA polymerase, 2 µL Mixture dNTP and dNNTP (dATP, dTTP, dCTP, dGTP), 0.5 µL primer (2.5 Pmol), 1 µL DNA and 3.35 µL H_2O (molecular biology grade). Sequencing of the same primers with PCR parameters were used; 33 cycles of denaturation at 94°C for 30 min; primer annealing at 54°C for 30 sec; extension at 72°C for 90 sec. The *rpoB* gene fragments of tuberculosis strains were sequenced using the Amersham auto sequencer and Amersham Pharmacia DYEnamic ET Terminator Cycle Sequencing Premix Kits. Alignment of the DNA fragments (*rpoB*) was carried out with the help of MEGA software (Gen bank_ PUBMED/BLAST). The data were assembled and edited with MEGA and DNAMAN programs.

Data analyzing of DNA sequencing. DNA sequences from *rpoB* gene were analyzed by "Blast"

program. In this manner, sequences of standard strains of H37RV, CDC1551 and M.T.210 (W Beijing) were used as control and compared with test strains. Comparison of all sequences, mutations were performed, by applying "Mega" and "DNA MAN" program. Alignment of the DNA fragments (*rpoB*) was carried out with the help of MEGA software (Gen bank_ PUBMED/BLAST). The obtained data were assembled and edited with DNAMAN programs.

RESULTS

Bacterial Strains

All samples were cultured and identified as *M. tuberculosis* by PCR method. 34 Rmp-r *M. tuberculosis* clinical isolates (including MDR strains) were subjected to DNA sequencing analysis of the hyper variable (hot-spot) *rpoB* region.

Drug Susceptibility

All 34 isolates examined were resistant to rifampicine, isoniazid (80%), streptomycin (90%) and 18 isolates (48%) were resistant to etambutol. In this study we found two strains mono-resistance to rifampicine.

PCR Amplification and DNA Sequencing Analysis

All 34 samples were cultured and identified as *M. tuberculosis* by PCR method which revealed 73 mutations in all stains (Figure 1,2). No mutations were found in the core region of the *rpoB* gene in 5 RIF-r *M. tuberculosis* (15%). Of 60 found mutations 6 silent and 54-were missense. Most of detected deletions were identified in codon 510 GAG/_AG. All silent mutations were localized in codon 507, missense mutations revealed 23 types of amino acid substitutions (Figure 2). Most frequent mutated codons in Iranian strains were 523 GGG/GG_, GGG/GCG and 526 CAC/TAC, CAC/CGC, CAC/AAC, CAC/TTC, CAC/CAA, CAC/_GC (six types of mutations, Table 1,2). Mutations in codons 510, 507, 531 were observed in 27%, 24%, 21% of isolates correspondingly and mutations in codon 523 resulted in Gly523Ala replacement and in 531 Ser531Leu and Ser531Phe.

We observed 6 alleles in codon 526, 3 alleles in triplets 507, 508, 513. 6 strains (19%) harbored single mutations placed in codons 526, 510,

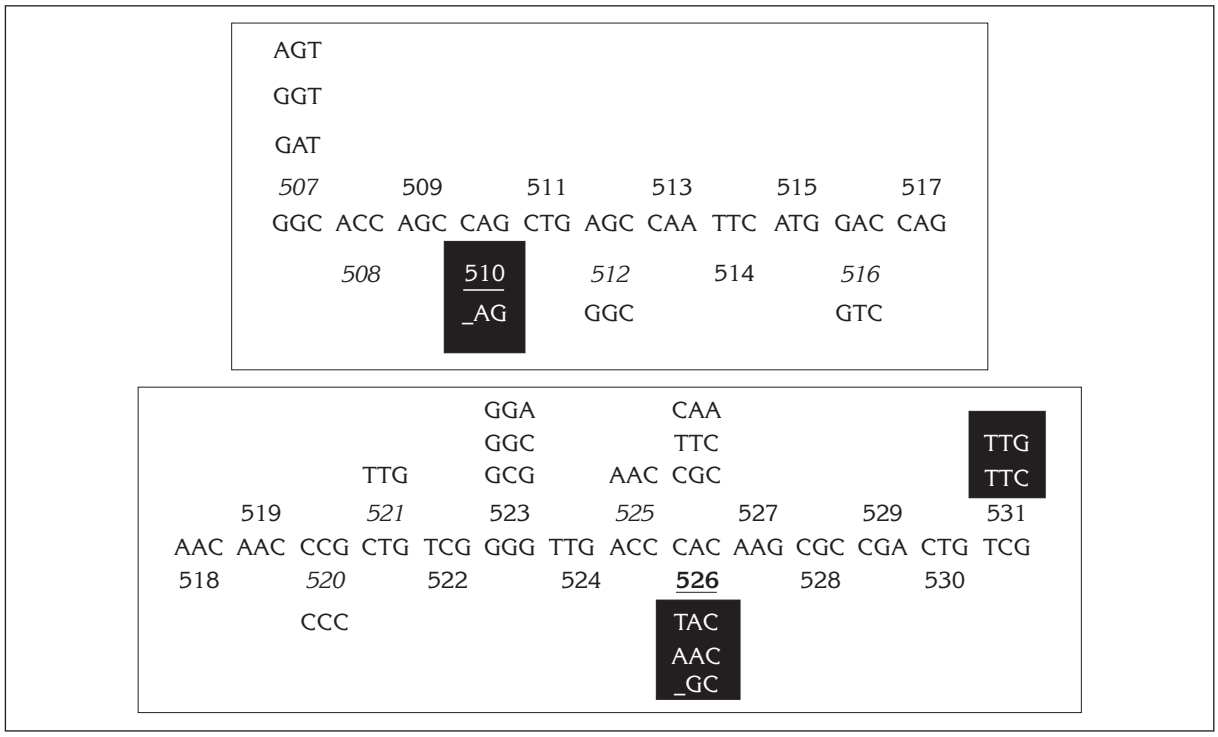

Figure 2. Location mutation in codon *rpoB* for isolates of Iran and type of mutation *M. tuberculosis*.

while isolates with multiple mutations reveled double 34%, triple 22% and quadruple 3% of the strains. 12% of strains harbored 5 mutations (Table 1,2).

DISCUSSION

Sequencing analysis of highly rifampicine-resistant (> 100 µg/mL) isolates were found to have deletion mutations in codons 510 and point mutations in 526, 523 and 531, which were most frequent in our study population. Other studies also indicated that these mutations are the most prevalent worldwide (1,5,6,9,17). Additionally, we observed two alleles in codon 531 that previously had not been described: Of 7 strains five TCG/TGG (Ser/Leu) and two TCG/TTC (Ser/Phe) exchanges were found. Mutations associated with nucleotide replacements in codons

Table 1. Frequency of amino acid and nucleotide changes of different codons in *rpoB* gene of 34 rifampicine-resistant strains of *M. tuberculosis* isolated in Iran.

Codon and amino acid change	Nucleotide change	Frequency	Number of isolates
531 Ser → Leu	TCG → TTG	5 (6.78%)	3708, 441, 163(2), 29(2), 710
531 Ser → Phe	TCG → TTC	2 (2.78%)	159, 163
526 His → Tyr	CAC → TAC	4 (5.5%)	3062, 108, 36, 159
526 His → Asn	CAC → AAC	1 (1.39%)	167
526 His → Stop	CAC → _GC	1 (1.39%)	165
526 His → Arg	CAC → CGC	3 (4.2%)	663, 600, 710
526 His → Phe	CAC → TTC	2 (2.78%)	36asli, 161
526 His → Stop	CAC → CAA	1 (1.39%)	163
510 Gln → Stop	CAG → _AG	9 (12.51%)	90, 633, 411, 73, 23, 3708, 441, 163(2), 29(2)
507 Gly → Ser	GGC → AGT	1 (1.39%)	3542
507 Gly → Gly	GGC → GGT	6 (8.3%)	19, 10, 33, 10(2), 163, 710
507 Gly → Asp	GGC → GAT	1 (1.39%)	159
508 Thr → Ala	ACC → GCC	1 (1.39%)	290
508 Thr → Pro	ACC → CCC	3 (4.2%)	3548, 3542, 663
508 Thr → His	ACC → CAC	2 (2.78%)	710, 163
509 Cys → Asp	AGC → GAC	1 (1.39%)	600
511 Leu → Ser	CTG → CCG	2 (2.78%)	303-281, 165
511 Leu → Val	CTG → GTG	1 (1.39%)	600
512 Ser → Tyr	AGC → GGC	2 (2.78%)	36asli, 710
512 Ser → Gly	AGC → GCC	1 (1.39%)	159
513 Gln → Asn	CAA → AAT	1 (1.39%)	36asli
513 Gln → Stop	CAA → TAA	1 (1.39%)	159
513 Gln → Stop	CAA → GAA	1 (1.39%)	600
516 Asp → His	GAC → CAC	1 (1.39%)	663
519 Asn → Lys	AAC → AAG	1 (1.39%)	600
520 Leu → Stop	CCG → C_G	1 (1.39%)	303-281
523 Gly → Ala	GGG → GCG	16 (22.24%)	167, 161, 290, 3548, 173, 23, 19, 10, 33, 10(2), 3708, 441, 163(2), 303-281, 165, 710
523 Gly → Stop	GGG → GG_	1 (1.39%)	29(2)
527 Lys → Stop	AAG → delation	1 (1.39%)	36asli

Table 2. Data for *rpoB* mutations (single, double, triple, quartile five) in rifampicine-resistance *M. tuberculosis* strains isolated from Iran.

Mutation	Number of codon	Number of isolates	Isolate number
Non mutation	-		23(2)-28-584, 103, 29
1 Mutation	526	3	3062, 108, 36
	510	3	90, 633, 411
2 Mutations	523-526	2	167, 161
	508-523	2	290, 3548
	510-523	2	173, 23
	507-508	1	3542
	507-523	4	19, 10, 33, 10(2)
3 Mutations	510-523-531	4	3708, 441, 163(2), 29(2)
	508-516-526	1	663
	511-520-523	1	303-281
	511-523-526	1	165
4 Mutations	507-508-526-531	1	163
5 Mutations	512-513-526-527-531	1	36asli
	507-508-512-523-526	1	710
	507-512-513-526-531	1	159
	509-511-513-519-526	1	600

510, 526, and 523 were associated with high-level of rifampicine resistance (> 100 µg/mL), whereas mutations in codon 516 were observed in low-level rifampicine resistance (p< 0.005) (Table 1). This finding is not in agreement with other authors who have reported different levels of high (18,20,23) and low (17,23) resistance association with specific nucleotide replacements. These differences reflect the complex and crucial interaction between the drug and its target at the molecular level, where the position of the affected allele seems to be variable.

This is the first report describing the genetic characteristics of multidrug-resistant *M. tuberculosis* strains isolated from TB-patients in Iran. Rifampicine is generally used as the first line drug for TB treatment. Mutations in *rpoB* gene of *M. tuberculosis* is indicative of multidrug resistance. Our finding of mutations is partially comparable and resemble to those reported strains from other countries (1,4,5,10,17). The *rpoB* codons 531, 526, 516 and 511 are the most frequently mutated sites worldwide, although variations in the relative frequencies of mutations in these codons have been described for isolates from different geographic locations (1-4,7,13). CAG mutation of codon 510 (deletion or CTG or CAC or CAT) is very seldom detected in other countries. However in our study (Table 1) we found much more mutation deletion (9 strains) in one base C (_AG). On the other hand, in other countries there are no changes in codon 510 (1,17,22). Mutation CAG → CAT was found in India (2), in Russia - CAG → CAT, in Belarus CAG → GAG, TAG were also found in this codon, in Lithuania CAG → GAG and in Poland CAG → GAG (1,9,17,22,25,26). Our result indicate Prominent findings which is in contrast with other reported investigations on codons 510 (12.51%), 523 (23.6%) and 526 (16.6%) which are the most frequent mutations bearing sites.

It should be mentioned that mutations in codons 531 and 526 are the most frequent in the world (TCG → TTG for codon 531, and CAC → TAC for 526) (1,2,8,17,23,27). There are other changes found in codons 531 (in India - TCG → TGG, TTG, in Russia - TCG → TGG, CAG or TGT, in China - TCG → TTG, in Japan- TCG → TTG, in Korea- TCG → TTG, in Taiwan- TCG → TTG and Ser → Gln) and 526 (in India - CAC → CTC, TAC, GAC, CGC or ACC in Russia - CAC → CTC, GAC, CAA, CAG, TGC, AAC, CGC or

CCC, in China - CAC → TAC, in Japan - CAC → TAC, in Taiwan CAC → TAC and CGC, in Korea CAC → TAC). In our study the changes in codon 531 TCG → TTG was found in 6.78% and change TCG → TTC in 2.78% of all isolates. The change CAC → TAC in codon 526 was found in four strains, in contrast we have not observed the change GAC, CTC in all isolates (8,10,12,23,24,29).

While mutation of CAC to GAC at codon 526 occurred at frequencies of 40.1% in Italian isolates (20) and 17.6% in Greece isolates (13), CAC to TAC at codon 526 was dominant in American isolates 27.9% (2,5,14) and Brazilian isolates 11% respectively. Our data shows very close relation to those observed in Asia (60%) while in contrast to Italian and Greece (His-526-Asp 40.1%) we have not found His-526-Asp among all strains (10,13,21,23).

Comparison of our data with other countries indicate fewer mutations in codon 531 (TCG → TTG) (2,18,31), and more mutations in codon 526 (CAC → TAC, CAC → _GC, CAC → CGC, CAC → AAC, CAC → TTC and CAC → CAA) (4,13,14,27) are found in Iran. Mutations in codon 526 (CAC → CAG) and Mutations in codon 516 (GAC → GTC) are not often seen in Iran, Poland and USA (6,14,22,32). We couldn't find any mutations in codon 511 which is one of frequent mutations worldwide. These findings demonstrate that the frequencies of particular mutations in rifampicine-resistant *M. tuberculosis* isolates from Iran are different from those that have been reported from other parts of the world.

Although the combination of two single point mutations has been described previously for rifampicine-resistant *M. tuberculosis* strains (4,8,17,30,31). The high percentage of double mutations found among Iranian strains (32%) differed clearly to the lower prevalence of double mutations in other studies (1,5,14,16,17,22,28,29). Prominent finding of this study indicate the high frequency of double mutations (32%) and triple (20%) and quatriple (2.9%) occurring in separate codons. We found (8.5%) nonsense mutations in 9 strains. Our data indicate five phenotypic rifampicine resistance strains revealing no mutations. We hope to continue our investigation on mutation frequency of other genes responsible for resistance to drugs and their association with virulence of *M. tuberculosis*.

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