ORIGINAL RESEARCH

Genetic Characterization Conferred Co-Resistance to Isoniazid and Ethionamide in *Mycobacterium tuberculosis* Isolates from Southern Xinjiang, China

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Background: Ethionamide (ETH), a structural analogue of isoniazid (INH), is used for treating multidrug-resistant tuberculosis (MDR-TB). Due to the common target InhA, INH and ETH showed cross-resistance in *M. tuberculosis*. This study aimed to explore the INH and ETH resistant profiles and genetic mutations conferring independent INH- or ETH-resistance and INH-ETH cross-resistance in *M. tuberculosis* circulating in south of Xinjiang, China.

Methods: From Sep 2017 to Dec 2018, 312 isolates were included using drug susceptibility testing (DST), spoligotyping, and whole genome sequencing (WGS) to analyze the resistance characteristics for INH and/or ETH.

Results: Among the 312 isolates, 185 (58.3%) and 127 (40.7%) belonged to the Beijing family and non-Beijing family, respectively; 90 (28.9%) were INH-resistant (INH^R) with mutation rates of 74.4% in *katG*, 13.3% in *inhA* and its promoter, 11.1% in *ahpC* and its upstream region, 2.2% in *ndh*, 0.0% in *mshA*, whilst 34 (10.9%) were ETH-resistant (ETH^R) with mutation rates of 38.2% in *ethA*, 26.2% in *inhA* and its promoter, and 5.9% in *ndh*, 0.0% in *ethR* or *mshA*; and 25 (8.0%) were INH-ETH co-resistant (INH^{R}ETH^R) with mutation rates of 40.0% in *inhA* and its promoter, and 8% in *ndh*. *katG* mutants tended to display high-level resistant to INH; and more *inhA* and its promoter mutants showed low-level of INH and ETH resistance. The optimal gene combinations by WGS for the prediction of INH^R, ETH^R, and INH^{R}ETH^R were, respectively, *katG*+*inhA* and its promoter (sensitivity: 81.11%, specificity: 90.54%), *ethA*+*inhA* and its promoter+*ndh* (sensitivity: 61.76%, specificity: 76.62%), and *inhA* and its promoter+*ndh* (sensitivity: 48.00%, specificity: 97.65%).

Conclusion: This study revealed the high diversity of genetic mutations conferring INH and/or ETH resistance among *M. tuberculosis* isolates, which would facilitate the study on INH^R and/or ETH^R mechanisms and provide clues for choosing ETH for MDR treatment and molecular DST methods in south of Xinjiang, China.

Keywords: cross-resistance, ethionamide, isoniazid, mutation, *Mycobacterium tuberculosis*

3117

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is one of the leading causes of death worldwide and caused 1.6 million deaths and 10.6 million new cases in 2021.[1] Despite decades of efforts to adopt measures to control TB, achieving the goal of ending TB has been delayed due to a variety of reasons, from drug-resistant TB to untimely diagnosis. The proportion of new and previously treated TB cases with multidrug-resistant or rifampicin-resistant tuberculosis (MDR/RR-TB, MDR-TB defined as resistance to at least rifampicin and isoniazid) globally was 3.6% and 18%, respectively, and the combined number of MDR/RR-TB plus extensively drug-resistant tuberculosis in 2021 increased by 6.4% over 2020.[1] The persistence of TB and drug-resistant TB was ascribed in part to the paucity of rapid diagnostic techniques; however, the new routes for implementing efficient and rapid diagnosis lie in the knowledge and analysis of the resistance rates and molecular characteristics of different drugs, which varied from different geographical regions.[2]

Ethionamide (ETH), a structure analog of the core drug of anti-TB regimens isoniazid (INH), was mostly used in MDR-TB.[3,4] As two prodrugs, both INH and ETH were activated by different mycobacterial enzymes for antibacterial effect.[5] After being activated by the catalase peroxidase KatG (encoded by *katG/Rv1908c*)[5] and NADPH-specific flavin adenine dinucleotide-containing monooxygenase EthA (encoded by *ethA/Rv3854c*),[6,7] respectively, both INH and ETH reacted with NAD^+ to yield INH-NAD or ETH-NAD adducts which were able to bind to and inhibit the same molecular target named NADH-dependent enoyl-acyl carrier protein reductase InhA,[8] leading to the disruption of mycolic acid biosynthesis and cell death.[9,10] So far, several genes involved in the INH and ETH antibacterial pathway have been found to perform important roles in the acquired resistance of INH and ETH (Figure 1).[11]

Resistance to INH and ETH in *Mycobacterium tuberculosis* was mainly attributed to mutations in the activators of the drugs coded by katG and ethA, respectively.[11] Mutations in the *inhA* or its promoter region which lead to the inhibition of InhA enzyme may cause cross-resistance to INH and ETH.[8] Mutation in *ndh* gene is another mechanism explained the cross-resistance between these two drugs. *ndh* encodes a type-II NADH dehydrogenase which oxidizes NADH into
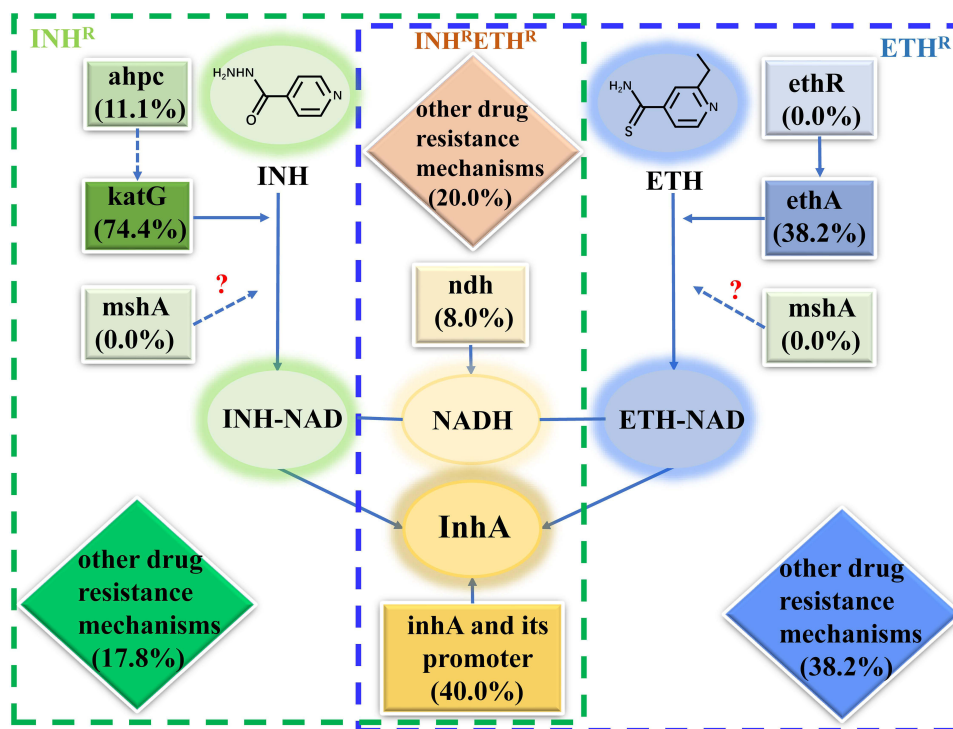

Figure 1 Action and resistance mechanisms of isoniazid and ethionamide in *Mycobacterium tuberculosis*.
Notes: The number in the blank means the contribution of mutations in this gene to isoniazid or ethionamide resistance. The ellipse indicates drug action mechanism, the square and diamond indicate genes associated with drug resistance. The dashed line pointed to an interaction that does not directly result in INH or ETH resistance. The question mark indicates an unclear effect on drug resistance.

NAD^+, mutations in *ndh* lead to an increase in NADH concentration, which acts as a competitive inhibitor to prevent the binding of INH-NAD and ETH-NAD adducts to InhA and consequently the activity of InhA enzyme is impacted, leading to the co-resistant to INH and ETH.[12,13] In addition, mutation in *mshA* may result in high-level in ETH resistance and low-level INH resistance in biochemical studies.[11] The *mshA* gene encodes the glycosyl transferase which involves in the biosynthesis of mycothiol, a main reducing and detoxifying agent in mycobacterial.[14] Finally, there are another two important regions or genes, the upstream region of *ahpC* and the *ethR gene*, involved in resistance to INH and ETH, respectively. *ahpc* encoded an alkyl hydroperoxide reductase, and when the upstream region of *ahpC* was mutated, it may render *M. tuberculosis* more susceptible to hydrogen and organic peroxides as *ahpC* expression increases.[11] Mutations in the *ahpC* were thought to function as compensatory mutations for the loss of KatG activity.[15] EthR, a transcriptional repressor, was the negative regulator of *ethA*,[16] and existing studies showed that *ethR* overexpression increased the resistance to ETH.[6]

Whole-genome sequencing (WGS) has been applied as a diagnostic, epidemiologic and research tool in the studies of *M. tuberculosis* since the complete genome sequence of *M. tuberculosis* was described in 1998.[17] Relying on the WGS, not only the prevalence of resistance-causing mutation SNPs was able to be grasped but also the specific deletions and SNPs peculiar to clinical strains could be identified. Additionally, it has the potential to demonstrate more extensive genetic variability.[18,19] Hence, it is of interest to investigate the molecular characteristics of INH-resistant and ETH-resistant *M. tuberculosis* isolates with WGS. However, in previous studies of INH and ETH resistance with WGS, either no cross-resistance studies of INH and ETH were performed or relatively few genetic regions were selected, or the overall resistance characteristics of the geographic region were not combined.[20–23] This study aimed to obtain the prevalence data and risk factors of INH and ETH resistance and cross-resistance through drug susceptibility testing (DST) and statistical analysis and investigate mutations in genes of *katG, ahpC* and its upstream that associated with only INH resistance, *ethA* and *ethR* that associated with only ETH resistance, and *inhA* and its promoter region, *ndh* and *mshA,* that associated with INH-ETH cross-resistance in *M. tuberculosis* isolates from southern region of Xinjiang, China.

Materials and Methods

Ethics Statement

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee of the Eighth Affiliated Hospital of Xinjiang Medical University (XJMU8HEC-20161215). Each potential participant was introduced to the nature of the research and provided with an information sheet. Participants were included in this study if their written informed consent was obtained.

Collection of *Mycobacterium tuberculosis* Clinical Isolates

A total of 312 *M. tuberculosis* complex isolates from TB patients were collected from four TB special hospitals in charge of the TB control in the southern region of Xinjiang, including Xinjiang Uygur Autonomous Region Chest Hospital, Kashi Lung Hospital, Kuche County Infectious Disease Hospital and Wushi County People Hospital, between Sep 2017 and Dec 2018. All of the pulmonary TB patients aged ≥16 years with positive cultures identified as *M. tuberculosis* complex (MTBC) and lived in southern Xinjiang, China, were interviewed and enrolled during the study period. The isolates collected from each participant were sub-cultured on the Lowenstein-Jensen (L-J) medium for DST and DNA extraction. The sub-culture, DST, the isolates' collection and inactivation for DNA extraction were performed in a biosafety level 3 laboratory. The demographic characteristics were collected from the electronic medical record. Mycobacterial species identification was performed using an in-house script by alignment of the WGS assembly data to the *16s rRNA* sequence of H37Rv (accession number: NC_000962.3), if identity >99%, the isolates were identified as *M. tuberculosis*, and all isolates in the present study were identified as *M. tuberculosis*.

Phenotypical Drug Susceptibility Testing

Each of the 312 *M. tuberculosis* isolates was tested for susceptibility to 12 anti-TB drugs, using the Sensititre® plates (Thermo Fisher Scientific Inc., Cleveland, Ohio, USA), and all steps were conducted strictly according to the

manufacturer's instructions by trained staff at the national tuberculosis reference laboratory of China. Briefly, the suspensions of *M. tuberculosis* strains were adjusted to 0.5 McFarland standard with sterile normal saline. The standardized suspensions were diluted 100-fold with Middlebrook 7H9-OADC broth (0.2% glycerol, 10% Middlebrook oleic acid-albumin-dextrose-catalase, and 0.05% Tween 80) for inoculation into the 96-well plates and then incubated at 37°C for 10–21 days to read the results using the Vizion Digital viewing System (Thermo Fisher Scientific Inc., Cleveland, Ohio, USA).[24] Minimum inhibitory concentration (MIC) was defined as the lowest concentration without apparent visible bacterial growth compared with positive controls and was measured by two readers.[24] The isolates with MICs greater than the following concentrations were identified as resistant: INH 0.25 μg/mL, rifampicin (RIF) 1.0 μg/mL, ethambutol (EMB) 5 μg/mL, streptomycin (SM) 1.0 μg/mL, amikacin (AMK) 1.0 μg/mL, ofloxacin (OFX) 2.0 μg/mL, moxifloxacin (MFX) 0.5 μg/mL, ETH 5.0 μg/mL, rifabutin (RFB) 0.5 μg/mL, kanamycin (KM) 5.0 μg/mL. Cycloserine (CS) and p-aminosalicylic acid (PAS) were excluded from our analysis because phenotypic DST results for these drugs are unreliable.[25,26] High level of resistance to INH or ETH was defined as MIC ≥1 μg/mL or ≥20 μg/mL, respectively, while low level of resistance to INH was defined as MIC between 0.25 and 0.5 μg/mL and to ETH was between 5 and 10 μg/mL.[27,28] The *M. tuberculosis* H37Rv (ATCC 27294) strain stored in the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, was used as a susceptible control.

DNA Extraction

The crude genomic DNA was extracted from fresh culture with the cetyltrimethylammonium bromide (CTAB) method as the previous document.[29,30] Three to four loops of *M. tuberculosis* colonies cultured on L-J medium for four weeks were harvested, followed by being heated in a water bath at 80°C for 30 min and then lysed with 0.1 mg/mL lysozyme at 37°C overnight. After treatment with the proteinase K, 10% sodium dodecyl sulfate (SDS), CTAB-NaCl (4.1% NaCl and 10% CTAB) and chloroform-isoamyl alcohol (24:1 [vol/vol]), the samples were centrifuged for 15 min at 13,000 × g. After being incubated at −20°C for 30 min in isopropanol, the genomic DNA samples were washed with 70% ethanol and resuspended with Tris-EDTA (TE, pH8.0). Finally, the DNA pellet was quantified with a NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) by measuring the absorption at 260 nm.

Spoligotyping and Data Analysis

Spoligotyping was performed using 43 covalently bound oligonucleotides derived from the spacer sequences of *M. tuberculosis* H37Rv and *Mycobacterium bovis* BCG P3 as previously described by Kamerbeek et al.[31] The results were entered in binary format into an Excel spreadsheet and compared with data in the SpolDB4 spoligotyping database (http://www.pasteurguadeloupe.fr:8081/SITVITDemo/index.jsp). In the present study, strains with spoligotype patterns characterized by deletion of spacers 1–34 or with additional deletion of one or more of the last nine spacers were classified as Beijing genotype, while others were defined as non-Beijing genotype.

Whole-Genome Sequencing

The gene mutation information in the present study was acquired from WGS data. Sequencing libraries were prepared with genomic DNA using kits as instructed by the manufacturer. DNA libraries were then selected to perform cluster growth and 150 bp paired-end sequencing on DNB SEQ-2000 instrument (Beijing Genomics Institute, Beijing, China). The raw FASTQ sequence reads were filtered by removing the adapter sequences, duplicate reads, and low-quality reads that had a quality score below 20 in more than 30% of the bases. The clean reads were mapped to the genome of H37Rv (accession number, NC_000962.2) using in-house softwares Bowtie2 (Version 2.3.4.1) and samtools (Version 1.7). VarScan (Version 2.4.4) was used for single-nucleotide polymorphism (SNP) finding. All genome-wide SNPs were identified by the VarScan software by parsing the mapping genome sequence data, and then the SNPs related to phylogeny or located in PE/PPE gene family regions were filtered out. An average of 15.4 million sequence reads was acquired per genome at a depth of 500× and with coverage of 98.0%.

According to several published literatures,[11,20,27,32,33] eight candidate genes and regions, including *katG, ahpC* and its upstream (INH), *ethA* and *ethR* (ETH), *inhA* and its promoter region, *ndh* and *mshA* (INH and ETH), were selected to

compare with the pan-susceptible reference genome (H37Rv, accession number: NC_000962.2) at the level of SNPs in promoter regions or intergenic regions, amino acids in genes, or insertions and deletions. The phenotypic and genotypic results were compared to determine the specificity and sensitivity for each gene with WGS to predict resistance.

Statistical Analysis

SPSS 16.0 (SPSS Inc., Chicago, IL, United States) was used to perform statistical analysis. Pearson chi-square test or Fisher's exact probability test and logistic regression analysis were used to determine the independent covariable associated with cross-resistance between INH and ETH (INH^{R}ETH^R), INH resistance, ETH resistance, and MDR group, respectively. A P value less than 0.05 was defined as significant. Sensitivity, specificity, odds ratio (OR) and confidence interval (CI) were calculated using $M_{ed}C_{alc}$ statistical software (MedCalc Software, Ostend, Belgium).

Result

Demographic Characteristics

Of the 312 TB patients with *M. tuberculosis* isolates, 159 (50.96%) were male and 153 (49.04%) were female. Age-wise analysis displayed that the ages of the participants ranged from 16 to 94, with a mean age of 54.47 years. Additionally, 164 (52.56%) were retreated cases (defined as new cases of tuberculosis treated with previous anti-tuberculosis drugs for more than one month, cases of relapse or cases of failure of initial treatment), 148 (47.44%) were new cases (defined as new cases not previously treated with anti-tuberculosis drugs or on drugs for less than one month).

We analyzed the distribution differences of treatment history between gender, age, occupation and education level and found treatment history among the age groups showed statistically significant difference ($P < 0.05$). We then performed a two-by-two comparison and found that the proportion of new cases was higher than the proportion of retreated cases in the <25 and 25–44 age groups, while the proportion of retreated cases was higher than the proportion of new cases in the 45–65 and >65 age groups. The results are shown in Table 1.

Drug Resistance Profiles in INH and/or ETH-Resistant Isolates

DST on the 312 *M. tuberculosis* isolates showed that there were 25 (8.0%) INH^{R}ETH^R, 65 (20.8%) INH^{R}ETH^S and 9 (2.9%) INH^{S}ETH^R isolates, and 213 (68.3%) isolates which were susceptible to both drugs. In total, 90 and 34 isolates

Table 1 Demographic and Genotyping Results of Patients with New Cases and Retreated Cases

		New Cases (N=164) (%)	Retreated Cases (N=148) (%)	Total (N=312) (%)	χ^2	P
Gender					1.234	0.267
	Male	89 (54.2)	71 (48.0)	160 (51.3)		
	Female	75 (45.7)	77 (52.0)	152 (48.7)		
Age (year)					22.455	<0.001
	<25	28 (17.1)	3 (2.0)	31 (9.9)		
	25–44	37 (22.6)	28 (18.9)	65 (20.8)		
	45–65	52 (31.7)	57 (38.5)	109 (34.9)		
	>65	47 (28.7)	60 (40.5)	107 (34.3)		
Occupation					1.041	0.308
	Farmer	111 (67.7)	108 (73.0)	219 (70.2)		
	Others[b]	53 (32.3)	40 (27.0)	93 (29.8)		
Education level					–	0.811[a]
	College/Bachelor	5 (3.1)	3 (2.0)	8 (2.6)		
	High school	9 (5.5)	6 (4.1)	15 (4.8)		
	Elementary/Junior High School	131 (79.9)	118 (79.7)	249 (79.8)		
	Illiterate	19 (11.6)	21 (14.2)	40 (12.8)		

Notes: [a]Acquired by Fisher exact test, while others acquired by chi-square test. [b]Occupations other than farmers.
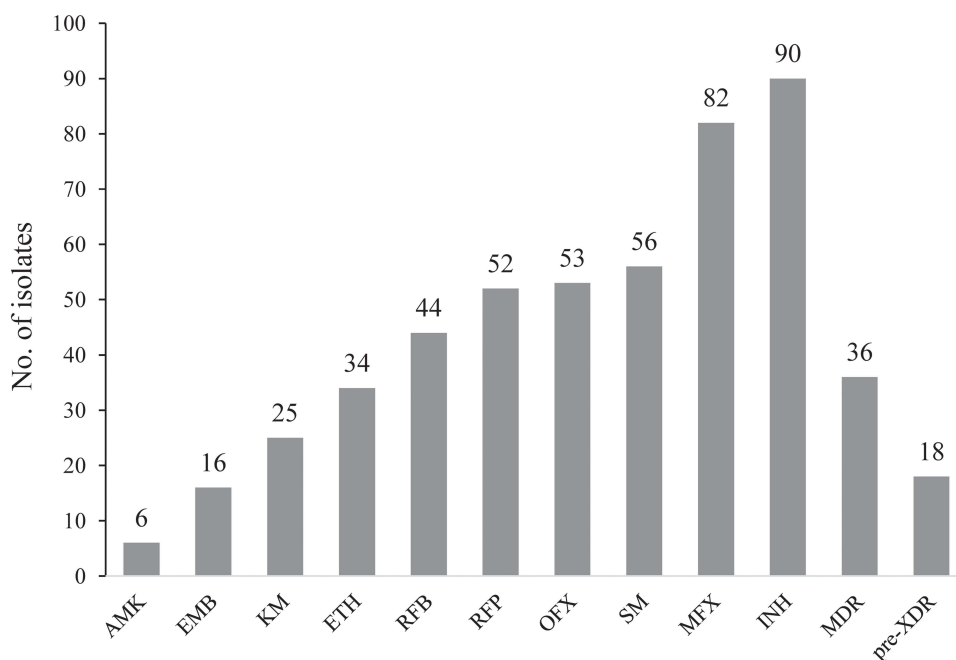
Figure 2 Drug resistance profiles of 312 *Mycobacterium tuberculosis* clinical isolates against 12 anti-tuberculosis drugs.
Abbreviations: INH, isoniazid; RIF, rifampicin; EMB, ethambutol; SM, streptomycin; AMK, amikacin; OFX, ofloxacin; MFX, moxifloxacin; ETH, ethionamide; RFB, rifabutin; KM, kanamycin; MDR, resistant to at least isoniazid and rifampicin; pre-XDR, resistant to rifampicin and any fluoroquinolone.

were resistant to INH and ETH, respectively; 18 (5.8%) pre-extensively drug-resistant TB (Pre-XDR-TB, defined as TB that is resistant to rifampicin and any fluoroquinolone) were found, of which 36 (11.5%) were MDR. Figure 2 shows the numbers of isolates resistant to each drug or special drug combinations.

Genotyping Results

Among the 312 isolates, 185 (58.3%) belonged to the Beijing family, while 127 (40.7%) belonged to the non-Beijing family. Of the Beijing genotypes, 55 (17.6%) were resistant to INH, 18 (5.8%) strains were resistant to ETH, and 14 (4.5%) strains were co-resistant. Meanwhile, 35 (11.2%) and 16 (5.1%) strains of the non-Beijing genotype were resistant to INH and ETH, respectively, and 11 (3.5%) strains were co-resistant.

Factors Associated with INH- and ETH-Resistance, and INH-ETH Co-Resistance

We tried to assess the association between INH, ETH, INH-ETH co-resistance or MDR and demographic characteristics and isolate genotype and only found that INH resistance and MDR were more likely to be associated with retreated cases with OR of 2.00 (95% CI: 1.18–3.40) and 2.53 (95% CI: 1.17–5.47), respectively. The results are shown in Figure 3.

Mutations in katG, inhA and Its Promoter Region, ahpC and Its Upstream Region, mshA and ndh in Isoniazid Resistant Isolates

We found that the mutation located in codon 463 (R463L) in *katG* was identified as the most prevalent with a frequency of 84.0% (262/312), followed by the A187V mutation in *mshA* (118/312). These two mutations were lineage markers so were excluded in the present study. In addition, 69 isolates (including 3 INH[R]ETH[R], 15 INH[R]ETH[S], 3 INH[S]ETH[R] and 48 INH[S]ETH[S] isolates) were with a nucleotide substitution of *ahpC*-88C>A, which was defined as a mutation unrelated to the resistance to INH and ETH according to the WHO document.[34] The mutation of *ahpC*-88C>A and other synonymous mutations except that i*nhA* G-154A (fabG1 L203L) that recorded as INH resistance associated[34] were all excluded during the analysis of mutations associated with drug resistance.
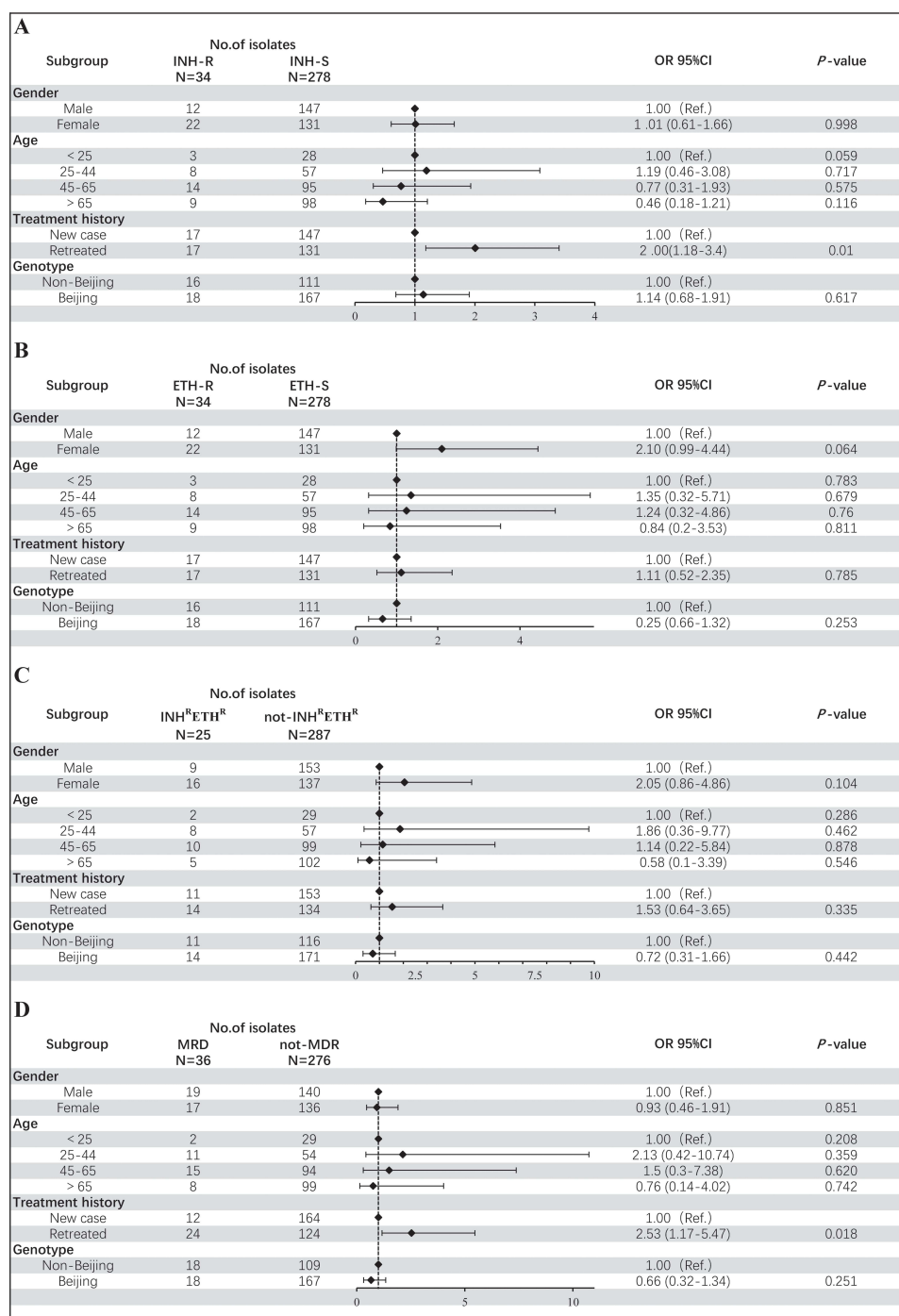
A

Subgroup	INH-R N=34	INH-S N=278	OR 95%CI	P-value
Gender				
Male	12	147	1.00 (Ref.)	
Female	22	131	1.01 (0.61-1.66)	0.998
Age				
< 25	3	28	1.00 (Ref.)	0.059
25-44	8	57	1.19 (0.46-3.08)	0.717
45-65	14	95	0.77 (0.31-1.93)	0.575
> 65	9	98	0.46 (0.18-1.21)	0.116
Treatment history				
New case	17	147	1.00 (Ref.)	
Retreated	17	131	2.00 (1.18-3.4)	0.01
Genotype				
Non-Beijing	16	111	1.00 (Ref.)	
Beijing	18	167	1.14 (0.68-1.91)	0.617

B

Subgroup	ETH-R N=34	ETH-S N=278	OR 95%CI	P-value
Gender				
Male	12	147	1.00 (Ref.)	
Female	22	131	2.10 (0.99-4.44)	0.064
Age				
< 25	3	28	1.00 (Ref.)	0.783
25-44	8	57	1.35 (0.32-5.71)	0.679
45-65	14	95	1.24 (0.32-4.86)	0.76
> 65	9	98	0.84 (0.2-3.53)	0.811
Treatment history				
New case	17	147	1.00 (Ref.)	
Retreated	17	131	1.11 (0.52-2.35)	0.785
Genotype				
Non-Beijing	16	111	1.00 (Ref.)	
Beijing	18	167	0.25 (0.66-1.32)	0.253

C

Subgroup	$INH^R ETH^R$ N=25	not-$INH^R ETH^R$ N=287	OR 95%CI	P-value
Gender				
Male	9	153	1.00 (Ref.)	
Female	16	137	2.05 (0.86-4.86)	0.104
Age				
< 25	2	29	1.00 (Ref.)	0.286
25-44	8	57	1.86 (0.36-9.77)	0.462
45-65	10	99	1.14 (0.22-5.84)	0.878
> 65	5	102	0.58 (0.1-3.39)	0.546
Treatment history				
New case	11	153	1.00 (Ref.)	
Retreated	14	134	1.53 (0.64-3.65)	0.335
Genotype				
Non-Beijing	11	116	1.00 (Ref.)	
Beijing	14	171	0.72 (0.31-1.66)	0.442

D

Subgroup	MRD N=36	not-MDR N=276	OR 95%CI	P-value
Gender				
Male	19	140	1.00 (Ref.)	
Female	17	136	0.93 (0.46-1.91)	0.851
Age				
< 25	2	29	1.00 (Ref.)	0.208
25-44	11	54	2.13 (0.42-10.74)	0.359
45-65	15	94	1.5 (0.3-7.38)	0.620
> 65	8	99	0.76 (0.14-4.02)	0.742
Treatment history				
New case	12	164	1.00 (Ref.)	
Retreated	24	124	2.53 (1.17-5.47)	0.018
Genotype				
Non-Beijing	18	109	1.00 (Ref.)	
Beijing	18	167	0.66 (0.32-1.34)	0.251

Figure 3 Univariate analysis of factors associated with INH resistance, ETH resistance, INH and ETH co-resistance and multidrug resistance. (**A**) Factors associated with resistant to INH. (**B**) Factors associated with resistant to ETH. (**C**) Factors associated with cross-resistant between INH and ETH. (**D**) Factors associated with multidrug resistance.
Abbreviations: OR, odds Radio; 95% CI, 95% confidence interval.

Of the 90 INH resistant isolates, 67, 11, 10 and two carried mutations in *katG, inhA* and its promoter, *ahpC* and its upstream region, and *ndh*, respectively. No mutation was detected in *mshA* (Figure 4A). The most prevalent mutation was observed at *katG* S315T, *inhA* −15C>T, *ahpC* −54C>T, *ndh* M370I, which were detected in 36, eight, three and two isolates, respectively. Of the 67 *katG* mutated isolates, 10 combined mutations in the *ahpC* and its upstream region; five
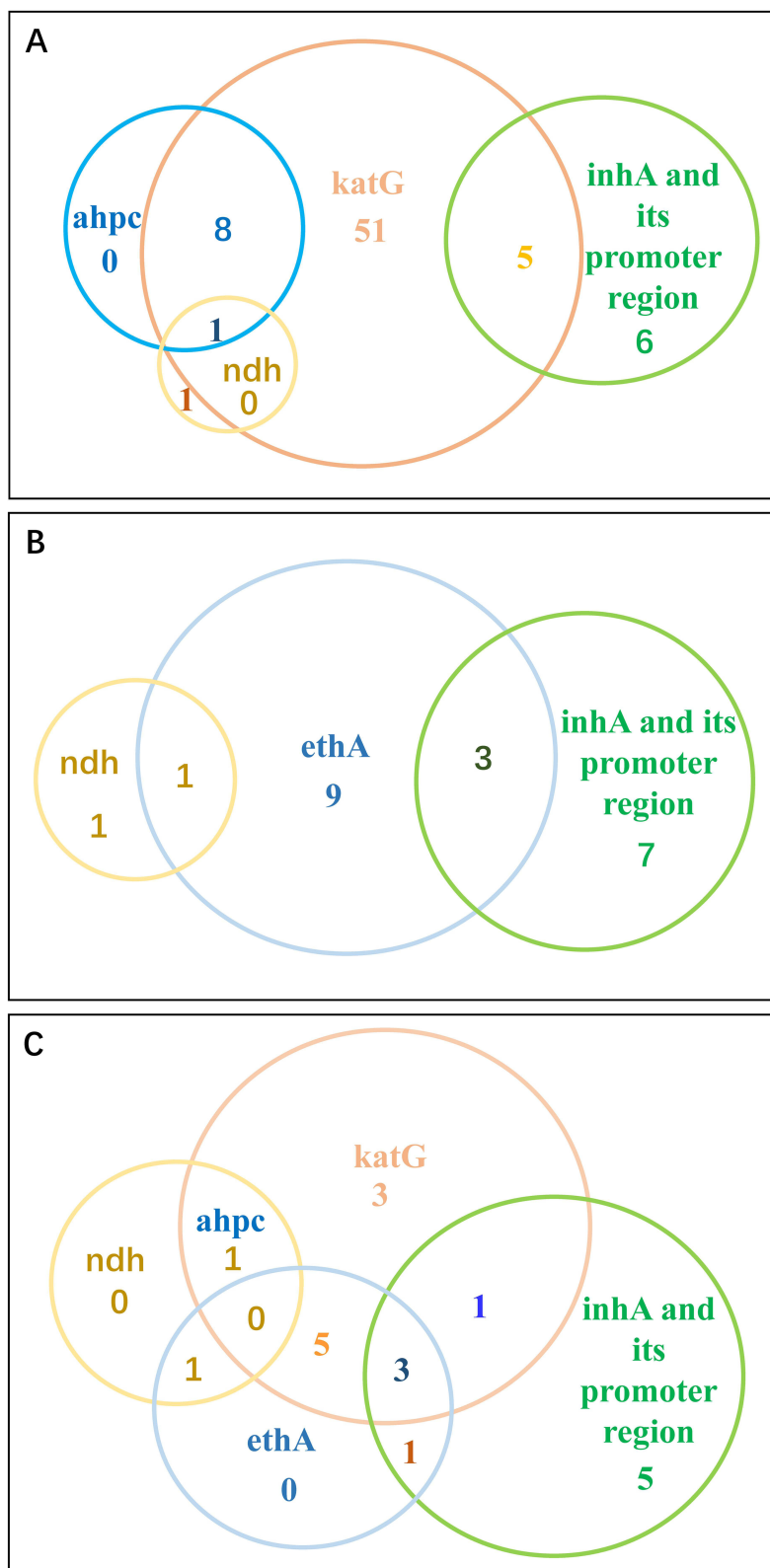
Figure 4 Distribution of INH and ETH resistant, and INH-ETH co-resistant isolates with different gene mutations. (**A**) Distribution of INH-resistant isolates with different gene mutations. (**B**) Distribution of ETH- resistant isolates with different gene mutations. (**C**) Distribution of cross-resistant to INH and ETH isolates with different gene mutations. The numbers within the circles represent the number of strains that have corresponding gene mutations.

combined mutations in the *inhA* promoter region (two with −8T>A, three with −15C>T); one carried M370I in *ndh* (Table 2).

Of the 222 INH susceptible isolates, the most frequent mutated gene was *katG* (17 isolates, 7.7%), followed by *inhA* and its promoter region (5 isolates, 2.3%), *ahpC* and its promoter region (10 isolates, 4.5%), and *mshA* (6 isolates, 2.7%) (Tables 2 and S1).

There were 11 novel mutations of *katG* R78P, W668C, L707P, 2186_2191delACAAGG, W728stop, 252dupG, −6575_376del and 553_558delTTCGGC, *ahpC* −77delT and D22H, and *ndh* M370I found only in INH resistant isolates, whilst 10 novel mutations found only in INH susceptible isolates: *katG* K143T and *katG* D513E, *inhA* A211T, −124G>A and −301A>G, *ahpC* V26A, F94C and −75T>G, *mshA* A187T and P368L (Table S1).

MIC Distributions of Isoniazid in *Mycobacterium tuberculosis*

MIC values showed that 67 INH resistant isolates were high-level resistant to INH, among which, 59 isolates carried mutations in *katG*, and eight isolates showed mutations in ahpc and its upstream region (Table 3). The prevalence of *katG* mutants in high-level INH resistant isolates was significantly higher than that in INH low-level resistant isolates (25.546, P < 0.001). In contrast, mutations in the *inhA* and its promoter region were statistically higher in low-level resistant isolates than in the high-level ones (8 vs 3 isolates, Fisher's exact test, P = 0.001). In addition, the most frequent mutation of *katG* S315T was found in 29 isolates, which all showed high-level resistant to INH (Table 2).

Mutations in ethA, ethR, inhA and Its Promoter Region, mshA and ndh in Ethionamide Resistant Isolates

Among 34 ETH resistant isolates, 13 carried mutations in *ethA*, 10 in *inhA* and its promoter, two in *ndh* (Figure 4B). Ten mutation types were found in the *ethA* (I34T, D56E, M59I, S266R, P334A, 245_902del, 740delC, 815delT, 1323_1329delCTCGCTG, 1405dupC), three types in the *inhA* promoter region (−8T>A, −15C>T, −154 G>A), and one in *ndh* (M37I). No mutation was detected in *ethR* and *mshA* in ETH resistant isolates. The most common mutation was *inhA* −15C>T detected in eight ETH resistant isolates (Table 2).

Of the 278 ETH susceptible isolates, the most frequent mutated gene was *ethA* (56 isolates, 20.1%), followed by *ethR* (seven isolates, 2.5%), *inhA* and its promoter region (six isolates, 2.2%), *mshA* (six isolates, 2.2%; three with R413N). Mutation of *ethA* S266R was the most frequent and found in 38 isolates.

Five novel mutations of *ethA* 815delT, 1405dupC, V439L, M59I and 245_902del were found only in ETH resistant isolates, while five novel mutations E36K, L268T, R306G, E400K, 734_735dupGC in *ethA* were found only in ETH susceptible isolates; the novel mutations *ethA* 740delC and P164L were found in both ETH resistant and susceptible isolates (Tables 2 and S1).

MIC Distributions of Ethionamide in *Mycobacterium tuberculosis*

The combination of mutation information and MIC values showed that among the 34 ETH resistant isolates, 25 were low-level resistant and nine were high-level resistant, and there was no statistical difference in the mutation rates of *ethA, ndh* or *inhA* and its promoter between the two resistant levels (Table 4).

Cross-Resistance Profiles Between Isoniazid and Ethionamide in *Mycobacterium tuberculosis*

There were 25 INH^R^ETH^R^ *M. tuberculosis* isolates in the present study, 13 (52.0%), nine (36.0%), 10 (40.0%), two (8.0%) and one (4.0%) had mutations in *katG, ethA, inhA* and its promoter mutations, *ndh* and *ahpC* upstream region, respectively (Table 2 and Figure 4C). In detail, of the 25 INH^R^ETH^R^ isolates, the most prevalent mutation was *inhA* promoter region −15C>T, occurring in 8 isolates. In addition, five out of 25 possessed both mutations in *katG* and *ethA*; two had mutation in *ndh* with a non-synonymous mutation (M370I), one of these two combined with other mutations of *katG* (R78P) and *ahpC* (−77delT), another one combined with mutation in *ethA* (I34T); three isolates only carried mutations in the *katG* (two with S315T, one with V151F).

Table 2 Mutations in *katG, ethA, inhA* Promoter Region, *inhA, ethR, ahpC* and Its Upstream Region, *mshA* and *ndh* Combined with MIC Values in Isoniazid or Ethionamide Resistant *Mycobacterium tuberculosis* Clinical Isolates

Drug Resistance		No. of Isolates	Resistance Genotype								MIC (µg/mL)	
			katG	ethA	inhA Promoter	inhA	ethR	ahpC and its Upstream	mshA	ndh	INH	ETH
INH^R	ETH^R	1	M105K, S315T	S266R	−8T>A	WT	WT	WT	WT	WT	1	5
		1	A139P, S140N	S266R	−15C>T	WT	WT	WT	WT	WT	0.25	5
		1	W191G	740delC*	−15C>T	WT	WT	WT	WT	WT	1	20
		1	S315T, K590E	815delT*	WT	WT	WT	WT	WT	WT	4	40
		1	S315T, K590E	1405dupC*	WT	WT	WT	WT	WT	WT	4	≥40
		1	S315T	D56E	WT	WT	WT	WT	WT	WT	4	≥40
		1	S315T	I305T	WT	WT	WT	WT	WT	WT	≥4	20
		1	S315G	V439L*	WT	WT	WT	WT	WT	WT	0.25	5
		1	R78P*	WT	WT	WT	WT	−77delT*	WT	M370I*	2	5
		1	V151F	WT	WT	WT	WT	WT	WT	WT	≥4	5
		2	S315T	WT	WT	WT	WT	WT	WT	WT	4	≥40
		1	S481L	WT	−15C>T	WT	WT	WT	WT	WT	0.25	5
		1	WT	I34T	WT	WT	WT	WT	WT	M370I*	0.5	5
		1	WT	WT	−15C>T	G3G^a	WT	WT	WT	WT	0.5	5
		2	WT	WT	−15C>T	WT	WT	WT	WT	WT	0.25	10
		1	WT	WT	−15C>T	WT	WT	WT	WT	WT	0.25	5
		1	WT	WT	−15C>T	WT	WT	WT	WT	WT	0.5	10
		1	WT	WT	−154G>A	WT	WT	WT	WT	WT	0.5	≥40
		2	WT	WT	WT	WT	WT	WT	WT	WT	0.25	5
		2	WT	WT	WT	WT	WT	WT	WT	WT	0.25	10
		1	WT	WT	WT	WT	WT	WT	WT	WT	4	10
INH^R	ETH^S	1	S315T	E400K*	−8T>A	WT	WT	WT	WT	WT	≥4	<5
		1	N138S	R306G*	WT	WT	WT	WT	WT	WT	4	<5
		1	R145P	S266R	WT	WT	WT	WT	WT	WT	≥4	<5
		1	F183L	S266R	WT	WT	WT	WT	WT	WT	1	<5
		1	W191G	740delC*	WT	WT	WT	WT	WT	WT	0.25	<5
		1	R249L	E36K*	WT	WT	WT	WT	WT	WT	≥4	<5
		1	S315T	734_735dupGC*	WT	WT	WT	WT	WT	WT	1	<5
		1	S315T	P164L*, S266R	WT	WT	WT	WT	WT	WT	4	<5
		1	S315T	S266R	WT	WT	WT	WT	WT	WT	4	<5
		1	S315N	S266R	WT	WT	WT	WT	WT	WT	4	<5
		1	W668C*	P334A	WT	WT	WT	−51G>A	WT	WT	≥4	<5

Cao et al

INH	ETH	No.	katG	ethA							INH MIC	ETH MIC
		1	W728R	S266R	WT	WT	WT	−72C>T	WT	WT	≥4	<5
		1	V1A	WT	WT	WT	WT	WT	WT	WT	≥4	<5
		1	N138S	WT	WT	WT	WT	WT	WT	WT	4	<5
		1	G234E	WT	WT	WT	WT	WT	WT	WT	2	<5
		1	G285V	WT	WT	WT	WT	WT	WT	WT	1	<5
		1	L298S	WT	WT	WT	WT	WT	WT	WT	0.5	<5
		1	D311G	WT	WT	WT	WT	−48G>A	WT	WT	1	<5
		1	S315N	WT	WT	WT	WT	WT	WT	WT	4	<5
		1	S315N	WT	WT	WT	WT	WT	A92A[a]	WT	4	<5
		1	S315R	WT	WT	WT	WT	D22H*	WT	K208K[a]	≥4	<5
		1	S315T	WT	WT	WT	N35S*	WT	WT	WT	4	<5
		3	S315T	WT	WT	WT	WT	WT	WT	WT	1	<5
		9	S315T	WT	WT	WT	WT	WT	WT	WT	2	<5
		10	S315T	WT	WT	WT	WT	WT	WT	WT	4	<5
		2	S315T	WT	WT	WT	WT	WT	WT	WT	≥4	<5
		1	L382R	WT	WT	WT	WT	WT	WT	WT	0.25	<5
		1	T394A	WT	WT	WT	WT	−77T>G	WT	WT	0.25	<5
		1	R632H	WT	WT	WT	WT	−57C>T	WT	WT	0.25	<5
		1	L707P*	WT	WT	WT	WT	WT	WT	WT	≥4	<5
		1	2186_2191delACAAGG* W728stop*	WT	WT	WT	WT	−54C>T	WT	WT	≥4	<5
		1	252dupG*	WT	WT	WT	WT	WT	WT	WT	≥4	<5
		1	−6575_376del*	WT	WT	WT	WT	−54C>T	WT	WT	≥4	<5
		1	553_558delTTCGGC*	WT	WT	WT	WT	−54C>T	WT	WT	≥4	<5
		1	WT	P334A	WT	WT	WT	WT	WT	WT	≥4	<5
		1	WT	P436S*	WT	WT	WT	WT	WT	WT	≥4	<5
		3	WT	WT	WT	WT	WT	WT	WT	WT	1	<5
		3	WT	WT	WT	WT	WT	WT	WT	WT	0.25	<5
		1	WT	WT	WT	WT	WT	WT	WT	WT	0.5	<5
		2	WT	WT	WT	WT	WT	WT	WT	WT	≥4	<5
INH^S	ETH^R	1	WT	M59I*	WT	WT	WT	WT	WT	WT	<0.25	5
		1	WT	P334A	WT	WT	WT	WT	WT	WT	<0.25	10
		1	WT	245_902del*	WT	WT	WT	WT	WT	WT	<0.25	10
		1	WT	1323_1329delCTCGCTG	WT	WT	WT	WT	WT	WT	<0.25	5
		1	WT	WT	WT	WT	WT	WT	WT	WT	<0.25	20
		4	WT	WT	WT	WT	WT	WT	WT	WT	<0.25	10

Notes: *Novel mutations in *katG, ethA, inhA* promoter region, *inhA, ethR, ahpC* and its upstream region, *mshA* and *ndh*. [a]These synonymous mutations were not analyzed as isoniazid or ethionamide resistance associated mutations.
Abbreviations: Ethionamide, ETH, Ethionamide; ETH^S, Ethionamide susceptible; ETH^R, Ethionamide resistant; INH, isoniazid; INH^S, isoniazid susceptible; INH^R, isoniazid resistant; R, resistant; S, susceptible; WT, wild-type.

Table 3 The Associations Between Isoniazid Resistance Levels and Four Gene Mutations in *Mycobacterium tuberculosis* Isolates

Genes	High Level of Resistance to INH, ≥1 µg/mL		Low Level of Resistance to INH, 0.1–0.5 µg/mL		χ^2	P-value
	With Mutation	Without Mutation	With Mutation	Without Mutation		
katG	59 (88.1%)	8 (11.9%)	8 (34.8%)	15 (65.2%)	25.546	<0.001
inhA and its promoter	3 (4.8%)	64 (95.5%)	8 (34.8%)	15 (65.2%)	-	0.001[a]
ahpc	8 (11.9%)	59 (88.1%)	2 (8.7%)	21 (91.3%)	-	1.000[a]
ndh	1 (1.5%)	66 (98.5%)	1 (4.3%)	22 (95.7%)	-	0.448[a]

Note: [a]Acquired by Fisher exact test, while others acquired by chi-square test.
Abbreviation: INH, isoniazid.

Table 4 The Associations Between Ethionamide Resistance Levels and Three Gene Mutations in *Mycobacterium tuberculosis* Isolates

Genes	High Level of Resistance to ETH ≥20 µg/mL		Low Level of Resistance to ETH, 5–10 µg/mL		χ^2	P-value
	With Mutation	Without Mutation	With Mutation	Without Mutation		
ethA	5 (55.5%)	4 (45.5%)	8 (32.0%)	17 (68.0%)	-	0.254[a]
inhA and its promoter	2 (22.2%)	7 (77.8%)	8 (32.0%)	17 (68.0%)	-	0.692[a]
ndh	0 (0.0%)	9 (100%)	2 (8.0%)	23 (92.0%)	-	1.000[a]

Note: [a]Acquired by Fisher exact test.
Abbreviation: ETH, ethionamide.

Table 5 The Differences in Mutation Rates of Genes Associated with Isoniazid or Ethionamide Resistance

Locus		INH-R	INH-S	χ^2	P-value	ETH-R	ETH-S	χ^2	P-value
katG	With mutation	67	17	145.183	<0.001	13	71	2.482	0.115
	WT	23	205			21	207		
ethA	With mutation	23	46	0.869	0.351	13	56	5.757	0.016
	WT	67	176			21	222		
inhA and its promoter	With mutation	11	5	-	0.001[a]	10	6	-	<0.001[a]
	WT	79	217			24	272		
ethR	With mutation	1	6	-	0.678[a]	0	7	-	1.000
	WT	89	216			34	271		
ahpC and its upstream	With mutation	10	10	4.659	0.031	1	19	-	0.709[a]
	WT	80	212			33	259		
mshA	With mutation	0	6	-	0.187[a]	0	6	-	1.000
	WT	90	216			34	272		
ndh	With mutation	2	0	-	0.026[a]	2	0	-	0.012[a]
	WT	88	222			32	278		

Note: [a]Acquired by Fisher exact test, while others acquired by chi-square test.
Abbreviations: Ethionamide, ETH, Ethionamide; ETH-S, Ethionamide susceptible; ETH-R, Ethionamide resistant; INH, isoniazid; INH-S, isoniazid susceptible; INH-R, isoniazid resistant; R, resistant; S, susceptible; WT, wild-type.

To elucidate which genes were associated with both INH and ETH resistance in this study, the differences in mutation rates of each gene between INH or ETH susceptible and resistant isolates were first analyzed by Pearson chi-square test or Fisher's exact test according to the sample number of isolates. Statistical analysis revealed that *inhA* and its promoter region and *ndh* mutations were associated with both isoniazid and ethionamide resistance (Table 5).

Table 6 The MICs Distribution of Isolates with *inhA* and Its Promoter Region in Cross-Resistant to INH and ETH

Resistance Group	Code	*inhA* and Its Promoter	INH		ETH	
			MIC (µg/mL)	Resistant Level	MIC (µg/mL)	Resistant Level
INH^R ETH^R	ICDCXJ19004	−8T>A	I	H	5	L
	ICDCXJ19008	−15C>T	0.25	L	5	L
	ICDCXJ19072	−15C>T	I	H	20	H
	ICDCXJ19112	−15C>T	0.25	L	5	L
	ICDCXJ19975	−15C>T	0.5	L	5	L
	ICDCXJ19797	−15C>T	0.25	L	10	L
	ICDCXJ191048	−15C>T	0.25	L	10	L
	ICDCXJ19664	−15C>T	0.25	L	5	L
	ICDCXJ19987	−15C>T	0.5	L	10	L
	ICDCXJ19805	−154G>A	0.5	L	≥40	H

Abbreviations: Ethionamide, ETH, Ethionamide; ETH^S, Ethionamide susceptible; ETH^R, Ethionamide resistant; INH, isoniazid; INH^S, isoniazid susceptible; INH^R, isoniazid resistant; R, resistant; S, susceptible; MIC, minimum inhibitory concentration; H, high-level resistant; L, low-resistant.

We further found that mutations in inhA and its promoter region were more frequent in low-level INH or ETH resistant isolates (both found with a ratio of 8/10) (Table 6).

In the present study, we only found that two isolates carried mutations in *ndh* M370I, one was high-level resistant to INH and low-level resistant to ETH, the other one was both low-level resistant to INH and ETH.

Prediction of Isoniazid and/or Ethionamide Resistance in *Mycobacterium tuberculosis* Based on Whole Genome Sequencing

As shown in Table 7, using the phenotypic data as reference, detection of mutation in *katG* for INH-resistance prediction exhibited a sensitivity of 74.44% and a specificity of 92.34%. When the combination of *katG, inhA* and its promoter region was evaluated, the sensitivity increased to 81.11%, and the specificity decreased to 90.54%; added with mutations in the *ahpC* upstream, the sensitivity was not improved, but the specificity was reduced to 88.74%; together with the *ndh* and *mshA* mutations, the sensitivity increased to 82.22%, and the specificity decreased to 86.49%.

Identification of *ethA* mutations for ETH-resistance prediction revealed a sensitivity of 38.24% and a specificity of 79.86%. While the combination of *ethA, ethR, mshA, ndh, inhA* and its promoter region mutations was assessed, the sensitivity was enhanced to 61.76% and the specificity was reduced to 75.18%, as shown in Table 8.

Table 7 The Prediction Values of Whole Genome Sequencing for Isoniazid Resistance in *Mycobacterium tuberculosis* Isolates

Locus	Isoniazid-Resistant		Isoniazid-Susceptible		Sensitivity (%; OR 95% CI)	Specificity (%; OR 95% CI)
	With Mutation	Without Mutation	With Mutation	Without Mutation		
katG	67	23	17	205	74.44 (64.16–83.06)	92.34 (88.02–95.48)
inhA/promoter	11	79	12	210	12.22 (6.26–20.82)	94.59 (90.75–97.18)
ahpc/ahpc upstream	10	80	5	217	11.11 (5.46–19.49)	97.75 (94.82–99.26)
mshA	0	90	6	216	0.00 (0.00–4.02)	97.30 (94.21–99.00)
ndh	2	88	0	222	2.22 (0.27–7.80)	100.00 (98.35–100.00)
katG+*inhA*/promoter	73	17	21	201	81.11 (71.49–88.59)	90.54 (85.90–94.05)
katG+*inhA*/promoter +*ahpc/ahpc* upstream	73	17	25	197	81.11 (71.49–88.59)	88.74 (83.83–92.58)
katG+*inhA*/promoter +*ahpc/ahpc* upstream+*mshA*+*ndh*	74	16	44	178	82.22 (72.74–89.48)	86.49 (81.27–90.69)

Abbreviations: OR, odds Radio; 95% CI, 95% confidence interval.

Table 8 The Prediction Values of Whole Genome Sequencing for Ethionamide Resistance in *Mycobacterium tuberculosis* Isolates

Locus	No. of Isolates				Sensitivity (%; OR 95% CI)	Specificity (%; OR 95% CI)
	Ethionamide-Resistant		Ethionamide-Susceptible			
	With Mutation	Without Mutation	With Mutation	Without Mutation		
ethA	13	21	56	222	38.24 (22.17–56.44)	79.86 (74.65–84.41)
inhA/promoter	10	24	6	272	29.41 (15.10–47.48)	97.84 (95.36–99.20)
ethR	0	34	7	271	0.00 (0.00–10.28)	97.48 (94.88–98.98)
mshA	0	34	6	272	0.00 (0.00–10.28)	97.84 (95.36–99.20)
ndh	2	32	0	278	5.88 (0.72–19.68)	100.00 (98.68–100.00)
ethA+*inhA*/promoter+*ndh*	21	13	65	213	61.76 (43.56–77.83)	76.62 (71.19–81.47)
ethA+*inhA*/promoter+*ethR* +*mshA*+*ndh*	21	13	69	209	61.76 (43.56–77.83)	75.18 (69.67–80.14)

Abbreviations: OR, odds Radio; 95% CI, 95% confidence interval.

Table 9 The Prediction Values of Whole Genome Sequencing for Isoniazid-Ethionamide Co-Resistance in *Mycobacterium tuberculosis* Isolates

Locus	No. of Isolates				Sensitivity (%; OR 95% CI)	Specificity (%; OR 95% CI)
	INH^R^ETH^R		INH^S^ETH^S			
	With Mutation	Without Mutation	With Mutation	Without Mutation		
inhA/promoter	10	15	5	208	40.00 (21.13–61.33)	97.65 (94.61–99.23)
mshA	0	25	8	205	0.00 (0.00–13.72)	97.65 (94.61–99.23)
ndh	2	23	0	213	8.00 (0.98–26.03)	100.00 (98.28–100.00)
inhA/promoter+*ndh*	12	13	5	208	48.00 (27.80–68.69)	97.65 (94.61–99.23)
inhA/promoter +*mshA*+*ndh*	12	13	10	203	48.00 (27.80–68.69)	95.31 (91.54–97.73)

Abbreviations: Ethionamide, ETH, Ethionamide; ETH^S, Ethionamide susceptible; ETH^R, Ethionamide resistant; INH, isoniazid; INH^S, isoniazid susceptible; INH^R, isoniazid resistant; R, resistant; S, susceptible.

For the prediction of the co-resistance between INH and ETH Detection, mutations in *inhA* and its promoter region and *ndh* showed a sensitivity of 48.00% and a specificity of 97.65%, plus that in the *mshA* showed a sensitivity of 48.00% and a specificity of 95.31%, as shown in Table 9.

Discussion

The present study gained a deep insight into the cross-resistance mechanism of INH and ETH, as well as the gene mutation characteristics associated with INH and ETH resistance in *M. tuberculosis* isolated from the South of Xinjiang, China, by integrating DST and WGS. As expected, high diversity of genetic mutation conferring INH and ETH resistance in *M. tuberculosis* was identified in our study.

In concordance with previous studies, analysis of INH resistant mutations showed a clear predominance of well-established mutations in *katG* genes accounting for 74.4% (67/90) in INH-resistant isolates in the present study (Figure 1). In addition, the most common mutation at codon 315 in *katG* was found in 44.4% (40/90) INH-resistant strains, which varied considerably across the geographic regions, ranging from 97% in South Africa,[35] 94% in north-western Russia,[36] 88% in Colombia,[37] 46% in Spain,[38] 28% in Japan.[39] The dominance of mutations at codon 315 in *katG* may be explained by the fact that *katG*315 mutants retained their catalase-peroxidase activity while showing a lowered ability to activate INH, which probably ensure a sufficient level of oxidative protection to have no bacterial

fitness cost and to maintain their virulence and transmissibility.[40,41] Notably, there were 29 mono-resistant INH isolates carrying the mutation of *katG* S315T, and all of these 29 INH-resistant isolates were high-level resistant to INH, which confirmed that mutations at amino acid position 315 of the *katG* gene were associated with high-level resistance to INH.[42,43] It is interesting to note that there were two isolates with mutations of *inhA* −8T>A combined with *katG* S315T, which was in line with Jessica's study conducted in clinical INH resistant isolates,[44] suggesting that the diagnostic significance of *inhA*-8T>A mutation may be compromised as it always co-occurred with *katG*315. Besides the canonical mutation in *katG*315, several mutations unreported previously were observed in our study, including three nonsynonymous mutations R78P, W668C, L707P, four frameshift mutations including 6575_376del, 553_558delTTCGGC, 252dupG, 2186_2191delACAAGG, and a silent mutation W728. The fact highlights the concern that a more widespread of use of existing molecular diagnostics that miss these mutations might impose an artificial selection process where the mutants with canonical mutations were detected and eradicated through proper chemotherapy, but those novel mutants could not be captured and continue to spread, probably resulting in the INH-resistant population evolution from a dominant single amino acid site mutation in *katG* to the whole-gene.

Nearly 42% of ETH-resistant strains harbored mutations in the *ethA*, in line with many other studies.[33,45,46] Five out of 15 *ethA* mutation types in ETH resistant isolates have not been previously published, this high diversity of *ethA* mutations associated with ETH resistance supported the idea that genetic mutations in *ethA* were distributed across the structural gene,[11,27] and unlike the dominant mutation *katG* S315T in INH-resistant variant, also differed from mutations located at a limited area named RR-determining region (RRDR) in RR isolates. The alternative hypothesis for lack of prominent mutations in ETH-resistant strains was that the presence of about 30 monooxygenase homologs in *M. tuberculosis* could prevent the bacteria from a loss of EthA enzyme activity.[6,45] The polymorphism of *ethA* gene mutation found in our study suggested that the development of a molecular test for rapid identification of ETH-resistance was unsuitable. MIC values for ETH-resistant isolates showed that six isolates with nonsynonymous mutations in *ethA* were low-level resistant to ETH, we speculated that nonsynonymous mutations in *ethA* might be associated with low-level resistance to ETH, and further research might be required to confirm their contribution in increasing the MIC for ETH.

There were certain proportions of INH susceptible or ETH susceptible isolates carried mutations in the tested genes in the present study, and some mutations have been reported in INH- or ETH-resistant isolates (Tables 2 and S1). This result may be influenced by the sample sources and geographical regions as well as the methods for DST. Especially, there was a high proportion (20.1%) of ETH susceptible isolates carried mutations in *ethA*. Despite the exact function of these mutations was uncertain, we speculated that these strains were prothionamide-resistant due to the cross-resistance between ETH and prothionamide, yet we did not perform DST for prothionamide in this study.

Our study demonstrated that mutations in *inhA* and its promoter region confer 40.0% of INH and ETH co-resistance, similar to several previous reports.[27,32,47] The most frequently mutation, *inhA* promoter −15C>T occurred in 32.0% (8/25) among INH^{R}ETH^R isolates, consistent with other reports indicating that the proportion of *inhA* −15C>T ranges from 21.1% to 55.3%,[27,32,33] suggesting that *inhA* promoter −15C>T may serve as a marker of cross-resistance to INH and ETH. When combining the MICs and mutation information, we found that *inhA* and *ndh* were indeed associated with cross-resistance in this study, and *inhA* and its promoter mutations were more prevalent in low-level INH or ETH resistant isolates (both 80%). For the *ndh* gene mutation, its contribution in cross-resistance between INH and ETH needed to be further investigated due to the limitation of sample size.

Besides the mutations in the canonical genes associated with INH or/and ETH resistance, we also investigated the mutations in *ndh*, *mshA*, *ahpC* and its upstream and *ethR*. In this study, we detected one unreported mutation in *ndh* (M370I) in two co-resistant strains. One of the two INH^{R}ETH^R isolates had the *ethA* (I34T) mutation to account for ETH resistance, but no other mutation to explain its resistance to INH, and another strain contained mutations both in *katG* and *ahpC* associated with INH resistance but no other mutation to explain its resistance to ETH. Thus, we presumed that the mutation M370I in *ndh* might have potential to define as a new mutation relevant with cross-resistance to INH and ETH. Nevertheless, further research should be conducted to elaborate the underlying mechanism for our observation. Few studies were reported on *mshA*. In the present study, only a synonymous mutation *mshA* A92A was found in an INH-resistant isolate; however, it was unlikely to confer resistance since this single-nucleotide polymorphism did not cause change in the structure of the

protein. As mentioned before, mutations in *ahpC* were more likely to appear together with *katG* mutations, supporting the fact that mutations in *ahpC* did function as compensatory mutation.[15] For *ethR*, we did not identify any strain with a mutation in ETH-resistant isolates, suggesting *that ethR* might play a minor role in ETH resistance in *M. tuberculosis* clinical isolates.[11] Notably, one INH^RETH^S strain displayed a rare mutation N35S in *ethR* but its effect was unknown. Taken together, the data indicated that the significance and mechanism of *ndh, mshA, ahpC* and *ethR* in INH- and ETH-resistance of *M. tuberculosis* should be discovered, simultaneously emphasizing the complexity of the mechanism underlying the resistance. More importantly, in our study, of the 90 INH-resistant isolates and the 34 ETH-resistant isolates, 14 (15.5%) and 10 (29.4%) showed no detectable mutation in the studied genes, suggesting that alternative mechanisms, such as drug efflux pump and decreased cell wall permeability to drugs, may also be related to drug resistance in *M. tuberculosis*.[48–50]

Compared with the phenotypic DST, the sensitivity and specificity for identifying INH-resistant isolates by WGS of combination of *katG, inhA* and its promoter region were 81.11% and 90.54%, respectively, consistent with data from a systematic review,[51] but lower than other findings.[32,37] The other combinations showed no superiority but reduced specificity in the present study (Table 7). To date, molecular diagnostics such as Hain GenoType MTBDR plus line probe assay (LPA) have vastly simplified and increased the speed of diagnosing INH-resistant TB.[43,52] However, the line-probe solely covered popular mutant loci (*katG* and *inhA*) rather than the full spectrum of probes to detect all INH resistance phenotypes, possibly resulted in a consequence of failures to detect resistant strains. Therefore, the combination of *katG* and *inhA* and its promoter region can significantly improve the sensitivity and specificity for INH resistance prediction by WGS. For MDR-TB with cross-resistance between INH and ETH, diagnosis of INH-resistant TB only using LPA by detecting INH resistance-associated mutations will likely lead to misuse of ETH in subsequent treatment regimens, ultimately leading to compromised treatment outcomes. Therefore, INH resistance results obtained using LPA should be accompanied by ETH resistance testing to avoid the misuse of ETH and delayed treatment. This fact motivated further exploration of mechanisms of ETH resistance and search for novel specific molecular targets to facilitate the development of specific methods for the detection of ETH resistance in the future.

Imperfectly, for the ETH resistance prediction, we found an optimal combination of *ethA, inhA* and its promoter and *ndh* by WGS showed sensitivity of 61.76 and specificity of 76.62%; the sensitivity was lower than the findings from France (78.6%)[53] and Colombia (84.2%),[37] and a possible explanation was that the isolates resistant to ETH (n = 34) collected in our study were relatively small so that limited the detection of the variety of gene variations. The sensitivity and specificity for detecting cross-resistance between INH and ETH by WGS of the combination of *inhA* and its promoter and *ndh* were 48.00% and 97.65%, respectively, which was the same as other findings.[27,32] Particularly, the addition of *ndh* mutations to that in *inhA* or its promoter region increased the sensitivity by 8.0% to predict cross-resistance between INH and ETH.

Another interesting finding of the present study was that INH resistance and MDR are more likely to be observed in retreated cases, as previously reported, emphasizing the crucial role of early diagnosis and timely, long-term and well-regulated drug administration, especially in patients treated within the hospital system in the region.

One major strength of this study is that we used whole-genome sequencing data to perform analysis based on SNP differences, which enables us to identify mutations more accurately. There are several limitations in our study. First, limited by the small sample size, we only collected a small number of drug-resistant strains so that we did not detect relevant mutations in the genes enrolled in the study. Second, we simply focused on genes known to confer resistance to INH and ETH in *M. tuberculosis*, and some other genes relevant to drug resistance are overlooked. Lastly, while we detect numerous novel mutations that have not been previously covered, research on the structure of protein in combination with mutagenesis and enzymatic studies directly in *M. tuberculosis* has not been launched to elucidate the specific molecular mechanisms and their effects on the resistance.

Conclusion

In summary, our results indicated that the prevalence of INH, ETH resistance and co-resistance between INH and ETH in the south of Xinjiang province were 28.9%, 10.9% and 8.0%, respectively. Furthermore, mutations in *katG, inhA* and its promoter, *ahpC* and its upstream, and *ndh* genes accounted for 74.4%, 12.2%, 11.1% and 2.2% of INH resistance, respectively. Mutations in *ethA, inhA* and its promoter, and *ndh* genes accounted for 38.2%, 29.4% and 5.9% of ETH resistance, respectively. Mutations

in *inhA* and its promoter and *ndh*, respectively, conferred 40.0% and 8% INH-ETH co-resistance. The optimal gene combinations by WGS for the prediction of INH and ETH resistance and INH-ETH co-resistance were respectively *katG*+*inhA* and its promoter region (sensitivity: 81.11%, specificity: 90.54%), *ethA*+*inhA* and its promoter region+*ndh* (sensitivity, 61.76%, specificity: 76.62%), and *inhA* and its promoter region+*ndh* (sensitivity, 48.00%, specificity: 97.65%). Mutations in *katG* were associated with high-level INH resistance, whilst mutations in *inhA* and its promoter were associated with both INH and/or ETH low-level resistance. The results found in this study would increase our understanding on the resistance mechanisms of INH and/or ETH and provide clues for choosing ETH for MDR treatment and molecular DST methods in this area.

Data Sharing Statement

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

This study obtained approval (XJMU8HEC-20161215) from the Ethics Committee of The Eighth Affiliated Hospital of Xinjiang Medical University. All methods were performed in accordance with the relevant guidelines and regulations. Written informed consent was obtained from individual or guardian participants.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

We have no conflicts of interest to declare.

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