High level isoniazid resistance correlates with multiple mutation in the *katG* encoding catalase proxidase of pulmonary tuberculosis isolates from the frontier localities of Iran

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ÖZET

İran sınır bölgesindeki pulmoner tüberküloz izolatlarında yüksek düzeyde izoniazid direnci katalaz-peroksidazı kodlayan katG'deki multipl mutasyonla koreledir

Bu çalışmanın amacı, katG genindeki multipl mutasyonların, nükleotid değişikliklerinin önemi ve İran'ın farklı coğrafik bölgelerinden rastgele toplanan primer ve sekonder aktif pulmoner tüberkülozlu 42 hastanın balgamında izoniazide yüksek düzeyde dirençli Mycobacterium tuberculosis izolatları ile ilişkisini araştırmaktı. İlaç duyarlılık testi, CDC standart konvansiyonel metod kullanılarak yapıldı. DNA ekstraksiyonu, katG gen amplifikasyonu ve DNA sekans analizi yapıldı. İzolatların 34 (%80)'ünde katG geninde multipl mutasyon (2-5 mutasyon) saptandı. İzoniazide yüksek düzeyde dirençli (MİK µg/mL ≥ 5-10) sekonder tüberkülozlu hastalarda daha sık olmak üzere, 315 (AGC→ACC), 316 (GGC→AGC), 309 (GGT→GTT) kodonlarında artmış predominant mutasyon ve nükleotid değişiklikleri saptandı. Ayrıca, sekonder infeksiyonlu hastalarda daha sık mutasyonu gösteren, 315, 316 ve 309 kodonlarında mutasyon kombinasyonları ve predominant nükleotid değişiklikleri gözlendi. Bu çalışmada, multipl mutasyonlu izolatların %62 (n= 21)'sinde, 315 (AGC→ACC), 316 (GGC→GTT), 309 (GGT→GGT) kodonlarında mutasyon kombinasyonları ile predominant nükleotid değişiklikleri olduğu bulundu ve bunun da izoniazide yüksek dirençli (≥ 5-10 µg/mL) sekonder infeksiyonlu hastaların izolatlarında daha sık olduğu saptandı.

Anahtar Kelimeler: *Multipl mutasyon, M. tuberculosis, yüksek düzeyde izoniazid direnci.*

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SUMMARY

High level isoniazid resistance correlates with multiple mutation in the katG encoding catalase proxidase of pulmonary tuberculosis isolates from the frontier localities of Iran

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The aim of this study was to investigate the significance of multiple-mutations in the katG gene, predominant nucleotide changes and its correlation with high level of resistance to isoniazid in Mycobacterium tuberculosis isolates that were randomly collected from sputa of 42 patients with primary and secondary active pulmonary tuberculosis from different geographic regions of Iran. Drug susceptibility testing was determined using the CDC standard conventional proportional method. DNA extraction, katG gene amplification, and DNA sequencing analysis were performed. Thirty four (80%) isolates were found to have multiple-mutations (composed of 2-5 mutations) in the katG gene. Increased number of predominant mutations and nucleotide changes were demonstrated in codons 315 (AGC→ACC), 316 (GGC→AGC), 309 (GGT→GTT) with a higher frequency among patients bearing secondary tuberculosis infection with elevated levels of resistance to isoniazid (MIC ≥ 5-10 µg/mL). Furthermore, it was demonstrated that the combination of mutations with their predominant nucleotide changes were also observed in codons 315, 316, and 309 indicating higher frequencies of mutations among patients with secondary infection respectively. In this study, 62% (n= 21) of multi-mutated isolates found to have combination of mutations with predominant nucleotide changes in codons 315 (AGC→ACC), 316 (GGC→GTT), 309 (GGT→GGT), and also demonstrated to be more frequent in isolates of patients with secondary infections, bearing higher level of resistance to isoniazid (≥ 5-10 µg/mL).

Key Words: *Multiple mutations, M. tuberculosis, high level resistant to izoniazid, Iranian isolates.*

Isoniazid is a first-line chemotherapeutic drug used in tuberculosis (TB) therapy (1-3). Resistance to isoniazid is associated with a variety of mutations affecting one or more genes such as that encoding catalase peroxidase (*katG*) (4,5). The *katG* gene is the most commonly targeted region of the *Mycobacterium tuberculosis* genome with the majority of mutations occurring in codon 315 in 30-90% of isoniazid-resistant strains depending on geographical distribution (6,7). Izoniazid resistance is the most frequently associated with a single mutation in *katG* gene, a gene that encodes catalase peroxidase enzyme in *M. tuberculosis* (8). Most isoniazid-resistant *M. tuberculosis* strains have not been reported to have a high proportion of *katG* deletions suggesting the need to more precisely analyze the structure of the *katG* gene in the resistant organisms. Further studies have revealed that *katG* gene deletions are very rare and this requires more detailed analysis of the *katG* its structure (4,5,9). Several groups have recently reported that many isoniazid-resistant strains contain missense and other types of mutations (4). Mutations at the Ser315 codon of *katG* have been reported to be associated with high-level of isoniazid resistance (10). Resistance to isoniazid has a second degree of magnitude in Iran, and combinations of mutations conferring *M. tuberculosis* resistance to isoniazid have been reported to be more common in the multidrug resistance tuberculosis (MDR-TB) than in monoresistant *M. tuberculosis* isolates, suggesting that isolates develop resistance to isoniazid by a stepwise accumulation of mutations (5,11).

In this study, we investigate the significance of multiple-mutations in the *katG* gene, its correlation with predominant nucleotide changes, and high level of resistance to isoniazid in 42 isolates of *M. tuberculosis* collected from patients with primary and secondary active pulmonary tuberculosis from different geographic regions of Iran.

MATERIALS and METHODS

Mycobacterial Strains

One hundred and sixty three *M. tuberculosis* were isolated from sputa of patients with active pulmonary tuberculosis collected from various geographic regions of Iran (Tehran, Zabol, Kermanshah, Mashad, Tabriz) from December 2007 to May 2008. Patients' history of tuberculosis, gender, clinical symptoms, radiography, tuberculin skin test (TST), etc. was recorded before collection of specimen. All isolates were cultured on Lowenstein-Jensen solid medium and grown colonies were identified to the species level using TCH (2-thiophene carboxylic acid) and PN99B (paranitrobenzoic acid) selective media using CDC standard biochemical procedures (12). Four sensitive isolates were selected and used as controls.

Susceptibility Testing

Anti-microbial drug susceptibility testing (AMST) was performed using the CDC standard conventional proportional method rifampicine (Rif) 40 µg/mL, isoniazid (INH) 2 µg/mL, ethambutol (EMB) 2 µg/mL, ethionamide (ETH) 20 µg/mL, streptomycin (SM) 4 µg/mL, and kanamycin (K) 20 µg/mL were used in slants and in addition to breakpoint concentrations for isoniazid 0.1 µg/mL, and rifampicin 2.0 µg/mL were also used in the BACTEC system (12). Four sensitive *M. tuberculosis* isolates and an H37Rv strain were used as negative controls. Mutations in the *katG* gene were identified on 42 isoniazid resistant isolates by sequencing methods and AMST was performed following sequencing to confirm resistance using different concentrations of isoniazid 2 5 and 10 µg/mL in the slant proportional method (12).

Standard PCR Identification and *katG* Gene Amplification

DNA extraction was done by Fermentase kit (K512), and DNA purification by Fermentase kit (k513-Graiciuno 8, Vilnius 2028, Lithuania). DNA extracted from *M. tuberculosis CDC1551*, *Mycobacterium H37RV* strains and from four sensitive isolates of *M. tuberculosis* was used as the negative control. A 209bp and 750bp segment of the *katG* gene were amplified by PCR using the following synthetic oligonucleotide primers *katG F* 5-GAAA-CAGCGGCGCTGGATCGT- 3, *katG R* 5- GTTGTCC-CATTTCGTCG GGG-3 for 209 bp and *katG F* 5 CGGGATCCG CTGGAGCAGATGGGC-3 and *katG R* 5-CGGAATTCCAGGGT GCGAATGACCT-3 for the 750bp fragment (13). PCR was carried out in 50 µL tube containing 2 µL KCl, 2 µL Tris (pH 8.0), 1.5 µL MgCl2, 5 µL dNTP, 1U *Taq* polymerase, 27 µL water (DDW molecular grade), 20 pmol of each primer and 6-10 µL of DNA template. The following thermocycling parameters were applied: initial denaturation at 95°C for 5 min; 36 cycles of denaturation at 94°C for 1 min; primer annealing at 56°C for 1 min; extension at 72°C for 1 min; and a final extension at 72°C for 10 min. The PCR product was amplified and purified again and controlled on the gel electrophoresis for purified segment. The final purified mycobacterial DNA obtained and was used for sequencing.

DNA Sequencing

The 209 bp and 750 bp fragments of the *katG* gene were amplified by PCR using forward or reveres primers mentioned above; 33 cycles of C for 45 sec; extension C for 30 sec; primer annealing at 48 denaturation at 94°C for 4 min. at 60 *katG* gene fragments were sequenced by using an Amersham auto sequencer and Amersham Pharmacia DYEnamic ET Terminator Cycle Sequencing Premix Kits.

Purified DNA of the *katG* fragment obtained from *M. tuberculosis CDC1551*, *Mycobacterium H37RV* strains and from four sensitive isolates was used as negative controls.

Analyzing of DNA Sequencing

Alignment of the DNA fragments (*katG*) were carried out using the MEGA and DNAMAN software (Gen bank PUBMED/BLAST) and was compared with the standard strains of CDC1551, H37RV and *M. tuberculosis 210*. The Blast 2 sequencing computer program was used for DNA sequence comparisons (http://www.ncbi.nlm.nih.gov/BLAST/). Alignment of the DNA fragments (*katG*) was carried out with MEGA 3.1 software (www.megasoftware.net/mega3.1/) and obtained data were analyzed and edited with DNAMAN software.

Definitions

In this study, primary cases (never-treated patients-new cases) refer to patients who did not have a previous history of tuberculosis disease or medical treatment. Secondary cases (previously treated patients) demonstrated a previous history of tuberculosis disease in their medical records

RESULTS

Mycobacterial Strains and Susceptibility

From 163 isolates, 42 were found to be resistant to isoniazid (100%), rifampicine (90%), streptomycin (90%), and ethambutol (28%). Mono-resistance to isoniazid was observed in 4 isolates (9.5%). In total, 42 isoniazid resistant and 121 sensitive isolates were identified.

Mutations were not detected for the four sensitive isolates to isoniazid in 209 bp and 750 bp regions of *katG* gene. Mutations were observed in affected codons 305, 306, 307, 309, 314, 315, 316, 321, 328 in 209 bp fragment and in 357, 454, and 463 of the 750 bp fragment of *katG* gene. In 40 isolates four types of mutations were identified in codon 315: AGC→ACC (n= 36) 80%, AGC→AGG (n= 1) 2.3%, AGC→AAC (n= 2) 4.7% and AGC→GGC (n= 1) 2.3%. One type of mutation was found in codon 316: GGC→AGC (n= 18) 41.4%, and in 15 isolates four types of mutations were demonstrated in codon 309: GGT→GTT (n= 7) 16.1%, GGT→GCT (n= 4) 9.2%, GGT→GTC (n= 3) 6.9%, GGT→GGG (n= 1) 2.7% (Table 1).

Predominant nucleotide changes were observed in 40 isolates as 315 (AGC→ACC) indicated to evolve (n= 28) 77% from secondary and (n= 8) 23% from primary cases, 316 (GGC→AGC) in which 77% (n= 14) from secondary and 23% (n= 4) from primary, and 309 (GGT→GTT) that 71% (n= 5) from secondary and 29% (n= 2) from primary cases, respectively (Table 2). Out of 105 mutations, predominant nucleotide changes were seen in codon 315 AGC→ACC (Ser→Thr) 36% (n= 40), 316 GGC→AGC (Gly→Ser) 17.7%, and in codon 309 GGT→GTT (Cys→Phe) 6.3% (n= 7) (Table 1,2).

Six isolates 14% (n= 6) were identified from secondary cases with predominant mutations observed in three codons 315, 316 (100% each) and in codon 309 (67%, n= 4), including non predominant mutation observed in 2 (33%) isolates of codon 309 (Table 2).

Twenty-six (62%) isolates demonstrated multiple mutations in at least two of the three codons (309, 315, 316) with predominant nucleotide changes in which nucleotide combination 315 (AGC→ACC), 316 (GGC→AGC) n= 12 (46%) all differentiated from secondary cases, and nucleotide combinations of 315 and 309 in which 315 (AGC→ACC) n= 9 (34.5%) was identified in 6 (23%) secondary and 3 (11.5%) primary cases, and a 309 mutation (GGT→GTT) found in secondary case (Table 2). Nucleotide combination in codon 316 and 309 were not found in primary or secondary cases (Table 2). Nucleotide combinations of 315 with others codons were observed in (n= 4) 15% of patient isolates including 3 secondary cases and 1 primary case (Table 2).

In two isolates, 2 types of mutations were found in codon 357 GAC→CAC and GAC→AAC. In addition two mutations which were also observed in codons 463 CGG→CTG and 454 GAG→CGA were found in secondary cases and did not correspond to high level resistance to isoniazid (MIC ≤ 2 µg/mL).

Isolates bearing a single mutation n= 8 (19%), double mutations n= 17 (40.46%), triple mutations n= 9 (21.42%), four mutations n= 4 (9.5%) and five mutations n= 4 (9.5%) were also observed among 42 resistant isolates (Table 1).

Silent Mutations

Three silent mutations were identified in four isolates in codons 306 (CCG→CCC), 309 (GGT→GGG) and 314 (ACC→ACG). These silent mutations did not show any effect on the susceptibility testing pattern (Table 1).

DISCUSSION

Isoniazid resistance is a surrogate marker for MDR *M. tuberculosis* (rifampin and isoniazid resistance) and is the result of mutation within certain region of the *katG* gene which encodes the catalase proxidase. It is important to understand the correlation of the clinical states of patients with tuberculosis to mutations and high resistance levels to isoniazid to determine wether the patients are initially infected with the MDR *M. tuberculosis* strain or whether the emergence of MDR *M. tuberculosis* was due to inadequate or inappropriate antibiotic treatment that resulted in the acquisition of mutations and antibiotic resistant. The known genes related to isoniazid-resistant are *katG, inhA, ahpC, kasA* (14). Several investigators have reported that *M. tuberculosis* resistance to isoniazid corresponds to amino acid changes in codon 315 (3,6,10). In our study, we have observed 95% of all isoniazid resistant isolates (n= 40) showed mutations in codon 315. Whereas 40% of all mutations (n= 105) conferring different types of nucleotide changes were found to be in codon 315: AGC→ACC (Ser→Thr) as predominant nucleotide changes 36%, and AGC→AGG (Ser→Arg) 0.9%, AGC→AAC (Ser→Asn) 1.8%, AGC→GGC (Ser→Gly) 0.9% were observed as non predominant. One type of mutation was found in codon 316: GGC→AGC (n= 18) 41.4%, and in 15 isolates four types of mutations were demonstrated in codon 309: GGT→GGT (n= 7) 16.1%, GGT→GCT (n= 4) 9.2%, GGT→GTC (n= 3) 6.9%, GGT→GGG (n= 1) 2.7% (Table 2). Predominant mode of acquisition of resistance via *katG* alterations is the selection of particular mutations that decrease the catalase activity but that maintain a certain level of the peroxidase activity of the enzyme in viable INH-resistant (INHr) organisms. The above data correlate with our findings that such mutations were found to be up to 85% of the INHr clinical isolates with decreased catalase activity. These mutations appear to provide the optimal balance between decreased catalase activity and a sufficiently high level of peroxidase activity in *KatG* (15).

Table 1. Frequency of amino acid and nucleotide change of different codons in the *katG* gene of 40 isoniazid-resistant strains of *M. tuberculosis* collected from active primary and secondary tuberculosis patients in the frontier localities of Iran.

No. of mutations [no.(%) of isolates]	Codon(s)	Change of nucleotide(s)	Change of amino acid(s)	MIC (µg/mL)	Frequency of change [no.(%)]
1 [8 (19%)]	315	AGC→ACC	Ser→Thr	≥ 5	5 (11.9)
		AGC→AAC	Ser→Asn		1 (2.38)
		AGC→GGC	Ser→Gly		1 (2.38)
	330	AAC→ATC	Asn→Ile	≥ 2	1 (2.38)
2 [17 (40.46%)]	305, 315	GGC→GCC, AGC→ACC	Gly→Ala, Ser→Thr	≥ 5-10	1 (2.38)
	309, 315	GGT→GTT, GTC, GCT, AGC→ACC	Gly→Cys, Phe, Ala, Ser→Thr	≥ 5-10	3 (7.14)
	314, 315	ACC→AAC, AGC→ACC	Thr→Asn , Ser→Thr	≥ 5-10	1 (2.38)
	311, 315	GAC→TTC, TAC AGC→ACC	Asn→Phe, Tyr Ser→Thr	≥ 5-10	2 (4.76)
	315, 316	AGC→ACC, AGG, GGC→AGC	Ser→Thr, Arg Gly→Ser	≥ 5-10	8 (19.4)
	357, 463	GAC→CAC, CGG→CTG	Asp→His, Arg→Leu	≥ 2	1 (2.38)
	357, 454	GAC→AAC, GAG→CGA	Asp→Asn, Glu→Arg	≥ 2	1 (2.38)
3 [9 (21.42%)]	309, 311, 315	GGT→GCT, GAC→TTC AGC→ACC	Gly→Ala, Asn→Phe Ser→Thr	≥ 5-10	1 (2.38)
	307, 309, 315	GGA→CGA, GGT→GGG AGC→ACC	Gly→Arg, Gly→Gly Ser→Thr	≥ 5-10	1 (2.38)
	305, 315, 321	GGC→GCC, AGC→ACC TGG→TTG	Gly→Ala, Ser→Thr Trp→Leu	≥ 5-10	1 (2.38)
	305, 309, 315	GGC→GCC, GGT→GCT AGC→ACC	Gly→Ala, Gly→Ser Ser→Thr	≥ 5-10	1 (2.38)
	315, 316, 328	AGC→ACC, GGC→AGC TGG→TGT	Ser→Thr, Gly→Ser Trp→Cys	≥ 5-10	1 (2.38)
	315, 316, 321	AGC→AAC, GGC→AGC TGG→TCC, TAG	Ser→Thr, Gly→Ser Trp→Ser, STOP	≥ 5-10	2 (4.76)
	309, 315, 316	GGT→GTT, AGC→ACC GGC→AGC	Gly→Cys, Ser→Thr Gly→Ser	≥ 5-10	2 (4.76)

Table 1. Frequency of amino acid and nucleotide change of different codons in the *katG* gene of 40 isoniazid-resistant strains of *M. tuberculosis* collected from active primary and secondary tuberculosis patients in the frontier localities of Iran (continued).

No. of mutations [no.(%) of Isolates]	Codon(s)	Change of nucleotide(s)	Change of amino acid(s)	MIC (µg/mL)	Frequency of change (no.[%])
4 [4 (9.42%)]	309, 311, 315, 316	GGT→GTT, GAC_TTC AGC→ACC,GGC→AGC	Gly→Cys, Asp→Phe Ser→Thr, Gly→Ser	≥ 5-10	2 (4.76)
	309, 315, 316, 328	GGT→GTC, AGC→ACC GGC→AGC, TGG→TGT	Gly→Phe, Ser→Thr Gly→Ser, Trp→Cys	≥ 5-10	1 (2.38)
	309, 315, 321, 328	GGT→GTT, AGC→ACC TGG→TTG, TGG→TGT	Gly→Cys, Ser→Thr Trp→Leu, Trp→Cys	≥ 5-10	1 (2.38)
4 [4 (9.42%)]	309, 311, 315, 316, 321	GGT→GTC, GAC→TAC, AGC→ACC, GGC→AGCTG G→TAG	Gly→Phe, Asp→Tyr, Ser→Thr Gly→Ser, Trp→STOP	≥ 5-10	1 (2.38)
	307, 309, 311, 314, 315	GGA→GCA, GGT→GCTGA C→TAC, ACC→ACG, AGC→ACC	Gly→Ala, Gly→Ser, Asp→Tyr Thr→Thr, Ser→Thr	≥ 5-10	2 (4.76)
	307, 309, 311, 314, 315	CCG→CCC, AGC→ACC, GGC→AGC, TGG→TGT, TGG→TGT	Pro→Pro, Ser→Thr, Gly→Ser Trp→Cys, Trp→Cys	≥ 5-10	1(2.38)

Table 2. Correlation between prevalent nucleotide and amino acid change among isolates with high levels of isoniazid resistance collected from active primary and secondary tuberculosis patients at the frontier localities of Iran.

Codon(s)	Change of nucleotide(s)	Change of amino acid(s)	No. of isolates	No. of primary cases	No. of secondary cases
315	AGC→ACC	Ser→Thr	35	8	27
316	GGC→AGC	Gly→Ser	18	4	14
Com. 315, 316	AGC→ACC, GGCÆAGC	Ser→Thr, GlyÆSer	12	0	12
315	AGC→ACC	Ser→Thr	35	8	27
309	GGT→GTT	Gly→Cys	9	2	7
Com. 315, 309	AGC→ACC, GGTÆGTT	Ser→Thr, GlyÆCys	9	3	6
315	AGC→ACC	Ser→Thr	35	8	27
330, 357, 463, 454	other codon change (Table 1)	Others change	5	4	1
315, other codon	AGC→ACC, other codon change	(Table 1) Ser→Thr, others	5	4	1
316	GGC→AGC	Gly→Ser	18	4	14
309	GGT→GTT	Gly→Cys	9	2	7
Com. 309, 316	GGT→GTT, GGCÆAGC	Gly→Cys, GlyÆSer	0	0	0
315	AGC→ACC	Ser→Thr	35	8	27
316	GGC→AGC	Gly→Ser	18	4	14
309	GGT→GTT	Gly→Cys	9	2	7
Com. 315, 316, 309	AGC→ACC, GGCÆAGC GGT→GTT	Ser→Thr, GlyÆSer Gly→Cys	2	0	2

Com: Combination.

In this study, nucleotide changes in codon 315: AGC→ACC (n= 36) 80%, 316: GGC→AGC (n= 18) 41.4% and 309: GGT→GTT (n= 7) 16.1% were more predominantly observed among isolates collected from secondary infection cases, and correlating to a higher frequency level of resistance to isoniazid MIC ≥ 5-10 µg/mL. This observation correlate with other studies reported that multi-drug resistance was found among 14% of the amino acid 315 mutants and 7% of the other INH-resistant strains (p> 0.05) (10), and reported that amino acid 315 mutants lead to secondary cases of tuberculosis as often as INH-susceptible strains (10). Distribution of isoniazid resistance associated mutations reported by other investigators to be different in isoniazid mono-resistant isolates when compared with multi-drug-resistant isolates, significantly fewer isoniazid resistance mutations observed in the isoniazid mono-resistant group and also mutations in *katG*315 were significantly more common in the multi-drug resistant isolates (16). Conversely, mutations in the *inhA* promoter were significantly more common in isoniazid mono-resistant isolates (16). It has been suggested that some drug resistance associated mutations occur at higher frequencies in MDR *M. tuberculosis* than in mono-isoniazid resistant clinical isolates (16). Whereas our data demonstrate that only 9.5% (n= 4) mono-resistant, 90% (n= 38) multi-drug resistant and 26 (62%) of isolates with multiple mutation conferring high level of resistance to isoniazid (MIC ≥ 5-10 µg/mL). Unfortunately, we have not completely examined the role of *inhA* promoter among mono and multi-drug resistant conferring multiple mutations in this study. Other studies reported that INHr strain showed a mutation in the *katG* gene in codon 314 as ACC→CCC (Thr→Pro) which has not been previously defined (17). However, in this study we found mutations in nearby similar segment of the *katG* gene in codons 309 and 316 which have very seldom been reported and associated with secondary MDR cases.

Our findings are in agreement with similar data reported in Lithuania where 95% of strains displayed mutation in codon 315 of the *katG* gene with the predominance of codon substitution AGC→ACC (Ser→Thr) (90%) (Bakonyte et al. 2003). However, we could not identify the mutation of AGC→ACA (Ser→Thr) which has been reported in Lithuania (5). In Poland 90% mutations are in the 315 AGC codon which corresponds to 5 types of mutations (ACC, ACT, ACA, AAC, and ATC) and resemble similar pattern of changes with our data including nucleotide ACC and AAC. However contrary to the data from Poland we did not observe nucleotide changes of ACT, ACA and ATC in

Iran (18). In Russia the highest proportion of nucleotide changes (70%) have been reported to be in the *katG* codon 315 AGC→ACC which is similar and in agreement with our data (2). Mutations at the Ser315 codon of *katG* have been reported to be associated with high-level isoniazid resistance (10) which is similar to our findings in 8 (19%) isolates bearing a single mutation at codon 315 and conferring resistance to isoniazid (MIC ≥ 5-10 µg/mL). This data suggests the alternative or complementary explanation that strains with mutations at codon 315 are more likely to gain increased resistance (10).

In our study, four types of mutations were detected in codon 309: GGT→GTT (Cys→Phe) 6.3%, GGT→GCT (Cys→Ser) (3.6%), GGT→GTC (Cys→Phe) (2.7%), GGT→GGG (Cys→Thr) (0.9%). Additionally, we identified a mutation in codon 316 GGC→AGC (Gly→Ser) (14.4%) which has not been reported previously. In this study, 75% of all isolates resistant to isoniazid (n= 42) demonstrated multiple types of mutations in codons 309 (n= 15, 34%) and 316 (n= 18, 41.4%) which might represent a second importance of mutations present in isolates of patients bearing secondary infection in Iran which has not been reported previously. In six isolates (14%) bearing a combination of multiple mutations in three codons (309, 315 and 316) and in 26 (61.9%) isolates that demonstrated having combinations of multiple mutations (in at least two of the three mentioned codons) were found to be MDR isolates having high frequency levels of resistance to isoniazid MIC ≥ 5-10 µg/mL. These finding indicate correlation of high level resistance due to mutation in codon 315 which has been shown by other authors (16). Higher frequency of combination of multiple mutations in *katG* gene (codon 315, 309 and 316) has not been previously reported in patients with secondary infection (Table 1,2). One explanation can be postulated that high population rate of alcoholism and incomplete treatment can be the cause of mutations. The other logical reason could be explained that high rate of immigrant transits of patients from high TB incidence areas like India, Afghanistan and China to Europe via center of eastern Europe (Iran) which can cause distribution, circulation, and interaction of numerous different molecular types of tuberculosis cases might lead to such combination of rare mutations. Other researchers have suggested that isolates develop resistance to isoniazid by a stepwise accumulation of mutations, which may be important for achieving the higher level of resistance or maintaining virulence in a human host. Inadequate prolonged treatment results in an accumulation of mutations, ultimately leading to *katG* and/or *inhA* mutations in virtually all strains. This finding is in agreement with our data regarding higher frequency of predominant nucleotide changes among secondary case infections. In contrast to our findings other investigators have not reported the association of multiple mutations and predominant nucleotide changes with high level resistance among patients with secondary infection cases.

In 2 isolates mutations were not detected in the 209bp fragment, therefore we sequenced the larger 750 bp fragment of *katG* gene for all isolates and have identified mutations in codons 463, 357, and 454, 357, which may indicate that this type of mutation is non-predominant colon in Iran when compared with neighboring countries (4,6,13,19). Other investigators have reported no silent mutations detected in the *katG* gene (9,20,21). Whereas, in our strain set three silent mutations (2.7%) in codons 306 (CCG→CCC), 309 (GGT→GGG) and 314 (ACC→ACG) were demonstrated which had no effect on the susceptibility testing pattern. The high percentage of double mutations found among the isolates of Iran clearly differed from the lower prevalence of double mutations in other studies (18,22-24). A prominent finding of this study was the high frequency of double (40.47%), triple (21.42%), quadruple (9.5%) and five nucleotide mutations (9.5%) occurring in separate codons indicating predominant nucleotide changes in codons 315, 316 and 309 to be more prevalent among secondary cases (Table 1).

In conclusion, this study demonstrates a correlation between the multiple mutations of the katg gene and the prevalent nucleotide changes in codon 309, 315 and 316. Notably, in multiple isolates bearing double, triple, quadruple and quintet mutations predominantly identified to be from secondary infection cases, high levels of isoniazid resistance (MIC ≥ 5-10 µg/mL) were seen. These data illustrate the need for further investigations to develop a more rapid and specific assay for the detection of MDR *M. tuberculosis* to be used as a screening method in areas where tuberculosis is highly endemic.

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CONFLICT of INTEREST

None declared.

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