Molecular Biology Reports (2022) 49:6135–6143
https://doi.org/10.1007/s11033-022-07404-2

ORIGINAL ARTICLE

Detection and characterization of mutations in genes related to isoniazid resistance in *Mycobacterium tuberculosis* clinical isolates from Iran

Pejman Bakhtiyariniya[1,2] · Azar Dokht Khosravi[1,2,3] · Mohammad Hashemzadeh[1,2] · Mohammad Savari[1,2]

Received: 22 December 2021 / Accepted: 17 March 2022 / Published online: 2 April 2022
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Abstract

Background The global rise in drug-resistant *Mycobacterium tuberculosis* (*M.tb*), and especially the significant prevalence of isoniazid (INH)-resistance constitute a significant challenge to global health. Therefore, the present study aimed to investigate mutations in prevalent gene loci—involved in INH-resistance phenotype—among *M.tb* clinical isolates from southwestern Iran.

Methods Drug susceptibility testing (DST) was performed using the conventional proportional method on confirmed 6620 *M.tb* clinical isolates, and in total, 15 INH-resistant and 18 INH-susceptible isolates were included in the study. Fragments of six genetic loci most related to INH-resistance (*katG*, *inhA* promoter, *furA*, *kasA*, *ndh*, *oxyR-ahpC* intergenic region) were PCR-amplified and sequenced. Mutations were explored by pairwise alignment with the *M.tb* H37Rv genome.

Results The analysis of gene loci revealed 13 distinct mutations in INH-resistant isolates. 60% (n = 9) of the INH-resistant isolates had mutations in *katG*, with codon 315 predominately (53.3%, n = 8). Mutation at *InhA* − 15 was found in 20% (n = 3) of resistant isolates. 26.7% (n = 4) of the INH-resistant isolates had *kasA* mutations, of which G269S substitution was the most common (20%, n = 3). The percentage of mutations in *furA*, *oxyR-ahpC* and *ndh* was 6.7% (n = 1), 46.7% (n = 7), and 20% (n = 3), respectively. Of the mutations detected in *ndh* and *oxyR-ahpC*, 5 were also observed in INH-susceptible isolates. This study revealed seven novel mutations, four of which were exclusively in resistant isolates.

Conclusions This study supports the usefulness of *katG* and *inhA* mutations as a predictive molecular marker for INH resistance. Co-detection of *katG* S315 and *inhA*-15 mutations identified 73.3% (11 out of 15 isolates) of INH-resistant isolates.

Keywords Iran · *Mycobacterium tuberculosis* · Isoniazid resistance · *katG* · *inhA* · *kasA* · *furA* · *oxyR-ahpC* · *ndh*

Introduction

Despite its long presence in human history, tuberculosis (TB) continues to invade the world, especially in developing countries, resulting in about 10 million new cases and 1.4 million deaths each year. According to the World Health Organization (WHO), *Mycobacterium tuberculosis* (*M.tb*), the causative agent of TB, currently infects about 25% of the world's population [1], although there are recent evidences suggesting that a great proportion of the *M.tb*-immunoreactive individuals cured the infection [2]. However on the basis of WHO latest report, TB was the leading cause of death from a single infectious agent until the coronavirus (COVID-19) pandemic [1].

Nowadays, an important challenge in TB control and treatment is the emergence of different forms of drug resistance among *M.tb* isolates. Compared to drug-susceptible cases, treatment for drug-resistant TB is more extended and far less successful [3]. *M.tb* can acquire antibiotic resistance via chromosomal mutations, but not through horizontal transfer of resistance genes [4]. These mutations occur mainly in single nucleotide polymorphisms, insertions, and deletions in genes encoding drug targets or drug-converting

✉ Azar Dokht Khosravi
azarkhosravi69@gmail.com

[1] Infectious and Tropical Diseases Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

[2] Department of Microbiology, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

[3] Iranian Study Group on Microbial Drug Resistance, Tehran, Iran

enzymes [5]. They confer different resistance levels by interfering with drug binding to the target, disrupting the drug activation pathway, or target overexpression [4].

Isoniazid (isonicotinic acid hydrazide, INH) is an essential component of treatment for active TB in combination regimens with rifampin (RIF), pyrazinamide (PZN), and ethambutol (EMB) and for latent TB infection as monotherapy [37]. It was not long after using INH in the 1950s that rapid development of resistance was reported [6]. The prevalence of INH resistance has increased in the last two decades [3]. In 2019, 13.1% of new cases and 17.4% of retreatment cases of TB worldwide were resistant to INH, which amounted to 1.42 million people. Globally, a quarter of INH-resistant *M.tb* isolates also had co-resistance to RIF, known as multidrug-resistant (MDR), and were inevitably more worrying [7].

The molecular basis of INH resistance is mutations in the *katG*, *inhA*, or *inhA* promoter genes of *M.tb*, to which about 80% of its phenotypic resistance in the world is attributed [8]. INH is a prodrug that diffuses passively into growing bacilli. The enzyme catalase-peroxidase (KatG), encoded by the *katG* gene, converts INH to the active form to exert its lethal effect by inhibiting mycolic acid biosynthesis, an essential component of the mycobacterial cell wall [9]. The enzyme InhA, or enoyl-acyl carrier protein (ACP) reductase, is encoded by the *inhA* gene and involved in mycolic acid biosynthesis. Activated INH binds to and inhibits this enzyme [10].

Several studies have reported mutations in *kasA*, *furA,* and *ndh* genes and the *oxyR-ahpC* intergenic region in INH-resistant isolates, the exact role of which in developing resistance requires further investigation [8]. The *kasA* gene encodes a β-ketoacyl-ACP synthase, an enzyme in mycolic acid's biosynthesis system and a potential target for activated INH [11]. The *furA* gene, which encodes the ferric uptake regulation protein, is known as the negative regulator of the *katG* gene [9]. The *ndh* gene encodes the enzyme NADH dehydrogenase II, which provides the necessary conditions for binding the activated form of INH to the active site of the enzyme InhA [10]. The *oxyR-ahpC* gene complex consists of an *ahpC* structural gene that encodes alkyl hydroperoxidase reductase (AhpC), and the upstream regulatory region includes the *oxyR* pseudogene and the intergenic region. The AhpC participates in response to oxidative stress and partially compensates for the reduced activity of the KatG enzyme [12].

Rapid diagnosis of INH-resistant TB and initiation of effective modified treatment can prevent the transmission of resistant strains and the development of secondary resistance [6]. Still, the gold standard for *M.tb* drug susceptibility testing (DST) is the culture-based phenotypic methods, which take several days to weeks [13]. Accelerating the diagnosis of drug resistance and initiating appropriate treatment requires shortening this process and employing reliable molecular methods to detect drug-resistant strains [8]. In the molecular approach, determination and collection of mutations in the genes associated with resistance to a given drug are valuable for developing diagnostic tools and, consequently, clinical decision-making [5].

The incidence of TB has been decreasing in recent decades in Iran [14]. In 2020, the incidence rate of all forms of TB reported in Iran was 6.74 cases per 100,000 population [15]. In a recent survey, the resistance rate of Iranian *M.tb* isolates to one or more anti-TB drugs was about 16%, which is lower than the rate of countries in the region [14]. In several studies performed by molecular detection method, the INH resistance has varied from 4 to 9% in different regions of Iran [16]. Even though TB's incidence and drug resistance rate in Iran is much lower than the regional and global average, proximity to countries with a high burden of drug-resistant TB may jeopardize the success of the TB control program in Iran [14].

With more than 4.5 million population, Khuzestan is at the fifth rank among the Iranian provinces regarding TB incidence [15]. To the best of our knowledge, limited genes were examined in previous studies of INH resistance in this region. The present study aimed to investigate the mutations in four genes (*katG*, *furA*, *kasA*, *ndh*) and two regulatory regions (*inhA* promoter and *oxyR-ahpC* intergenic region) to establish a more rapid and precise detection of INH resistance in *M.tb* clinical isolates.

Materials and methods

Sample collection and bacterial isolates

A total of 6620 *M.tb* complex isolates from all regions of Khuzestan Province were sent to the Regional TB Reference Laboratory, Ahvaz, Iran, during 7 years, from April 2014 to March 2021. Most isolates were detected in sputum samples of patients with suspected pulmonary TB by direct microscopy and referred for further diagnostic procedures according to the indications mentioned in the national guideline for TB control [17]. Isolates were phenotypically identified as *M.tb* using acid-fast staining, culture on Löwenstein–Jensen (LJ) medium, and biochemical tests, including nitrate reductase, niacin accumulation, and catalase at 37 °C and 68 °C [18]. The *M.tb* H37Rv (ATCC® 27294) strain was used as the control strain. The Research Ethics Committee of Ahvaz Jundishapur University of Medical Sciences approved the preliminary proposal of the present study (IR.AJUMS.REC.1399.183), and the necessary permission was granted for sample collection.

Antimicrobial susceptibility testing

Susceptibility testing for first-line anti-TB agents was performed by proportion method on LJ medium according to the recommendations of the WHO [13]. The susceptibility testing was only performed for confirmed *M.tb* isolates. The final concentrations of INH, RIF, and EMB (Sigma-Aldrich, Germany) were 0.2, 40, and 2 µg/mL, respectively. If the ratio of the number of colonies on the medium containing an anti-TB agent to the number of colonies on the drug-free control medium was less than 1%, the isolate was determined as susceptible. The isolate was considered resistant if this ratio was equal to or greater than 1%.

All *M.tb* isolates resistant to INH (n = 15) and 18 INH-susceptible isolates were included in the study. Susceptible isolates consisted of 14 isolates resistant to other first-line anti-TB drugs and 4 pan-susceptible isolates. The latter isolates were selected using the random numbers from the pan-susceptible isolates received in the last 2 months of the study.

DNA extraction

Genomic DNA was extracted from each *M.tb* isolate by the boiling method described earlier with some modification [19]. In brief, two or three fresh bacterial colonies were harvested from the LJ slopes and dissolved in TE (Tris–EDTA) buffer and were boiled twice, each time for 15 min at 100 °C, with 5-min refrigeration at − 21 °C between two boils. Then, after 5 min of centrifugation at 13,000 rpm, the supernatant containing DNA was used as a template for PCR amplification.

PCR amplification and sequencing

All isolates were initially assessed for the *IS6110* gene to confirm belonging to the *M.tb* complex. In the next step, fragments of four genes (*katG*, *furA*, *kasA*, *ndh*) and two regulatory regions (*inhA* promoter and *oxyR-ahpC* intergenic region) were amplified in confirmed *M.tb* isolates with phenotypic resistance or sensitivity to INH. All oligonucleotide primers used for PCR amplification had been designed and described in previous studies and are listed in Table 1.

The PCR mixture contained the following components: 12.5 µL of Taq DNA polymerase 2 × Master Mix RED, 1.5 mM $MgCl_2$ (Ampliqon, Denmark), 1 µL of the template (65 ng/µL) except for *IS6110* and *inhA* promoter, which were 5 and 0.5 µL, respectively, 10 pmol of each primer, and double-distilled water adjusted to a final volume of 25 µL.

DNA was amplified in a thermal cycler (Bio-Rad, USA) under the following condition: initial denaturation at 95 °C for 5 min, followed by 35 cycles consisting of denaturation at 95 °C for 30 s, annealing for 30 s, and extension at 72 °C for 30 s, and ultimately a final extension at 72 °C for 5 min. The thermocycler parameters used for the amplification of *ndh* and *furA* were different as follows: initial denaturation at 95 °C for 10 min, followed by 30 cycles of 94 °C for 45 s, annealing for 30 s, and 72 °C for 50 s, ended with a final extension at 72 °C for 7 min. The optimum temperatures for the annealing of primer sets obtained in this study are also presented in Table 1.

After electrophoresis on 1.5% agarose gel for 60 min to evaluate the quality and ensure the absence of unneeded bands, PCR products were sent to the Cardiogenetic Research Center (Tehran, Iran) Laboratory for sequencing. It was performed in forward and reverse directions using the same primers to amplify gene fragments.

Table 1 Oligonucleotide primers used in this study

Gene	Primer sequence	Product size (bp)	References	Annealing temperature (°C)
katG	F: 5′-CTCGGCGATGAGCGTTAC-3′ R: 5′-TCCTTGGCGGTGTATTGC-3′	458	[19]	63
inhA promoter	F: 5′-TGCCCAGAAAGGGATCCGTCATG-3′ R: 5′-ATGAGGAATGCGTCCGCGGA-3′	455	[19]	69
oxyR-ahpC intergenic	F: 5′-GCTTGATGTCCGAGAGCATCG-3′ R: 5′-GGTCGCGTAGGCAGTGCCCC-3′	701	[20]	65
furA	F: 5′-GCGATCGGGTCCTAGCAG-3′ R: 5′-TTCATATGACCCACGACGG-3′	641	[21]	61
kasA	F: 5′-GTTCAGGCGAGGCTTGAG-3′ R: 5′-GCGATGTCGTGCTTCAGTAA-3′	1293	[21]	61.6
ndh	F: 5′-ATCACCACCGCCGCTGAAGC-3′ R: 5′-GTTCGGGTACCCGGGAATG-3′	1134	[21]	65
IS6110	F: 5′-CTCGTCCAGCGCCGCTTCGG-3′ R: 5′-CCTGCGAGCGTAGGCGTCGG-3′	130	[22]	63

Data analysis

The reporting sequences were analyzed using the BLAST algorithm available at https://blast.ncbi.nlm.nih.gov/Blast.cgi: BLASTn and BLASTx for nucleotide and translated nucleotide sequences, respectively [23, 24]. The sequences were compared by pairwise alignment with the corresponding wild gene sequences in the *M.tb* H37Rv genome, available in the GenBank (RefSeq sequence: NC_000962.3). A search was done in the Comprehensive Antibiotic Resistance Database, available at https://card.mcmaster.ca [25], and the TB mutations catalog [26] to determine if a mutation found has already been described.

Z test was applied to compare the proportion of isolates with specific characteristics. Chi-square (or as the case may be Fisher's exact) test was used to check the relationship or independence of the two categorized quantities. In all statistical tests, the *p*-value was considered less than 0.05.

Table 2 Detected mutations in four genes and two regulatory regions in INH-resistant (n = 15) and INH-susceptible (n = 18) *M.tb* isolates

Locus	Mutation		Frequency	
	Nucleotide	Amino acid	INH-R	INH-S
katG	1280 G>C	S315T	7	0
	1280 G>A	S315N	1	0
	1501 del C	M242W fs[a]	1	0
inhA promoter	− 15 C>T	–	3	0
furA	+4 A>G		1	0
kasA	805 G>A	G269S	3	0
	752 ins A	E252G fs	1	0
Intergenic	− 46 G>A	–	6	7
	− 34 T>G	–	1	0
oxyR	37 C>T	–	1	1
ahpC	–	–	0	0
ndh	1300 G>T	K31N	2	1
	1037 C>G	A119G	1	1
	999 G>A	A132T	1	1

Mutations are shown as amino acid (single-letter designations) for all proteins encoded by the corresponding genes except for the *oxyR-ahpC* regulatory and *inhA* promoter regions by nucleotide. The position of the nucleotide deletion or insertion is indicated

INH-R isoniazid-resistant, *INH-S* isoniazid-susceptible, *del* deletion, *ins* insertion, *fs* frameshift

[a]Leading to the termination codon

Results

In the phenotypic drug susceptibility test, a total of 29 isolates showed resistance to at least one of the first-line anti-TB drugs, 51.7% (n = 15) of which were resistant to INH. Due to the presence of *IS6110*, all 29 resistant isolates belonged to the *M.tb* complex. Among INH-resistant isolates, 80% (n = 12) also had resistance to RIF (i.e., MDR), and the rest were mono-resistant. The isolates were all detected in sputum samples from patients with pulmonary TB. The average age of the patients was 39 years.

All INH-resistant isolates had mutations, at least at one of the loci under study. Thirteen single-nucleotide mutations were identified, including one insertion, one deletion, six non-synonymous substitutions (NSS), and five simple nucleotide changes (SNCs). Of these, five were also observed in INH-susceptible isolates (Table 2). 66.7% (10/15) of isolates carried mutations at more than one locus (Table 3). Out of 13 mutations in 6 gene loci, 7 were not previously reported (Table 4). We used "non-synonymous substitution" (NSS) for structural gene mutations that alter a nucleotide and subsequently replace an amino acid in a protein chain. "Simple nucleotide change" (SNC) is a type of nucleotide substitution used only for mutations in regulatory regions or non-coding genes.

Mutations in the *katG* gene

Three mutations, including two NSSs and one single-nucleotide deletion, were identified in 60% (n = 9) of the resistant isolates. Each isolate had only one mutation at this locus. 58.3% of MDR isolates (7/12) and 66.7% of mono-resistants (2/3) had mutations in the *katG* structural gene, which was not significantly different (*p*-value = 0.7949). The most common NSS occurred at codon 315 and resulted in serine amino acid changes in 53.3% (8/15) of isolates: S315T (n = 7; 46.7%, including 6 MDR isolates and a mono-resistant); S315N (n = 1; 6.7%, a mono-resistant). The deletion was found in an MDR isolate that, while frameshifting, led to a termination codon in the next 16th codon. None of the susceptible isolates had a mutation in this gene. The relationship between *katG* locus mutations, in general, and resistance to INH was statistically significant (*p*-value = 0.0001). This significant association was also established for *katG* codon 315 mutations alone and INH resistance (*p*-value = 0.0005).

Mutation in the *inhA* promoter region

The primer amplified the promoter region of the *inhA* operon and 284 nucleotides (equivalent to 93 amino acids) of the *fabG1* gene. Two MDR isolates (2/12, 16.7%) and one mono-resistant isolate (1/3, 33.3%) had an SNC in the *inhA* promoter region, but the difference was not significant (*p*-value = 0.5157). Substitution of T for C at the − 15 position upstream of *fabG1* was observed in those isolates (3/15; 20%). None of the isolates carried mutations in *katG*, but all three mutated at least one other locus. No

Table 3 Results of sequencing analysis in 15 INH-resistant *M.tb* isolates

Isolate ID	*katG*	*inhA* promoter	*kasA*	*ndh*	*furA*	*oxyR-ahpC*
AHZ-R1	–	SNC	–	–	SNC	–
AHZ-R3	DEL	–	–	NSS	–	–
AHZ-R5	–	–	INS	–	–	SNC
AHZ-R6	NSS	–	–	–	–	SNC
AHZ-R7	NSS	–	–	–	–	SNC
AHZ-R9	NSS	–	–	–	–	–
AHZ-R14	–	SNC	NSS	–	–	–
AHZ-R18	–	–	–	NSS	–	SNC
AHZ-R21	NSS	–	NSS	–	–	–
AHZ-R22	–	SNC	–	NSS	–	SNC
AHZ-R25	NSS	–	NSS	–	–	SNC
AHZ-R26	NSS	–	–	–	–	–
AHZ-R27	NSS	–	–	–	–	–
AHZ-R30	NSS	–	–	–	–	–
AHZ-R31	–	–	–	–	–	SNC

DEL deletion, *INS* insertion, *NSS* non-synonymous substitution, *SNC* simple nucleotide change, – no detectable mutation

Table 4 Distribution of mutations (n = 13) between INH-resistant and INH-susceptible *M. tb* isolates

	Specific to INH-R		Both INH-R and INH-S		Total
	Known[a]	Novel[b]	Known	Novel	
katG	2	1	0	0	3
inhA	1	0	0	0	1
furA	0	1	0	0	1
kasA	1	1	0	0	2
oxyR-ahpC	0	1	2	0	3
ndh	0	0	0	3	3
Total	4	4	2	3	13

INH-R isoniazid-resistant, *INH-S* isoniazid-susceptible

[a]Reported in previous studies

[b]Not found in the literature

mutation was found in the *fabG1* gene fragment. The rest of the isolates had no mutations in this regulatory region.

Mutations in the *furA* gene

The amplified fragments included the entire length of the *furA* gene and the *furA-katG* intergenic region. Mutations in this locus were detected in 6.7% (n = 1) of resistant isolates. Only one MDR isolate had an SNC in the intergenic region (position 4 downstream of *furA* and 34 upstream of *katG*). This isolate also mutated in the *inhA* promoter region.

Mutations in the *kasA* gene

Four INH-resistants (26.7%), all MDR, had mutations in *kasA*. All but one had additional mutations in *katG* or *inhA* promoter. The NSS G269S was observed in 20% (3/15) of the INH-resistant isolates. An isolate had nucleotide insertion, leading to frameshift. No mutation was found in the study gene segment in other isolates, whether resistant or susceptible. The relationship between *kasA* locus mutations and INH resistance was statistically significant (p-value = 0.0334).

Mutations in *oxyR-ahpC* region

The amplified fragments contained 289 nucleotides from the *ahpC* gene, 105 nucleotides of the intergenic region, and 244 nucleotides from the *oxyR* pseudogene. Eight resistant isolates had no mutations in this complex region, and the mutations identified in the other 46.7% (n = 7) isolates occurred at three polymorphic sites. Six isolates (including five MDRs and one mono-resistant) had a total of seven SNCs in the intergenic region. The A nucleotide replaced the G at position 46 upstream of the *ahpC* in these isolates. This mutation was also found in seven susceptible isolates, statistically nonsignificant (p-value = 0.9442). One of those MDR isolates also had a G replacement for T in position − 34. The latter isolate carried the *katG* S315T mutation. In one of the MDR isolates with no mutations in other loci, a point mutation occurred in position 37 of the *OxyR* pseudogene that was also present in a susceptible isolate. Mutations in the *ahpC* structural gene were not detected in any resistant and susceptible isolates.

Mutations in the *ndh* gene

Three resistant isolates (20%), including two MDRs and one mono-resistant, had mutations in this gene locus. One of the MDR isolates had two NSSs, and each of the other two isolates had one NSS. All substitutions were observed in three INH-susceptible isolates without significant difference (p-value = 0.8026).

Discussion

This study analyzed sequences of 4 genes and 2 regulatory regions associated with INH resistance in 15 resistant and 18 susceptible isolates to detect possible mutations. Mutations of *katG*, *inhA* promoter, *kasA*, and *furA* loci were observed only in resistant isolates. The most frequent mutations in resistant isolates were detected at *katG* and *oxyR-ahpC* loci. Mutations in *katG* and *kasA* loci were significantly associated with INH resistance.

With 9.72 cases per 100,000 population, Khuzestan province is one of the regions with a high incidence of TB in Iran [15], and according to a previous study, the average resistance to INH was 6.25% [27]. Thus, about 27 cases of INH-resistant TB were to occur annually in this province. The following reasons can partially explain the significant gap between incidence and identification. The broad therapeutic range of INH makes it possible to achieve high drug concentrations in tuberculous lesions. Such a concentration overcomes many cases of low-level resistance [6]. As a result, they respond to conventional combination therapy without being recognized. On the other hand, the fundamentally different treatment of RIF resistance is a wise reason to prioritize the detection of RIF-resistant isolates [7]. In recent years, detection has become faster by implementing GeneXpert technology (which is capable of directly identifying *M.tb* isolates and detecting RIF resistance in sputum samples) in TB reference laboratories. However, the focus on RIF resistance has also increased. The presence of only three INH-mono-resistants among the resistant isolates under study confirms the relative neglect of INH resistance, which of course, is more or less the case worldwide [6].

Mutations in *katG* may confer INH resistance by a mechanism that blocks the pathway of prodrug activation [3]. According to a systematic study, the frequency of *katG* mutations in INH-resistant isolates is slightly more than 64% [28]. The present study result (60%) was close to this frequency. The most common mutation in this gene was S315T replacement (46.7%), present in half of MDR and one-third of mono-resistant isolates. Almost all similar studies show the superiority of this mutation, although with greater frequency, from 67.6 to 89.7% [5, 29]. The higher prevalence of this mutation in MDRs compared to mono-resistants is consistent with studies involving both isolates [37]. This finding supports the theory that S315T mutation predates RIF resistance [8].

There is a long list of NSS mutations for the *katG* gene [10], but the reason for the abundance of S315T mutation in INH-resistant *M.tb* strains is its comparative advantage for bacteria. Despite this mutation, the catalase/peroxidase activity of the enzyme is maintained but has less ability to activate INH [3, 30]. The association of S315T substitution with INH resistance—although moderate-level—has been confirmed [25, 26, 31, 32], including in our study. S315N substitution is also a resistance-conferring mutation [25, 26] that occurs at a lower frequency. Its prevalence in the present study (6.7%) is closer to that of India (5.9%), according to Munir et al. [29]. Part of the difference in the frequency of S315T mutation in this study with most other reports is due to one deletion in the *katG* gene of 6.7% of INH-resistant isolates. This mutation, which has not been previously reported in any study, probably disrupted or stopped the enzyme's activity by altering its structure. The occurrence in an MDR isolate without other confirmed resistance-conferring mutations could indicate its influential role in resistance.

Mutations in the *inhA* gene may lead to INH resistance by a mechanism of drug target alteration. Drug targets are highly conserved [3]. Therefore, the prevalence and diversity of *inhA* structural gene mutations, with a frequency of about 4% and mainly at two polymorphic sites, are not significant in clinical isolates [12, 33]. Instead, the *M.tb* takes a different path. Acquisition of mutations in the *inhA* promoter region, through overexpression of the drug target, causes resistance, albeit at a lower level [3]. In this study, 20% of resistant isolates contained − 15 C>T mutation in the promoter region. This mutation is considered to confer resistance [25, 26], and a systematic study has reported an average frequency of about 19% [28], which is consistent with our finding. The − 15 C>T mutation, by itself, creates low-level resistance; However, if it coincides with a mutation in *katG*, especially S315T, it will cause high-level resistance [31, 32]. None of the three isolates carrying − 15 C>T mutation had a mutation in the *katG* gene. Mutations in other loci of the isolates harboring *inhA* promoter mutations may synergize, requiring further investigation.

The *furA* gene is known as the negative regulator of the *katG* gene; so a mutation in the *furA* gene or the *furA-katG* intergenic region may develop a level of INH resistance by reducing the *katG* expression [10]. Some studies have not found mutations in this gene in INH-resistant isolates [31, 34], and others reported a small number of mutations at low frequencies from 2.3 to 3.7% [9, 32]. Few of them have decreased *katG* expression and may be associated with low-level resistance [10]. The present study identified an SNC (− 34 A>G) in the intergenic region not

mentioned in the relevant documents. Its possible role in developing resistance requires further investigation, especially since the isolate's *inhA* − 15 C>T mutation.

M.tb upregulates some genes, including *kasA*, when exposed to INH. Therefore, *kasA* has been suggested as a potential target for INH [10], and its mutations may cause resistance in the *M.tb* by the mechanism of drug target alteration. The *kasA* mutation rate in this study (26.7%) was consistent with a study from Poland (24%) [12] but higher than the frequency reported in other studies. This difference was partly due to an insertion not reported in previous studies. That insertion may disrupt the structure and function of the encoded enzyme by creating a frameshift. Regardless of the insertion, the 20% frequency of G269S substitution, which occurs only in MDR isolates, is consistent with some studies [37]. Most studies have identified G269S as the most common *kasA* mutation [9]. Some have identified this mutation in INH-sensitive isolates, so its role in conferring resistance is unclear [10]. We did not find G269S substitution in INH-susceptible isolates, but all three isolates carrying the mutation also had mutations in the *katG* 315 codon or *inhA* promoter region. In addition, the relationship between *kasA* mutation and INH resistance was statistically significant in this study. Consequently, it is difficult to judge the effect of *kasA* mutation on INH resistance with these findings, and it should not be ruled out altogether.

Among the resistant isolates studied, 46.7% had mutations in the *oxyR-ahpC* complex region, almost unprecedented and about 2.5 times the highest rate reported by other studies [32]. Two mutations (*oxyR* 37 C>T and *ahpC* − 46 G>A) were also detected in INH-susceptible isolates, consistent with previous studies [10, 20]. So, they probably did not play a role in INH resistance. The only specific mutation for resistant isolates was an SNC in the intergenic region (− 34 T>G) detected in an MDR isolate with a *katG* mutation. Mutations at this site have been previously reported [10], but G nucleotide substitution is novel. None of the mutations found in this study are on the list of resistance-associated mutations [25, 26]. Mutations in the *oxyR-ahpC* gene complex are not conducive to resistance but rather compensatory [8]. Clinical isolates with some *katG* mutations also carry mutations in the *oxyR-ahpC* region that cause AhpC overexpression and partially restore the fitness cost due to reduced *katG* activity [3]. Acquisition of these compensatory mutations can be a factor in the survival of bacteria and the evolution of transmissible resistant strains that are hazardous for public health [4, 20]. The mutations found in the *oxyR-ahpC* gene complex, with one exception, were not exclusive to resistant isolates. Therefore, it may be hoped that reduced fitness will prevent the predominance of INH-resistant isolates in the circulating *M.tb* population in the province.

Theoretically, if a mutation in *ndh* causes a defect in encoded enzyme activity, increased NADH levels can lead to resistance by competitively inhibiting the binding of active INH to the InhA enzyme and the peroxidation required for INH activation [35]. The frequency of *ndh* mutations in this study (20%) was higher than the maximum previously reported rate in INH-resistant isolates (9.5%) [36]. Three substitutions were found for the *ndh* gene both in INH-resistant and -susceptible isolates. Not only are these substitutions missing from the list of resistance-associated mutations [25, 26, 28], but they are not found in any other studies to the best of our knowledge. The definitive association of none of the few substitutions reported in previous studies with INH resistance has been established. Co-occurrence of two detected *ndh* substitutions with *katG* or *inhA* promoter mutations in the isolates under study reduces the likelihood of their involvement in INH resistance.

No resistance-conferring mutations were found in four of the INH-resistant isolates. Thus, either the mutations were acquired in loci other than those studied or a mechanism other than the mutation initially caused resistance. An important limitation of the present study was the small sample size. Nevertheless, from another perspective, enrolment of all resistant isolates identified in the last few years in a region with a population of 4.5 million may be a strength.

Conclusion

The present study characterized the mutations in 6 gene loci, previously reported to be associated with INH resistance, in 33 clinical isolates. This study indicated that the combination of *katG* 315 and *inhA* − 15 mutations could detect 73.3% of INH-resistant isolates. One deletion that resulted in a frameshift was identified in the *katG* gene of a resistant isolate, which lacked the *katG* 315 and *inhA* − 15 mutations. This finding may demonstrate the complexity of the molecular basis and the unknown aspects of INH resistance. Analysis of the remaining gene loci (other than *katG* and *inhA* promoter) did not significantly detect INH resistance, although the frequency of mutations in *ndh* and *oxyR-ahpC* were considerable. Our study revealed seven novel mutations in the studied genes, of which one insertion, one deletion, and two SNCs were observed exclusively in INH-resistant isolates.

Acknowledgements This research is part of the Ph.D. Dissertation of Pejman Bakhtiyariniya, which was approved and conducted at the Infectious and Tropical Diseases Research Center of the University, and was financially supported by a Grant (No. OG-9930) from Research affairs, Ahvaz Jundishapur University of Medical Sciences (AJUMS), Ahvaz, Iran. We sincerely appreciate the Regional Tuberculosis Reference Laboratory of Khuzestan Province Staff, the Infectious and Tropical Diseases Research Center Staff, and the Department of

Microbiology Staff to provide the necessary equipment and a friendly atmosphere for experiments.

Author contributions PB: Methodology, Investigation, Resources, Formal analysis, Writing—Original draft preparation, Writing—Review and Editing. ADK: Conceptualization, Supervision, Funding acquisition, Methodology, Writing—Review and Editing. MH: Data curation, Methodology, Validation, Writing—Review and Editing. MS: Data curation, Methodology, Project administration, Writing—Review and Editing. All authors read and approved the final manuscript.

Funding This work was supported by the Vice-Chancellor of Research Affairs of the Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (Grant No. OG-9903).

Data availability All analyzed data within this study can be obtained from the corresponding author on request.

Code availability Not applicable.Consent for publication Not applicable.

Declarations

Conflict of interest The authors have no conflict of interest.

Ethical approval The ethical approval statement for this study was obtained from the Research Ethics Committee of the Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (IR.AJUMS.REC.1399.183), in accordance with the Declaration of Helsinki.

Informed consent Before the study, written informed consents were obtained from all patients.

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