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Original Article

Characterization of isoniazid resistance and genetic mutations in isoniazid-resistant and rifampicin-susceptible *Mycobacterium tuberculosis* in China

Dongxin Liu[a], Bing Zhao[b], Yang Zheng[b], Xichao Ou[b], Shengfen Wang[b], Yang Zhou[b], Yuanyuan Song[b], Hui Xia[b,*], Qiang Wei[a,*], YanLin Zhao[b,*]

[a] National Pathogen Resource Center, Chinese Center for Disease Control and Prevention, Beijing 102206, China
[b] National Tuberculosis Reference Laboratory, National Center for Tuberculosis Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China

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ABSTRACT

Background: Patients with tuberculosis resistant to isoniazid but susceptible to rifampicin (H^r-R^s TB) remain a neglected demographic, despite a high disease burden and poor outcomes of these patients. The aim of this study was to investigate the characteristics of isoniazid-resistance-related mutations in *Mycobacterium tuberculosis* and resistance rates to drugs included in WHO-recommended regimens for H^r-R^s patients.

Methods: *Mycobacterium tuberculosis* isolates (n = 4922) obtained from national tuberculosis drug-resistance surveillance were subjected to whole-genome sequencing to identify H^r-R^s strains. The minimal inhibitory concentrations (MICs) were established for the H^r-R^s strains to determine the isoniazid resistance levels. We also identified drug-resistance-associated mutations for five drugs (fluoroquinolones, ethambutol, pyrazinamide, streptomycin, and amikacin) in the H^r-R^s strains.

Results: Of the 4922 strains, 384 (7.8 %) were H^r-R^s. The subculture of seven strains failed, so 377 (98.2 %) strains underwent phenotypic MIC testing. Among the 384 genotypic H^r-R^s strains, 242 (63.0 %) contained the *katG* Ser315Thr substitution; 115 (29.9 %) contained the -15C>T in the promoter region of the *fabG1* gene; and 16 (4.2 %) contained Ser315Asn in the *katG* gene. Of the 239 strains with the Ser315Thr substitution, 229 (95.8 %) had MIC ≥ 2 μg/mL, and of the 114 strains with the -15C>T mutation, 103 (90.4 %) had 0.25 μg/mL ≤ MIC ≤ 1 μg/mL. The genotypic resistance rates were 0.8 % (3/384) for pyrazinamide, 2.3 % (9/384) for ethambutol and fluoroquinolones; 39.6 % (152/384) of the strains were resistant to streptomycin, but only 0.5 % (2/384) of the strains were resistant to amikacin.

Conclusion: Ser315Thr in *katG* was the predominant mutation conferring the H^r-R^s phenotype, followed by the *fabG1* -15C>T mutation. The combination of rifampicin, pyrazinamide, ethambutol, and levofloxacin should be effective in the treatment of patients with H^r-R^s tuberculosis because the resistance rates for these drugs in China are low.

1. Introduction

Drug resistance is still an enormous challenge for the control and prevention of tuberculosis (TB) in China. Isoniazid is one of the cornerstone drugs for the treatment of rifampicin-susceptible pulmonary TB. An appropriate regimen design based on drug-resistance patterns is crucial for improving the treatment outcomes in TB patients. Failure rates are low in patients infected with pan-sensitive strains who receive the standard first-line treatment regimen, whereas failure rates increase in patients with mono-resistance to isoniazid, ranging from 18

Abbreviation: TB, tuberculosis; H^r-R^s, isoniazid-resistant and rifampicin-susceptible; MDR-TB, multidrug-resistant tuberculosis; MDR/RR-TB, multidrug- or rifampicin-resistant tuberculosis; WGS, whole-genome sequencing; MIC, minimal inhibitory concentration; WHO, World Health Organization.

* Corresponding authors.
E-mail addresses: xiahui@chinacdc.cn (H. Xia), weiqiang@chinacdc.cn (Q. Wei), zhaoyl@chinacdc.cn (Y. Zhao).

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% to 44 %, and importantly, the rate of acquired resistance is significantly higher in these patients than in drug-susceptible patients [1,2].

In China, susceptibility testing for isoniazid and second-line drugs is usually performed on patients demonstrating rifampicin resistance on Xpert® MTB/RIF or patients for whom TB treatment fails. Consequently, patients with isoniazid-resistant and rifampicin-susceptible (H^r-R^s) TB will be missed in settings where diagnostic algorithms prioritize the detection of rifampicin resistance, and will not receive appropriate treatment. Therefore, during the continuation phase of standard first-line drug therapy for rifampicin-susceptible TB, those patients with isoniazid resistance will effectively receive only rifampicin monotherapy, so their treatment will be poor and they may also develop resistance to rifampicin [1]. Jacobsen et al. reported that 61 % of mono-isoniazid-resistant patients who received standard therapy for new and previously treated TB progressed to multidrug-resistant TB (MDR-TB) in rural South Africa [3]. Therefore, the development of isoniazid resistance is a common first step in the evolution of MDR-TB [4]. As we know, multiple genetic mutations confer resistance to isoniazid, and different resistance variants cause different levels of resistance [5,6]. The level of resistance significantly affects the formulation of treatment regimens, as using higher-dose of relatively less toxic and more widely available drugs, such as rifampicin and isoniazid, could prevent switching to a more toxic second-line drugs [7]. Whole-genome sequencing (WGS) can be used to identify known mutations conferring drug resistance, and WGS-based detection methods have proven capable of detecting levels of isoniazid resistance with high accuracy.

Globally, isoniazid resistance rates in patients with rifampicin-susceptible TB range from 5.4 % in settings of low-level (< 1.47 %) MDR-TB prevalence to 14.5 % in settings of high-level (≥ 4.58 %) MDR-TB prevalence [8]. In 2019, 11 % (range, 6.5 %–15 %) of global incident cases of TB were H^r-R^s TB, which translated into an estimated 1.1 million (range, 0.6–1.5 million) cases [9]. China has always been a country with a high burden of drug-resistant TB, and the proportion of multidrug- or rifampicin-resistant tuberculosis (MDR/RR-TB) patients was 3 % in new patients and 20 % in retreated patients in 2022 [10]. Therefore, we speculate that the prevalence of H^r-R^s TB is high in China. The National Tuberculosis Program focuses on the diagnosis and treatment of rifampicin-resistant TB, but little attention has been paid to H^r-R^s TB patients. The Chinese guidelines recommend a 6–9-month treatment regimen composed of rifampicin, ethambutol, pyrazinamide, and levofloxacin for patients with H^r-R^s TB. Another two injectable drugs, streptomycin and amikacin, are also widely used in China. However, no molecular-based commercial assay for the detection of pyrazinamide resistance is available and assays for the detection of resistance to other drugs are only available in a few laboratories in China. Therefore, there is lack of representative data on the resistance rates to these drugs among H^r-R^s TB patients, and susceptibility to these drugs is not routinely tested at the initial examination. Therefore, determining the prevalence of resistance to these drugs at the population level is critical when clinical decisions must be made on the treatment of H^r-R^s TB patients in China.

2. Material and methods

2.1. Isolate sources

From 4922 *Mycobacterium tuberculosis* isolates that were isolated in 2013 from 70 national drug-resistance surveillance sites (Supplementary Table 1) in China, with qualified WGS results, 384 isolates with isoniazid-resistance-related mutations and a wild-type *rpoB* gene were analyzed retrospectively. The strains were cultured on Löwenstein-Jensen medium. The subculture of seven strains failed, so 377/384 (98.2 %) strains were available for minimal inhibitory concentration (MIC) testing.

2.2. WGS analysis

Genomic DNA was extracted with the cetyltrimethylammonium bromide (CTAB) method [11]. WGS with 2 × 150 paired-end reads was performed with the Illumina HiSeq 2500 platform (Illumina, Inc., San Diego, CA). TB Profiler v.3.0.8 (https://jodyphelan.gitbook.io/tb-profiler/) was used to predict genotypic drug susceptibility. In the present study, the mutated loci were defined as resistant according to the grading of “associated with resistance (Assoc w R)” and “associated with resistance–interim (Assoc w RI)”, as described by the World Health Organization (WHO). All *M. tuberculosis* genomic data were deposited at the China National Microbiology Data Center (NMDC) under accession number NMDC10018526.

2.3. MIC testing

The concentration range for testing the MIC for isoniazid was 0.03–4 μg/mL. The growth of *M. tuberculosis* in wells containing isoniazid was evaluated visually with the Vizion System (Thermo Fisher Scientific, Waltham, MA, USA) and compared with its growth in the drug-free control wells after incubation of 14 or 21days. Resistance was classified according to a breakpoint for isoniazid ≥ 0.25 μg/mL, recommended by the Clinical Laboratory and Standards Institute [12].

Table 1
Characteristics of isoniazid-resistance-conferring mutations in H^r-R^s strains.

	Mutation type	Frequency *n* (%)	Confidence grading#
1	*katG*_p.Ser315Thr	242 (63.0)	Assoc w R
2	*fabG1*_c.-15C>T	115 (29.9)	Assoc w R
3	*katG*_p.Ser315Asn	16 (4.2)	Assoc w R
4	*fabG1*_c.-8T>C	5 (1.3)	Assoc w RI
5	*fabG1*_c.-8T>A	4 (1.0)	Assoc w RI
6	*katG*_p.Ser315Thr, *fabG1*_c.-15C>T	1 (0.3)	–
7	*katG*_p.Ser315Thr, *fabG1*_c.-8T>C	1 (0.3)	–
Total		384 (100)	–

Note: # confidence grading refers to WHO document [14].
Abbreviations: Assoc w R: associated with resistance; Assoc w RI: associated with resistance–interim

2.4. Transmission of H^r-R^s isolates

Transmission clusters were defined by applying a threshold of ≤ 12 pairwise single-nucleotide polymorphisms (SNPs) between sequences, as previously described [13]. A minimum spanning tree was generated with Phyloviz available at http://www.phyloviz.net/tutorials.html. The genomic cluster represents the transmission of H^r-R^s strains. For non-clustered strains, it indicate that the H^r-R^s TB is through transmitted if H^r-R^s strains were isolated from new patients who have not received anti-tuberculosis treatment.

2.5. Statistical analysis

Descriptive data were analyzed. Categorical variables are presented as frequencies and proportions. The frequencies of categorical variables were compared with Pearson's χ^2 test or Fisher's exact test as appropriate. The data were analyzed with SPSS software, version 22.0 (IBM, Armonk, NY, USA).

3. Results

3.1. Characteristics of isoniazid-resistance-conferring mutations in H^r-R^s strains

Based on WGS, 384/4922 (7.8 %) strains of *M. tuberculosis* were H^r-R^s. Among these 384 strains, the Ser315Thr mutation in *katG (*242, 63.0 %) was the most frequent change, followed by -15C>T in the promoter region of *fabG1* (115, 29.9 %) and Ser315Asn in *katG* (16, 4.2 %). Other mutation types were less frequent in the H^r-R^s strains, ranging in frequency from 0.3 % to 1.3 % (Table 1).

3.2. Determination of isoniazid resistance levels

The MIC values for isoniazid for the genotypic H^r-R^s strains ranged from 0.06 to > 4 μg/mL. Of the 239 strains with the most commonest mutation, Ser315Thr, 229 (95.8 %) had MIC ≥ 2 μg/mL. Of the 114 strains with the -15C>T mutation, 103 (90.4 %) had 0.25 μg/mL ≤ MIC ≤ 1 μg/mL, and only five (4.4 %) had MIC ≥ 2 μg/mL. Of the 13 strains with the Ser315Asn mutation, 11 (84.6 %) had MIC ≥ 2 μg/mL. Of the nine strains with the -8T>A or -8T>C mutation, eight (88.9 %) had MIC < 0.25 μg/mL and were interpreted as susceptible, based on the critical concentration of 0.25 μg/mL. Strains with combined Ser315Thr and other mutations had MICs of 4 μg/mL (Fig. 1).

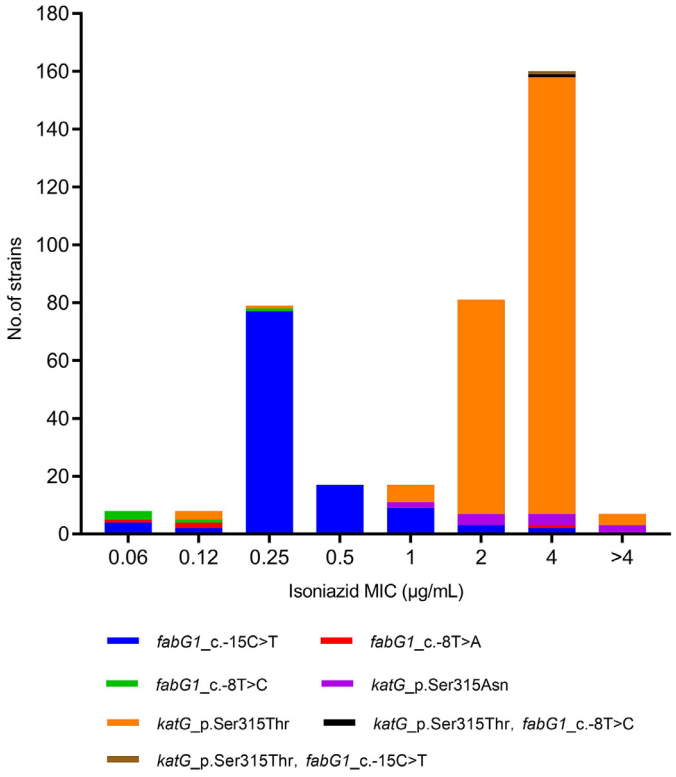

Fig. 1. MIC distribution stratified by different isoniazid-resistance-related mutation types.

3.3. Resistance to other drugs based on WGS

Among the 384 H^r-R^s isolates, three (0.8 %), nine (2.3 %), and nine (2.3 %) were resistant to pyrazinamide, ethambutol, and fluoroquinolone, respectively, based on WGS. Streptomycin had the highest resistance rate (152/384, 39.6 %), whereas only two strains (0.5 %) were resistant to amikacin. The mutation types associated

Table 2
Mutation types associated with resistance to other drugs.

Drugs and mutation types	Frequency	Confidence Grading	Isoniazid MIC ranges (μg/mL)
Pyrazinamide			
*pncA*_p.Ala146Thr	1	Assoc w R	
*pncA*_p.Asp49Gly	1	Assoc w R	
*pncA*_p.Thr47Ala	1	Assoc w R	
Subtotal	3		2 to > 4
Ethambutol			
*embB*_p.Gln497Lys	1	Assoc w R	
*embB*_p.Gly406Ser	1	Assoc w R	
*embB*_p.Met306Ile	3	Assoc w R	
*embB*_p.Met306Val	4	Assoc w R	
subtotal	9		0.25–4
Fluoroquinolone			
*gyrA*_p.Ala90Val	2	Assoc w R	
*gyrA*_p.Asp94Ala	3	Assoc w R	
*gyrA*_p.Asp94Gly	2	Assoc w R	
*gyrB*_p.Ala504Val	1	Assoc w R	
*gyrB*_p.Asp461Asn	1	Assoc w R	
subtotal	9		0.25 to > 4
Streptomycin			
*rpsL*_p.Lys43Arg	113	Assoc w R	
*rpsL*_p.Lys88Arg	11	Assoc w R	
*rrs*_r.514a>c	23	Assoc w R	
*rrs*_r.517c>t	4	Assoc w R	
*rpsL*_p.Lys43Arg, rrs_r.888g>a	1	Assoc w R	
Subtotal	152		0.06–4
Amikacin			
*rrs*_r.1401a>g	2	Assoc w R	2 to > 4

with particular drugs are shown in Table 2. The Lys43Arg mutation in *rpsL* gene was prevalent in 20 % (23/115) of -15C>T mutant H^r-R^s isolates and in 34.3 % (83/242) of Ser315Thr mutant H^r-R^s isolates ($p < 0.01$), $\chi^2 = 7.633$.

A simultaneous *katG* Ser315Thr substitution was detected in 3/3 (100 %), 8/9 (88.9 %), 7/9 (77.8 %), and 116/152 (76.3 %) of pyrazinamide-, ethambutol-, fluoroquinolone-, and streptomycin-resistant isolates, respectively.

3.4. Transmission power of H^r-R^s isolates

Among 384 H^r-R^s isolates, 92 clustered into 36 WGS clusters (Fig. 2 nd Supplementary Table 2) and 292 were unique genomic isolates. Of these unique genomic isolates, 204 were isolated from new treatment-naïve patients and 66 from retreated patients. The 22 unique genomes from patients with no recorded treatment history were excluded from the analysis. When we combined the gene clusters and treatment histories, at least 81.8% (296/362) of cases of H^r-R^s TB were probably caused by the transmission of H^r-R^s isolates in China.

4. Discussion

Isoniazid is a critical antituberculosis drug. On the one hand, excluding isoniazid from treatment regimens based solely on Xpert® MTB/RIF results indicating resistance to rifampicin would deprive too many patients of this crucial anti-TB drug. On the other hand, adding isoniazid to the regimen for H^r-R^s patients would pose a risk of treatment failure and additional acquired rifampicin resistance. Therefore, knowing the prevalence of H^r-R^s is essential. In this study, 7.8 % of strains collected from 70 counties in China showed H^r-R^s based on WGS. The *katG* Ser315Thr mutation (63.0 %) was the most frequently observed change among all the H^r-R^s strain, followed by -15C>T in the promoter region of the *fabG1* gene (29.9 %). Other studies have shown similar results, insofar as *katG* Ser315Thr was the commonest mutation underlying isoniazid resistance, followed by *fabG1* -15C>T [15,16]. Most mutations or deletions in *KatG* seriously compromise its catalase/peroxidase function and thereby the virulence of the strain, and it has been proposed that these losses can be compensated by the increased expression of AhpC, an alkylhydroperoxidase. However, no compensatory mutations for the *fabG1* mutation have been proposed, which may be why the *katG* Ser315Thr mutation accounts for the greatest proportion of isoniazid resistance. Of the Ser315Thr mutant strains, 95.8 % had MIC ≥ 2 μg/mL, indicating high-level resistance to isoniazid, whereas 90.4 % of strains with the -15C>T mutation were associated with low-level resistance (0.25 μg/mL ≤ MIC ≤ 1 μg/mL). The prevalence of drug resistance to pyrazinamide, ethambutol, or levofloxacin, which constitute the recommended treatment regimen for H^r-R^s TB patients, is very low.

The prevalence of H^r-R^s TB varies in different countries, e.g., 11.4 % in Ho Chi Minh City (2008–2011) [17], 13.8 % and 21.1 % in South Africa (2011 and 2014, respectively) [18], 8.7 % in India (2016–2019) [19], 8 % in Lima, Peru (2010–2011) [20], and 3.9 %–4.6 % in two eastern provinces of China [21,22]. In this study,

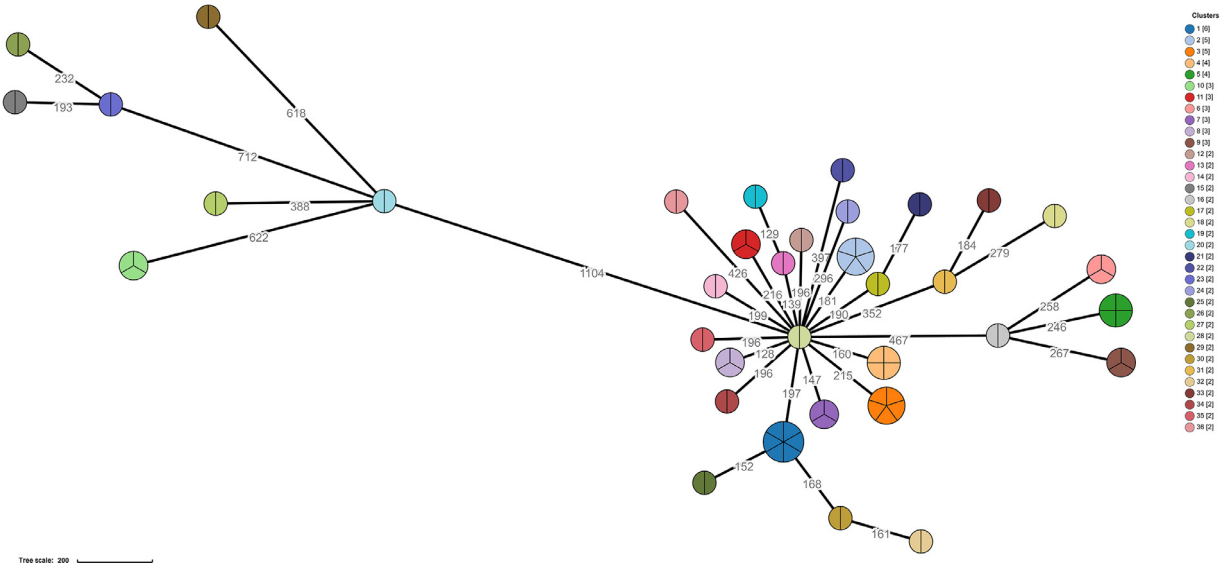

Fig. 2. WGS clusters of H^r-R^s TB isolates. Ninety-two H^r-R^s isolates clustered into 36 WGS clusters. We defined genetic distances within 12 SNPs isolates were clusters, only clustered isolates were retained. Each circle represents a cluster. The color of each circle represents different cluster. The size of the circle represents the number of isolates in the cluster.

the prevalence of H^r-R^s TB in China was 7.8 % at the population level, which is a moderate rate of resistance compared with those of other countries. Considering the poor outcomes of H^r-R^s patients treated with standard first-line therapy, more attention must be paid to rapidly testing for isoniazid susceptibility in rifampicin-susceptible patients. We also found that the single -15C>T mutation in the promoter region of *fabG1* accounted for approximately 30 % of genotypic isoniazid resistance, higher than the proportions reported elsewhere [23,24]. The differences between MDR-TB and H^r-R^s patients in the frequency distributions of the *katG* (76.1 % and 41.2 %, respectively) and *fabG1* mutations (7.6 % and 30.2 %, respectively) were also significant in Pakistan [25]. Some research has indicated that the *katG* Ser315Thr substitution is associated with MDR-TB, which has important implications for the transmission of MDR-TB, and entails a higher risk of generating a secondary resistant cases than isoniazid-resistant strains with other mutations. The sucessful transmission of MDR-TB with *katG* Ser315Thr mutation may also be caused by mutations that compensate for *katG* gene mutations [26–28]. In this study, at least 81.8 % of H^r-R^s strains were already resistant when infected rather than acquired resistance within the patient, so the transmission of H^r-R^s TB in China is extremely severe.

In the present study, > 95 % of the Ser315Thr *katG* mutant isolates and > 90 % of the -15C>T *fabG1* mutant isolates showed high- and low-level resistance, respectively, consistent with previous findings [29,30]. Patients infected with isolates carrying the -15C>T mutation, confirmed by genotypic assay, may therefore benefit from high-dose (10–15 mg/kg per day) isoniazid therapy, as described in WHO's guideline and other research [31,32]. A previous study reported that the accurate diagnosis and tailored treatment of isoniazid-resistant TB, based on the line probe assay, resulted in a relative reduction of nearly 50 % in acquired MDR-TB compared with the Xpert® MTB/RIF strategy, which focuses on the detection of rifampicin resistance [33]. Therefore, accurate molecular-based MDR-TB assays, such as the line probe assay, are essential for the treatment and control of H^r-R^s TB.

Our study has shown that, based on WGS, genotypic resistance rates were very low for the drugs of interest, ethambutol, pyrazinamide, fluoroquinolone and amikacin, but not for streptomycin. Previous studies have shown that cases of MDR/RR-TB in China are more likely resistant to ethambutol, streptomycin, fluoroquinolones, and pyrazinamide [34–36], ranging in frequency from 30 % to 50 %. The higher resistance rates to these drugs among MDR-TB strains than among H^r-R^s TB strains can be explained by the high transmissibility and long duration of transmission of MDR-TB strains. During the transmission process, resistance to other drugs is acquired. In Pakistan, the rates of resistance to levofloxacin and pyrazinamide in patients with H^r-R^s pulmonary TB were 25.2 % and 12.2 %, respectively [25]. The low resistance rates to the drugs comprising the H^r-R^s regimen in this study indicate a higher likelihood of successful treatment using WHO recommended regimen on H^r-R^s patients. Although the inclusion of second-line injectable drugs into the regimen is not recommended, in some areas, such as two regions of Uzbekistan, patients diagnosed with H^r-R^s TB have been treated with a 9-month regimen that includes injectable second-line drugs during the first 3 months, with an 80 % successful treatment rate [37]. Given the extremely low rate of resistance to amikacin in China (only 0.5 %), we assume that if an effective treatment regimen cannot be established due to drug resistance, side effects,

or an unsatisfactory response to the regimen, amikacin may be used as an alternative option. Approximately 40 % of isolates showed resistance to streptomycin, supporting the notion that streptomycin should not be included in the treatment regimens for H^r-R^s TB, consistent with the WHO guidelines.

In this study, we have determined the background prevalence of H^r-R^s *M. tuberculosis* strains and resistance rates to drugs included in the TB treatment regimen at the population level. The main limitation of the study was that it focused only on the characteristics of the strains from a laboratory perspective, and did not analyze the relevant risk factors or follow-up the treatment outcomes. Identifying the risk factors for H^r-R^s TB will allow the prioritization of patients for isoniazid susceptibility testing to rule out isoniazid resistance, and the provision of the tailored therapies for H^r-R^s patients when no rapid test for isoniazid resistance is available.

In conclusion, the *katG* Ser315Thr mutation was the predominant mutation in H^r-R^s isolates of *M. tuberculosis*, followed by -15C>T in the promoter region of *fabG1*. Rapid diagnostic assays and an effective diagnostic algorithm must be introduced to identify cases of H^r-R^s TB in China, and to provide evidence for their appropriate treatment. Because the rate of drug resistance in China is low, the combination of rifampicin, pyrazinamide, ethambutol, and levofloxacin should be effective in treating patients with H^r-R^s TB.

Isoniazid is one of the critical drugs for the treatment of rifampicin-susceptible pulmonary tuberculosis (TB). Patients with isoniazid-resistant and rifampicin-susceptible (H^r-R^s) TB are always missed in settings where diagnostic algorithms prioritize the detection of rifampicin resistance (such as GeneXpert® MTB/RIF). Failure rates and acquired rifampicin resistance are higher in H^r-R^s patients treated with standard first-line therapy than in pan-sensitive patients. However, there are no nationally representative data on the prevalence of H^r-R^s TB or the resistance rates to drugs comprising the H^r-R^s regimen in China. In this study, we demonstrate a modest prevalence of H^r-R^s TB and low resistance rates for pyrazinamide, ethambutol, and fluoroquinolones. These findings suggest a program should rapidly test the susceptibility to isoniazid of rifampicin-susceptible patients, and provide appropriate regimen for H^r-R^s patients.

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Author contributions

HX, DL, Y Zhao, and QW contributed to the study design; DL, BZ, YZ, XO, SW, and YS collected the data and performed the laboratory testing; Y Zhou helped with the bioinformatic analysis; HX and DL analyzed the data and prepared the report for publication.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability statement

All *M. tuberculosis* genomic data were deposited at the China National Microbiology Data Center (NMDC) under accession number NMDC10018526. Other data that support the findings of this study are available on request from the corresponding author, the data are not publicly available due to privacy or ethical restrictions.

Ethics statement

The isolates in this study were collected during 2013 national drug-resistance surveillance, which was approved by the Ethical Committee of the Chinese Center for Disease Control and Prevention. Ethics approval for the present study was waived because all the isolates were from this 2013 surveillance program and no identifiable human subject data or intervention measures were involved.

Informed consent

All patients provided written informed consent at the time of entering this study.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.imj.2024.100129.

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