Original Article

Novel mutation in efflux pump *Rv1258c* (Tap) gene in drug resistant clinical isolates of *Mycobacterium tuberculosis* in Iran

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Abstract
Introduction: Tuberculosis (TB) remains a serious public health problem worldwide. Drug-resistant TB is considered a major and growing global threat. Despite the great variety of described mutations in *Mycobacterium tuberculosis* (MTB) resistance genes, the mechanisms of drug resistance are still controversial. Recently, a report on the role of efflux pump genes in drug resistance added to this complexity. Therefore, a thorough understanding of efflux pump genes in drug-resistant TB clinical isolates is needed.
Methodology: We performed molecular analysis of the efflux pump gene (*Rv1258c*) in 33 drug-resistant and 20 drug-sensitive clinical MTB isolates by sequencing the amplicons' targets in both the forward and reverse directions.
Results: A novel mutation of the *Rv1258c* gene was identified at G442A (Ala148Thr) in rifampicin mono-resistant clinical strain, as compared to the H37Rv reference strain. In addition, a cytosine nucleotide insertion was found between the positions 580 and 581 (denominated Tap580) in two drug-sensitive strains at identical gene positions.
Conclusions: These results indicated the possibility of mutation in the efflux pump genes and the important role of Tap efflux pump genes in drug-resistant MTB isolates. However, further research is required to determine the direct association of these mutations with resistant MTB.

Introduction

Mycobacterium tuberculosis (MTB) is an obligate parasite and the causative agent of tuberculosis (TB). There are ten million new cases of TB per year, and about 1.4 million deaths were reported in 2019 due to TB. Tuberculosis is still one of the main causes of morbidity and mortality in humans worldwide, especially in developing countries [1].

Drug-resistant TB is a major threat to global health. Simultaneous resistance to front-line drugs, such as isoniazid (INH) and rifampin (RIF) is defined as multi drug-resistant tuberculosis (MDR-TB). Moreover, extensively drug-resistant tuberculosis (XDR-TB) is a type of MDR-TB that is resistant to isoniazid and rifampin, along with any fluoroquinolone and at least one of the three injectable second-line drugs (i.e., amikacin (AMK), kanamycin (KAN), or capreomycin (CAP)) [2].

Generally, MDR-TB and XDR-TB are difficult to manage, and the use of less effective second-line injectable drugs (SLIDs) with major side effects is inevitable [3]. Therefore, researchers have attempted to find other approaches to increase the effectiveness of antibacterial drugs, considering the emergence of drug resistance [4].

So far, the horizontal transfer of resistance genes has not been reported in MTB isolates [5]. Evidence suggests that spontaneous mutations in chromosomal genes in MTB are the cause of drug resistant TB [6].

Research shows that efflux pumps (EPs) play an important role in intrinsic resistance. EPs are associated with virulence, biofilm formation, pathogenicity, and coordination of gene expression in dense bacterial populations [7]. The interaction between the decreased cell wall permeability and the activity of EPs, along with the increased expression of genes encoding EPs, is believed to constitute the first step in the development of drug-resistant phenotypes [8].

The EPs of MTB belong to different classes, including the ATP-binding cassette (ABC) superfamily of EPs which are the primary transporters encoded by 2.5% of the MTB genome. The secondary transporters include the major facilitator superfamily (MFS), small multidrug resistance (SMR), multidrug and toxic

compound extrusion (MATE), and resistance-nodulation-division (RND) transporters [9].

Rv1258c is one of the MFS EPs, which was primarily identified in *Mycobacterium* species, with low-level resistance to tetracycline and aminoglycosides [10]. A study showed that EPs encoded by *Rv1258c* can extrude various chemical classes and antibiotics, but to a lesser extent in MTB isolates [11]. Although many researchers have described the overexpression of EP genes and their reactions to anti-TB drugs, there is limited information about their gene mutations. Some ion channel blockers have been shown as potential inhibitors of the efflux system of MTB, such as phenothiazines (e.g., thioridazine), chlorpromazine, and phenylalkylamines (e.g., verapamil) [12]. The combination of EP inhibitors (EPIs) and antibiotics may provide an effective adjuvant for the conventional chemotherapy regimens [13].

In this regard, Garima *et al.* recognized single-nucleotide polymorphisms (SNPs) in ten EP genes by comparing an MDR-TB strain sequence with H37Rv. They found that the same genes were overexpressed in response to INH and RIF treatment [14]. Moreover, in 2017, a study on XDR-MTB clinical strains analyzed the whole genome sequence (WGS) and showed nonsynonymous single nucleotide polymorphisms (SNPs), mainly in the ABC and MFS of EP genes [15]. In this regard, Machado *et al.* showed the strong correlation of these inhibitors as adjuvants in TB treatment [13].

The overexpression of *Rv1258c EP*, also called *Tap* or *P55*, has been shown to be involved in the resistance of *Mycobacterium smegmatis* and *Mycobacterium bovis* Bacille de Calmette et Guérin (BCG) to acriflavine and tetracycline drugs [16]. Another study reported that the overexpression of *Rv1258c* gene in BCG could cause resistance to INH, RIF, ethambutol (EMB), and pyrazinamide (PZA) [16]. In addition, overexposure to INH and RIF could lead to the overexpression of *Rv1258c* gene, and consequently, cause drug resistance in MDR-TB clinical isolates [15].

Recently, various SNPs have been found in the *Rv1258c* EP genes in clinical XDR-TB isolates [15]. However, the correlation of these SNPs in clinical isolates with drug resistance has not been clarified yet. In this regard, Liu *et al.* found SNPs within the *Rv1258c* (*Tap*) gene of clinical MTB isolates. To evaluate the importance of these SNPs in clinical isolates, related to drug resistance, they evaluated point mutations (V219A and S292L) in the genome of isogenic MTB isolates. The sequencing results of the *Rv1258c* gene showed

that these mutations could cause drug resistance [17]. Also, the *Rv1258c* gene may lead to cross-resistance to aminoglycosides [18]. The sequence analysis of *Rv1258c* EP gene indicated a novel amino acid change of Y177H in the XDR isolates [19]. Moreover, Machado *et al.* illustrated that MDR, especially resistance to INH, increased in laboratory MTB isolates, which is possibly related to the activity of EPs [8].

In our previous studies, resistance associated genes evaluated in the 33 drug resistant clinical isolates were; *katG* (codon 315), *inhA* promoter, *rpoB*, *embB* [20], and other regions of *KatG*, *inhA*, *ahpC-oxyR*, and *ubiA* genes [21], In this study, the authors aimed to conduct sequence analysis of an EP gene (*Rv1258c*) among drug-resistant and susceptible clinical MTB isolates to get more information on these TB clinical isolates.

Methodology

Bacterial isolation, biochemical and molecular identification

A total of 53 pulmonary tuberculosis (PTB) isolates were collected from the Mollahadi Sabzevari Tuberculosis Center in Isfahan, Iran during 2017-2020. Out of these, 33 were resistant to the first-line drugs (INH, RIF, EMB and PZA), and 20 were susceptible.

Laboratory identification was done with the microscopic Ziehl Neelsen staining method and on egg-based Lowenstein-Jensen (LJ) medium culture. MTB isolates were validated by the production of niacin and nitrate [22]. Demographic data, phenotypic and genotypic characteristics of the PTB patients are presented in Table 1.

Molecular detection of IS6110 insertion element was performed by the polymerase chain reaction (PCR) assay. The samples were collected from PTB patients following the previously described method [23]. The PCR assay conditions for IS6110 insertion element were as follows: denaturation at 95 °C (15 minutes), followed by 30 denaturation cycles at 95 °C (30 seconds), annealing at 57 °C (30 seconds), elongation at 72 °C (30 seconds), and a final elongation at 72 °C for 5 minutes.

Drug-susceptibility testing (DST)

DST of the MTB complex (MTBC) clinical isolates was performed by the proportion method on LJ medium, according to the World Health Organization (WHO) guidelines for the first-line drugs (INH, RIF, EMB and PZA) [24], The critical concentrations of drugs used on the LJ medium were as follows: INH (0.2 μg/mL), RIF (40 μg/mL), EMB (2 μg/mL) [24].

Genomic DNA extraction

The cetyltrimethylammonium bromide (CTAB) method was used for DNA extraction of the clinical isolates [25]. A NanoDrop One Spectrophotometer (Thermo Fisher Scientific Inc. Waltham, Massachusetts, U.S.) was used for evaluation of DNA concentration and purity.

DNA amplification and sequencing

The *Rv1258c* gene were amplified from all DNA samples of MTB clinical isolates using PCR. All the

Table 1. Demographic data, phenotypic and genotypic characteristics identified of the pulmonary tuberculosis patients.

ID	Genus	City	Nationality	Phenotypic characteristics					Genotypic characteristics (mutation)							
				MDR	INH	RIF	EMB	PZA	*Rv1258c*	*katG*	*inhA* promotor	*inhA*	*rpoB*	*oxyR-ahpC* intergenic region	*embB*	*ubiA*
1	Male	Isfahan	Iranian	-	R	S	S	S	-	*	-	-	-	*	-	-
2	Male	Isfahan	Iranian	-	R	S	S	S	-	*	-	-	-	*	-	-
3	Male	Arak	Iranian	*	R	R	S	S	-	*	-	-	*	*	-	-
4	Female	Qom	Iranian	-	R	S	S	R	-	*	-	-	-	*	-	-
5	Female	Isfahan	Afghan	*	R	R	S	S	-	*	-	-	*	-	-	-
6	Female	Isfahan	Afghan	*	R	R	S	S	-	*	*	-	*	-	-	-
7	Male	Yazd	Iranian	*	R	R	R	S	-	*	-	-	*	*	-	-
8	Female	Isfahan	Afghan	-	S	S	S	R	-	-	-	-	-	-	-	-
9	Male	Isfahan	Iranian	-	S	R	S	S	-	-	-	-	-	-	-	-
10	Male	Tehran	Iranian	*	R	R	S	S	-	*	-	-	*	*	-	-
11	Male	Isfahan	Iranian	*	R	R	S	R	-	*	-	-	*	*	-	-
12	Male	Arak	Iranian	*	R	R	S	R	-	-	-	*	*	-	-	-
13	Male	Isfahan	Iranian	-	S	R	S	S	Ala148Thr	-	-	-	-	-	-	-
14	Female	Isfahan	Iranian	-	S	R	S	S	-	-	-	-	-	-	-	-
15	Female	Isfahan	Iranian	-	S	R	S	S	-	-	-	-	-	-	-	-
16	Female	Isfahan	Iranian	-	S	R	S	S	-	-	-	-	-	-	-	-
17	Male	Isfahan	Afghan	-	S	R	S	S	-	-	-	-	-	-	-	-
18	Male	Arak	Iranian	-	S	R	S	S	-	-	-	-	*	-	-	-
19	Female	Isfahan	Iranian	-	R	S	S	S	-	*	-	-	-	-	-	-
20	Male	Isfahan	Iranian	-	R	S	S	R	-	*	-	-	-	-	-	-
21	Female	Isfahan	Iranian	-	R	S	S	S	-	*	-	-	-	*	-	-
22	Female	Isfahan	Iranian	-	R	S	S	R	-	-	-	-	-	*	-	-
23	Male	Isfahan	Iranian	-	R	S	S	S	-	*	*	-	-	*	-	-
24	Male	Isfahan	Iranian	-	S	S	R	S	-	-	-	-	-	-	-	-
25	Male	Isfahan	Iranian	-	R	S	S	S	-	*	-	-	-	-	-	-
26	Male	Kashan	Iranian	-	R	S	S	R	-	*	*	-	-	*	-	-
27	Male	Tehran	Iranian	-	R	S	S	S	-	*	-	-	-	-	-	-
28	Male	Isfahan	Iranian	*	R	R	S	S	-	*	-	-	*	*	-	-
29	Female	Isfahan	Iranian	-	R	S	S	S	-	*	-	-	-	*	-	-
30	Male	Arak	Iranian	-	R	S	S	S	-	*	*	-	-	*	-	-
31	Male	Isfahan	Iranian	-	R	S	S	S	-	*	-	-	-	*	-	-
32	Male	Isfahan	Iranian	-	S	S	R	S	-	-	-	-	-	-	-	-
33	Male	Isfahan	Iranian	-	S	S	R	S	-	-	-	-	-	-	-	-
34	Male	Isfahan	Iranian	-	S	S	S	S	-	-	-	-	-	-	-	-
35	Male	Isfahan	Iranian	-	S	S	S	S	-	-	-	-	-	-	-	-
36	Female	Isfahan	Afghan	-	S	S	S	S	-	-	-	-	-	-	-	-
37	Female	Isfahan	Afghan	-	S	S	S	S	-	-	-	-	-	-	-	-
38	Female	Isfahan	Iranian	-	S	S	S	S	-	-	-	-	-	-	-	-
39	Male	Isfahan	Iranian	-	S	S	S	S	-	-	-	-	-	-	-	-
40	Female	Isfahan	Iranian	-	S	S	S	S	-	-	-	-	-	-	-	-
41	Male	Isfahan	Iranian	-	S	S	S	R	-	-	-	-	-	-	-	-
42	Female	Isfahan	Iranian	-	S	S	S	S	-	-	-	-	-	-	-	-
43	Male	Isfahan	Iranian	-	S	S	S	S	-	-	-	-	-	-	-	-
44	Female	Isfahan	Afghan	-	S	S	S	S	-	-	-	-	-	-	-	-
45	Female	Isfahan	Iranian	-	S	S	S	S	-	-	-	-	-	-	-	-
46	Male	Isfahan	Afghan	-	S	S	S	S	-	-	-	-	-	-	-	-
47	Male	Isfahan	Iranian	-	S	S	S	S	-	-	-	-	-	-	-	-
48	Male	Isfahan	Iranian	-	S	S	S	S	-	-	-	-	-	-	-	-
49	Male	Isfahan	Iranian	-	S	S	S	S	-	-	-	-	-	-	-	-
50	Male	Isfahan	Iranian	-	S	S	S	S	-	-	-	-	-	-	-	-
51	Male	Isfahan	Iranian	-	S	S	S	S	-	-	-	-	-	-	-	-
52	Female	Isfahan	Iranian	-	S	S	S	S	Tap^{580}	-	-	-	-	-	-	-
53	Female	Isfahan	Iranian	-	S	S	S	S	Tap^{580}	-	-	-	-	-	-	-

INH: isoniazid; RIF: rifampin; EMB: ethambutol; PZA: pyrazinamide, MDR: multi-drug resistant; R: resistant, S: susceptible, *: positive, -: negative; Tap^{580}: cytosine nucleotide insertion was found between positions 580 and 581.

Table 2. Primers used for sequencing.

Gene	Sequence of primer	Product size (bp)
Rv1258c	Forward: CGGCATTCCTGATCCTGTT Reverse: CGTGTGGTCGGTGAAGTATT	700 bp
IS6110 insertion element	Forward: GAACCGTGAGGGCATCGAGG Reverse: GCGTAGGCGTCGGTGACAAA	249 bp

amplicons were visualized on 1.5% agarose gel stained with SYBR Safe DNA gel stain by electrophoresis, followed by purification and sequencing the target amplicons. The sequencings were performed in both the forward and reverse directions by using the Sanger sequencing method [26].

PCR amplification and sequencing of *Rv1258c* gene were carried out from clinical INH, RIF, EMB, and PZA resistant and 20 sensitive clinical isolates. Table 2 presents a list of primers used for sequencing in this study. Amplification was done in a PCR reaction tube (50 µL). It contained 25 µL of 2× Master Mix (1.5 mM MgCl$_2$; Ampliqon, Odense, Denmark), 1.5 µL of each primer (10 pmol) Microsynth Company, Balgach, Switzerland, 20.5 µL of PCR-grade H$_2$O, and 1.5 µL of template DNA. The conditions of PCR assay were as follows: denaturation at 95 °C (15 minutes), followed by 30 cycles of 95 °C (30 seconds), annealing at 60 °C (30 seconds), 72 °C (30 seconds), and a final elongation step at 72 °C for 5 minutes [19].

Bioinformatics analysis

The sequences were analyzed for the presence of mutations by comparing with the published sequences of H37Rv, using CLC Genomic Workbench version 20. All sequences with the reference sequence were compared and analyzed using the Basic Local Alignment Search Tool (BLAST) (https://www.ncbi.nlm.nih.gov/).

Statistical analysis

Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS) software, version 26 (IBM Corp., USA).

Results

Bacterial isolates and drug susceptibility testing

Among the 33 PTB resistant isolates that were collected, 22 (66.7%) were from males, and 11 (33.3%) from females. Drug susceptibility tests indicated resistance to INH (22, 66.7%), RIF (15, 45.4%), EMB (4, 12.1%) and PZA (7, 21.2%). Moreover, 8 (24.2%) out of 33 PTB isolates were MDR, and 20 (60.6%) were mono-drug resistant, including 10 (30.3%) INH mono-resistant, 7 (21.2%) RIF mono-resistant and 3 (9.1%) EMB mono-resistant isolates. Out of 33 isolates, one isolate (3.1%) was not only resistant to INH and RIF, but also resistant to EMB, and two of them (6.1%) were simultaneously resistant to 3 drugs (INH, RIF and PZA). No isolate was resistant to all 4 drugs (INH, RIF, and EMB and PZA) (Table 1 and Table 3).

Molecular analysis

All 53 resistant and susceptible isolates were sequenced for the analysis of the *Rv1258c* gene. Out of

Table 3. Frequency of resistance patterns and *Rv1258c* efflux pump gene mutation among first-line drugs resistance and susceptible *Mycobacterium tuberculosis* clinical isolates.

Resistance (33)	Frequency n (%)	*Rv1258c* mutation n (%)	Nucleotide change	Amino acid change
Patterns				
INH	22 (66.7%)	-	-	-
RIF	15 (45.4%)	-	-	-
EMB	4 (12.1%)	-	-	-
PZA	7 (21.2%)	-	-	-
Mono INH	9 (27.3%)	-	-	-
Mono RIF	7 (21.2%)	1 (3%)	G442A[a]	Ala148Thr
Mono EMB	3 (9.1%)	-	-	-
Mono PZA	-	-	-	-
MDR (INH/RIF)	8 (24.2%)	-	-	-
INH/RIF/EMB	1 (3.1%)	-	-	-
INH/RIF/PZA	2 (6.1%)	-	-	-
Susceptible (20)	20 (37.7%)	2/20 (10%)	C nucleotide insertion 580-581	Frameshift
Total (53)		3/53 (5.7%)		

[a] Mutations that were not previously reported. INH: isoniazid; RIF: rifampin; EMB: ethambutol; PZA: pyrazinamide; MDR: multi-drug resistant; C: cytosine nucleotide.

33 clinical MTB isolates, 22 (66.7%) showed first-line drug resistance patterns, one isolate had a novel mutation in the *Rv1258c* efflux pump gene. Interestingly, none of the isolates with INH resistance displayed a mutation in the *Rv1258c* gene (Table 1, Table 3). In addition, among EMB (12.1%), PZA (21.2%) and MDR (24.2%) resistance isolates, the *Rv1258c* gene analysis revealed no mutation despite mutations in other genetic regions (Table 1). However, in RIF resistant isolates (45.4%), one isolate (RIF mono resistant) had a novel amino acid change (Ala148Thr) within the *Rv1258c* efflux pump, with no mutations in the studied drug target genes (*katG*, *inhA*, *inhA* promotor, *oxyR-ahpC* intergenic region, *rpoB*, *embB* and *ubiA*). This mutation was not found in any of the susceptible isolates (Table 3). Molecular analysis of the *Rv1258c* loci gene in 20 susceptible isolates showed a cytosine nucleotide insertion mutation between positions 580 and 581 in the identical position of the *Rv1258c* gene (Table 3).

Sequence analysis Rv1258c genes

All 33 first-line drug-resistant isolates and 20 susceptible isolates were analyzed to compare the *Rv1258c* gene. A novel mutation (Ala148Thr) was observed in 1/33 (3%) first-line drug-resistant isolates (RIF mono resistant isolate), as compared to the H37Rv reference strain and a cytosine nucleotide insertion mutation (denominated Tap580) in 2/20 (10%) drug susceptible (DS) isolates that was not found in any of the resistant isolates (Table 1 and Table 3).

Accession numbers

The obtained sequences were deposited in GenBank with the following accession numbers: MW132165 for *Rv1258c* efflux pump gene G442A (Ala148Thr) and MW586861 and MZ209419 for cytosine nucleotide insertion (denominated Tap580).

Discussion

Although drug resistance in MTB is mainly related to the presence of mutations within the drug target genes, EP activity has been lately identified to play a remarkable role in the increased drug resistance of MTB. Recent research has focused on EPs and their correlation with drug resistance. A study by McMurry *et al.* was the first report of the involvement of active EPs in resistance to antibiotics [27]. Moreover, Liu and Xie found non-synonymous mutations within 20 known EP genes of MDR, pre-XDR, and XDR-TB MTB isolates that were not detected in the H37Rv reference strain [28].

Generally, EPs play a key role in bacterial drug resistance. Overall, drug resistance is induced when an organism is exposed to a sublethal dose of anti-TB drugs [8]. In this regard, Calgin *et al.* demonstrated that the overexpression of EPs could lead to high minimum inhibitory concentrations (MICs) of anti-TB drugs during the process of treatment [29]. It has been also shown that the *Rv1258c* EP gene is overexpressed in the presence of drugs, suggesting its role in drug resistance phenotypes [13].

Various studies have shown the overexpression of EP genes following exposure to drugs in MDR/XDR MTB isolates as well as RIF-resistant isolates [30,31], Similarly, a study by Yamchi *et al.* indicated a significant difference in the expression level of EP genes between drug-susceptible and MDR/XDR MTB clinical isolates [30]. In this study, sequencing of *Rv1258c* gene was performed in MTB isolates resistant to first-line drugs (INH, RIF, EMB, and PZA), as well as pan-sensitive isolates of MTB. We found the novel *Rv1258c* mutation at G442A (Ala148Thr) in only 1/33 isolates resistant to first-line drugs that RIF mono-resistant isolate without rpoB mutation, and other genes mutations were sequenced, while no such mutation has been observed in pan-sensitive isolates. Also, no other study has reported the G442A mutation. The encoding of a tetracycline/aminoglycoside resistant-like EP by *Rv1258* [32] and its upregulation occur under RIF pressure in MDR-TB isolates [33].

According to previous studies, mutations within the *Rv1258c* gene were detected in drug-resistant clinical isolates that lacked the known mutations [15,19]. Moreover, Malinga *et al*. reported the Y177H mutation in the *Rv1258c* gene in one XDR-TB isolate with AMK/KAN/CAP cross-resistance. Jia *et al* reported the newly identified T297P and I328T mutations in *Rv1258c* of XDR-TB isolates that improved resistance to ethambutol and capreomycin [34].

Overall, the *Rv1258c* gene region is stable, and emergence of a mutation may lead to the over expression of EPs [19]. So far, the G442A mutation has not been detected in drug-resistant or drug-susceptible clinical isolates. Kanji *et al.* found several novel low-frequency SNPs, which might be related to drug resistance in multiple EP genes, including the *Rv1258* gene. The *Rv1258* G391R and P369T SNPs were also found in XDR-TB clinical isolates [15]. Another study indicated the overexpression of *Rv1258c* gene as a result of G133C mutation and revealed its correlation with cross-resistance to streptomycin (STR) and KAN [18].

Our sequencing data showed that in pan-sensitive clinical isolates, there was a cytosine nucleotide insertion between positions 580 and 581 of *Rv1258c* gene, which was not found in any of the drug-resistant clinical isolates. Villellas *et al.* demonstrated two different mutations in the *Rv1258c* gene of STR-resistant clinical strains. A single nucleotide deletion (adenine) was found at position 13, followed by a frameshift mutation resulting in a stop codon after only ten amino acids. Similar to our study, Villellas *et al.* observed a cytosine nucleotide insertion between positions 580 and 581 within the *Rv1258c* gene in four strains. This frameshift mutation resulted in a shorter protein from codon 194 onwards (231 amino acids). This protein (perhaps without functional activity as a membrane transporter) contained only six transmembrane segments (TMS), as compared to the normal and full-length 12-TMS proteins (419 amino acids). It should be noted that bacterial drug EPs of MFS have 12 or 14 TMS, which are essential for transport activity [35].

Since Tap580 mutation in the *Rv1258c* gene can lead to a truncated structure and inactivate proteins, harboring a nucleotide insertion seems to be a disadvantage for drug-resistant isolates, as compared to isolates with an active and entirely functional *Rv1258c* EP.

To define the role of *Rv1258c* point mutations in drug resistance, Liu *et al.* constructed point mutations (V219A and S292L) in the *Rv1258c* gene by site-directed mutagenesis and found the correlation of these point mutations with PZA, INH and STR resistance; this may explain the unknown drug resistance in the clinical strains [17]. Additionally, in previous studies, the overexpression of EP genes in drug-resistant isolates, without mutations in drug target genes, showed that the possible combination of SNPs in EP genes may be another factor affecting drug resistance in MDR/XDR isolates [36]. The upregulation of *Rv1218* and *Rv1273* genes in MDR isolates, without *rpoB* or *katG* mutations [30], strengthened the assumption that EPs are one of the causes of drug resistance. Shahi *et al.* evaluated multiple EPs among MDR-TB clinical isolates and showed that *Rv1258c* mediate the efflux of antibiotics and was overexpressed upon RIF or INH exposure [37].

On the other hand, some researchers have shown that EPs can induce MDR-TB susceptibility to drugs by inhibition of EPs and suggested consideration of EPs as adjuvants for TB treatment regimens [38–40]. In the presence of EPIs, knockout mutants (e.g., *Rv1258c* knockout mutant) showed greater susceptibility to chemical compounds [11]. Other studies reported a decrease in the resistance level of clinical MTB strains to first- and second-line anti-TB drugs, including verapamil, thioridazine, and chlorpromazine as EPIs [13,41]. Moreover, Louw *et al.* and Li *et al.* found a reduction in the MIC of RIF upon exposure to carbonyl cyanide m-chlorophenyl hydrazone (CCCP) and reserpine as EPIs in RIF-resistant isolates [42,43]. Considering the significant potential of EPIs in reversing antibiotic resistance, they can be used as antibiotic adjuvants in the future [44]. Overall, the use of EPIs seems to be an effective strategy, as the available therapeutic agents have many challenges.

Conclusions

Our results demonstrate that point mutations in *Rv1258c* gene can play a potential role in increasing drug resistance in isolates. Overall, it is essential to prioritize the improvement of molecular detection of EP mutations and drug target mutations in drug-resistant MTB isolates, because the exact mechanism of mutation in EPs is not yet fully described. Such efforts can shed light on some formerly unknown causes contributing to drug-resistance. We suggest further in silico and in vitro studies on natural and synthetic compounds having EPI activities that can prevent the function of EPs by impeding the energy required by the EPs and affecting the membrane energetics of MTB to prevent EPs. However, despite the usefulness of efflux inhibitors in vitro studies, in most cases, their application in vivo is restricted due to toxicity. It seems that the sequence, protein structure, and function of EPs evolve in clinical isolates during the acquisition of drug resistance. Therefore, further large-scale studies are needed with a larger sample size to highlight the mechanisms of first- and second-line antibiotic resistance, mediated by mutations in the *Rv1258c* gene in clinical isolates.

Authors' contributions
Bahram Nasr Esfahani: supervision, funding acquisition, project administration, writing, reviewing, and editing; ShimaSadat Farzaneh: investigation, formal analysis, software, data curation, and writing original draft; Fatemeh

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