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Compensatory Mutations of Rifampin Resistance Are Associated with Transmission of Multidrug-Resistant *Mycobacterium tuberculosis* Beijing Genotype Strains in China

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Mycobacterium tuberculosis **can acquire resistance to rifampin (RIF) through mutations in the *rpoB* gene. This is usually accompanied by a fitness cost, which, however, can be mitigated by secondary mutations in the *rpoA* or *rpoC* gene. This study aimed to identify *rpoA* and *rpoC* mutations in clinical *M. tuberculosis* isolates in northern China in order to clarify their role in the transmission of drug-resistant tuberculosis (TB). The study collection included 332 RIF-resistant and 178 RIF-susceptible isolates. The majority of isolates belonged to the Beijing genotype (95.3%, 486/510 isolates), and no mutation was found in *rpoA* or *rpoC* of the non-Beijing genotype strains. Among the Beijing genotype strains, 27.8% (89/320) of RIF-resistant isolates harbored nonsynonymous mutations in the *rpoA* ($n = 6$) or *rpoC* ($n = 83$) gene. The proportion of *rpoC* mutations was significantly higher in new cases ($P = 0.023$) and in strains with the *rpoB* S531L mutation ($P < 0.001$). In addition, multidrug-resistant (MDR) strains with *rpoC* mutations were significantly associated with 24-locus mycobacterial interspersed repetitive-unit–variable-number tandem-repeat clustering ($P = 0.016$). In summary, we believe that these findings indirectly suggest an epistatic interaction of particular mutations related to RIF resistance and strain fitness and, consequently, the role of such mutations in the spread of MDR *M. tuberculosis* strains.**

The worldwide emergence of drug-resistant tuberculosis (TB), especially multidrug-resistant (MDR) TB, is seriously threatening public health and poses a major threat to global TB control. China is one of the countries with a high burden of TB, accounting for about 20% of the global burden of MDR TB (1). According to a national survey of drug-resistant TB in China conducted in 2007 and 2008, 5.7% of patients with new cases of TB and 25.6% of patients with previously treated cases had MDR TB, presenting a serious challenge to TB control (2).

There are many factors contributing to the transmission of drug-resistant TB, including ineffective control programs, the presence of comorbidities, and numerous patient-related factors (3–6); however, the bacterial forces behind the spread of drug-resistant TB are not completely understood. Mathematical models predicted that the future of the drug-resistant TB epidemic would depend mostly on the fitness of drug-resistant *Mycobacterium tuberculosis* strains relative to that of drug-susceptible strains (7). Recent studies have shown that the drug-resistant strains can acquire one or more secondary mutations that will improve their fitness during evolution (8, 9).

Rifampin (RIF) is one of the important first-line anti-TB drugs and serves as a surrogate marker of MDR TB. RIF targets the β subunit of the RNA polymerase, encoded by the *rpoB* gene, and *M. tuberculosis* can acquire resistance to RIF through mutations in this gene, especially in the 81-bp RIF resistance-determining region (RRDR) (10–12). Gagneux et al. (13) found that the acquisition of RIF resistance in laboratory-derived strains is usually accompanied by a fitness cost. However, in some clinical isolates harboring *rpoB* mutations, no fitness cost was found when the fitness of the isolates was compared with that of their parent wild-type isolates. Subsequent studies showed that some secondary mutations in the *rpoA* or *rpoC* gene can mitigate the initial fitness cost caused by an *rpoB* mutation (14, 15). In China, these putative compensatory mutations in *rpoA* and *rpoC* have not been studied yet, and it is unknown whether they contribute to the spread of drug-resistant TB in China.

In this study, we aimed to investigate the characteristics of the *rpoA* and *rpoC* mutations in clinical *M. tuberculosis* strains isolated in northern China and their role in the transmission of drug-resistant *M. tuberculosis* strains.

MATERIALS AND METHODS

Study population. *M. tuberculosis* isolates were recovered from patients admitted to the Beijing Chest Hospital from January 2011 to December 2013. All strains isolated from patients living in Beijing and the adjacent provinces of Tianjin and Hebei found to be RIF resistant according to the initial drug susceptibility testing (DST) results were included in this study.

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TABLE 1 Primers used for DNA sequencing

Gene	Position[a] (bp) in reference to start position	Orientation	Oligonucleotide sequence (5′–3′)	T_m[b] (°C)
rpoB RRDR	916 to 1572	Forward	GGTCGCTATAAGGTCAACAAGAAG	59
		Reverse	GTACACGATCTCGTCGCTAACC	61
Complete *rpoB*				
rpoB1	−148 to 477	Forward	TTGCGGCTCAGCGGTTTA	60
		Reverse	CGTGCCCTTCTCGGTCATC	61
rpoB2	369 to 1027	Forward	GCGGCTCCACTGTTCGTCA	63
		Reverse	GCAAGCGGACCAGAT	53
rpoB3	1503 to 2114	Forward	CCGTTCGGGTTCATCG	55
		Reverse	CCGTCGTCAGTACAGGGA	58
rpoB4	2010 to 2666	Forward	CGGTCCAACCACGGCACTT	63
		Reverse	TCACGCCCTTGTTGCC	63
rpoB5	2592 to 3268	Forward	GTGTATGTGGCTCAGAAACG	57
		Reverse	TGTCATCGGACTTGATGGTC	57
rpoB6	3047 to 3534 (82 bp downstream)	Forward	GGTCACGGTTGGCTACA	59
		Reverse	CCGATGCGGAGTTCA	60
rpoA gene				
rpoA1	−30 to 595 (30 bp upstream)	Forward	TGGCGGACGTCGAAAGGAAGAA	66
		Reverse	TGGTCTCCACGTCCAGGATCAGC	67
rpoA2	537 to 1068 (34 bp downstream)	Forward	TCCATCTACTCACCGGTGCTCAAA	63
		Reverse	CATAGCTGACGCTCCTGTCTGGAT	62
rpoC gene				
rpoC1	1030 to 1650	Forward	ACCGCAGGGTGATCAACCGC	66
		Reverse	TTCGGCGCTCAAAGGCAGGT	66
rpoC2	1589 to 2299	Forward	GGCGTTCAATGCCGACTTCG	65
		Reverse	GGTTCAAAGCGCCACGCTGG	67

[a] Position in reference to start position.
[b] T_m, melting temperature.

RIF-susceptible strains were selected using a random-number table. This study was approved by the Health Research Ethics Committee of Beijing Chest Hospital, Capital Medical University.

DST. The isolates were cultured on Lowenstein-Jensen medium for 4 weeks at 37°C. Testing for susceptibility to four first-line anti-TB drugs and four second-line drugs was performed by the absolute concentration method. The concentrations of the different drugs in the medium were as follows: isoniazid, 0.2 μg/ml; RIF, 50 μg/ml; streptomycin, 10 μg/ml; ethambutol, 5 μg/ml; ofloxacin, 2 μg/ml; levofloxacin, 2 μg/ml; amikacin, 30 μg/ml; and capreomycin, 40 μg/ml.

DNA extraction and sequencing. DNA was extracted from mycobacterial cultures by the lysozyme, proteinase K, and cetyltrimethylammonium bromide procedure. Oligonucleotide primers were designed for PCR amplification and DNA sequencing using Primer software (v.3) (16) (Table 1). In this study, the entire *rpoA* gene (*Rv3457c*), the portion of the *rpoC* gene encoding the RpoA-RpoC interaction site (*Rv0668*; amino acids 351 to 760; previously shown to be prone to the acquisition of compensatory mutations [15]), and the core fragment of the *rpoB* gene (amino acids 395 to 598) were amplified and sequenced. The amplifications of *rpoA* and *rpoC* were performed using the following conditions: 5 min of denaturation at 95°C, followed by 35 amplification cycles (in which each cycle consisted of 94°C for 50 s, 64°C for *rpoA2* and 67°C for *rpoA1*, *rpoC1*, and *rpoC2* for 30 s, and 30 s of extension at 72°C) and an elongation step of 10 min at 72°C. The PCR conditions for *rpoB* were as follows: 3 min of denaturation at 95°C, followed by 35 amplification cycles (in which each cycle consisted of 94°C for 45 s, 62°C for 45 s, and 35 s of extension at 72°C) and an elongation step of 10 min at 72°C. DNA sequencing was carried out at the Tian Yi Hui Yuan Biotechnology Company (Beijing, China). Gene polymorphisms were identified by comparing the sequence of each isolate to the sequence of the corresponding gene of the H37Rv reference strain (GenBank accession no. NC_000962) using GeneDoc (v.3.2) software.

Genotyping. Members of the Beijing family of strains were identified by the RD105 multiplex PCR. The standard 24-locus variable-number tandem-repeat (VNTR) method (17) was used to genotype the *M. tuberculosis* isolates. Two or more isolates from different patients who shared the same 24-locus VNTR genotype patterns were considered clustered. Other isolates were classified as unique. We assumed that high-resolution 24-locus genotypic clustering represented the recent transmission of *M. tuberculosis* isolates.

Statistical analysis. The VNTR genotyping data were analyzed by use of the mycobacterial interspersed repetitive-unit (MIRU)–VNTR MIRU-VNTRplus web application (http://www.miru-vntrplus.org/MIRU/index.faces). Statistical analysis was performed with SPSS (v.16.0) software (SPSS Inc.). Univariate analysis of categorical variables was performed using chi-square and Fisher exact tests, as appropriate. Variables with a P value of less than 0.05 in the univariate analysis were further used in the multivariable logistic regression analysis.

RESULTS

Characteristics of study population and strains. Among the total of 575 isolates selected, DST, genotyping, and DNA sequencing results were available for 510 isolates. The median age of the patients was 47 years (range, 8 to 90 years), and most patients (72.1%) were male. Of the 493 patients for whom data on treatment history were available, 260 (52.7%) had been previously treated. Among the 510 isolates, only 24 (4.7%) were non-Beijing family isolates, and none of the 12 non-Beijing family RIF-resistant strains harbored an *rpoA* or *rpoC* mutation. For this reason, the subsequent analysis was performed with 486 (95.3%) Beijing genotype isolates. According to the DST results, 320 Beijing family isolates were RIF resistant. Among these, 298 (93.1%) were MDR isolates, including 84 (26.3%) MDR *sensu stricto*, 138 (43.1%) pre-extensively drug-resistant (pre-XDR), and 76 (23.8%) XDR isolates.

Mutations in *rpoA* and *rpoC*. All strains were analyzed for mutations in the *rpoA* gene and the RpoA-RpoC interaction site in

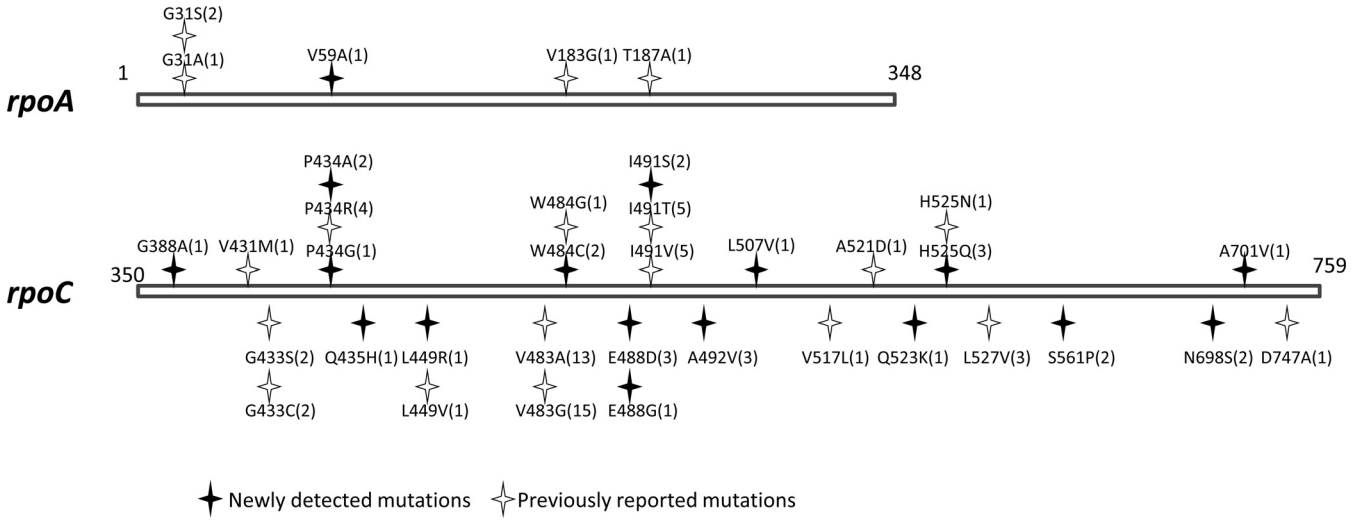

FIG 1 Nonsynonymous mutations identified in *rpoA* and *rpoC* of RIF-resistant isolates from China. The number of isolates is shown in parentheses.

the *rpoC* gene. Among 320 Beijing family strains, 27.8% (89/320 strains) harbored nonsynonymous mutations in the *rpoA* or *rpoC* gene (a single mutation per strain). A synonymous mutation in *rpoC* (D580D) was found in one isolate, and a synonymous mutation in *rpoA* (S30S) was found in two isolates. The mutations in *rpoC* occurred more frequently, accounting for 93.3% (83/89) of all the nonsynonymous mutations in *rpoA* and *rpoC* genes. A total of 31 different *rpoC* mutant variants were found, and 16 of these have not been described previously, to the best of our knowledge (Fig. 1). The most common mutated codons were *rpoC* codon 483 (33.7%, 28/83) and *rpoC* codon 491 (14.4%). Only six isolates (6.7%) showed nonsynonymous mutations in *rpoA* (in which four codons were concerned). In addition, 2 of 178 RIF-susceptible isolates harbored a nonsynonymous mutation in *rpoC* (T721C). Given that mutations in *rpoA* were extremely rare, only *rpoC* mutations were included in the further analysis.

The characteristics of RIF-resistant isolates with *rpoC* mutations. Among the 83 RIF-resistant isolates with an *rpoC* mutation, 97.6% (81/83 isolates) were MDR isolates (including pre-XDR and XDR isolates) (Table 2). The proportion of pre-XDR and XDR isolates with an *rpoC* mutation was marginally higher than the proportion of MDR *sensu stricto* or RIF-monoresistant isolates with such a mutation (29.9% versus 20.2% and 9.1%, respectively; $P = 0.066$). Some characteristics of the patients infected with the strains with the *rpoC* mutation were further compared (Table 2). The isolates with an *rpoC* mutation were found more frequently in patients with newly diagnosed TB than in patients being retreated (37.2% versus 21.8%; $P = 0.023$).

TABLE 2 Factors associated with nonsynonymous mutations in the *rpoC* gene in Beijing genotype isolates[a]

Characteristic or variant	No. (%) of isolates with:		OR (95% CI)	*P* value for OR	Adjusted OR (95% CI)	*P* value for adjusted OR
	rpoC mutation	No *rpoC* mutation				
Age (yr)						0.143
≥47	25 (18.5)	110 (81.5)	1		1	
25–47	38 (30.6)	86 (69.4)	1.94 (1.09–3.47)	0.023	1.23 (0.63–2.37)	
≤25	18 (39.1)	28 (60.9)	2.83 (1.36–5.90)	0.005	2.33 (0.99–5.43)	
Unknown	2	13				
Treatment history						0.023
Retreatment	46 (21.8)	165 (78.2)	1		1	
New treatment	35 (37.2)	59 (62.8)	2.13 (1.25–3.62)	0.005	2.01 (1.10–3.65)	
Unknown	2 (12.5)	14 (87.5)				
Resistance type						0.066
RIF monoresistant	2 (9.1)	20 (90.9)	1		1	
MDR *sensu stricto*	17 (20.2)	67 (79.8)	2.54 (0.54–11.93)	0.225	1.21 (0.28–5.29)	
Pre-XDR and XDR	64 (29.9)	150 (70.1)	4.27 (0.97–18.79)	0.038	2.57 (0.65–10.21)	
Mutation in *rpoB*						<0.001
Other mutations	7 (4.8)	138 (95.2)	1		1	
rpoB S531L	76 (43.4)	99 (56.6)	15.13 (6.69–34.23)	<0.001	12.79 (5.54–29.56)	

[a] OR, odds ratio; CI, confidence interval.

TABLE 3 Factors associated with transmissibility of Beijing genotype clinical isolates

Characteristic	No. (%) of isolates[a]		OR (95% CI)[b]	*P* value
	In the genotypic cluster	Not in the genotypic cluster		
Resistance type				
RIF monoresistant	9 (40.9)	13 (59.1)	1	
MDR	114 (38.3)	184 (61.7)	0.90 (0.37–2.16)	0.805
XDR	31 (40.8)	45 (59.2)	0.99 (0.38–2.61)	0.992
Mutation type in MDR isolates				
No *rpoC* mutation	74 (34.1)	143 (65.9)	1	
rpoC mutation	40 (49.4)	41 (50.6)	1.89 (1.12–3.17)	0.016
rpoB S531L mutation alone	26 (29.9)	61 (70.1)	1	
rpoB S531L and *rpoC* mutation	37 (49.3)	38 (50.7)	2.28 (1.19–4.35)	0.011

[a] Defined by 24-locus MIRU-VNTR analysis.
[b] OR, odds ratio; CI, confidence interval.

Association between mutations in *rpoC* and the *rpoB* RRDR. Sequencing of the *rpoB* gene (codons 395 to 598) from 320 RIF-resistant strains revealed that 308 (96.3%) isolates had at least one mutation in the RRDR, and these mainly involved codons 531 (61%), 526 (21.8%), and 516 (7.8%). The *rpoB* S531L mutation was the most common mutation (56.8%, 175/308 strains); in 12 strains, it was combined with another RRDR mutation. None of the 178 RIF-susceptible isolates harbored a mutation in the RRDR.

The *rpoC* mutations were significantly more often found to be associated with the *rpoB* S531L mutation than with the other *rpoB* mutations (43.4% versus 4.8%; $P < 0.001$; Table 2). A broad range of *rpoC* mutations was detected among isolates with the *rpoB* S531L mutation, in which a mutation in *rpoC* codon 483 was the most common (34.2%, 26/76). Among seven isolates with *rpoC* and other *rpoB* mutations, four harbored an *rpoB* mutation at codon 526, one harbored an *rpoB* mutation at codon 513, and one harbored an *rpoB* mutation at codon 533. In addition, one isolate with an *rpoC* W484G mutation did not show any mutation in the RRDR. The rest of the *rpoB* gene was then sequenced for this isolate, and the *rpoB* V146F mutation, which was previously reported to be associated with RIF resistance (18), was found.

***rpoC* mutations and transmission capacity of isolates.** Genetic clustering (by highly discriminatory VNTRs or IS*6110*-based restriction fragment polymorphism analysis [RFLP]) has been used extensively in molecular epidemiological studies as a measure of recent TB transmission. The underlying assumption was that TB patients infected with genotypically unique isolates represent cases of reactivation of latent infection, whereas patients with genetically clustered isolates are epidemiologically linked and represent chains of TB transmission.

In this study, three groups of isolates (RIF-monoresistant, MDR, and XDR isolates) did not differ with regard to clustering (Table 3). Since 97.5% of the isolates with *rpoC* mutations were MDR, further analysis focused on the MDR group. Out of 298 Beijing family MDR isolates, 114 were clustered isolates and 184 were orphans. Among the clustered isolates, 35.1% (40/114 isolates) harbored *rpoC* mutations. The most common mutations were in *rpoC* at codon 483 (35%, 14/40) and *rpoC* at codon 491 (22.5%, 9/40). Among the orphan isolates, 22.3% (41/184) harbored *rpoC* mutations, and those in *rpoC* at codon 483 were the most common (34.1%, 14/41). An *rpoC* mutation was found more frequently in clustered than nonclustered isolates (35.1% versus 22.3%; $P = 0.016$). We also detected *rpoC* mutations in 57.5% (23/40) of clustered isolates. The isolates in five and two of these clusters showed identical and different *rpoC* mutations, respectively. The other 16 clusters included isolates with and without *rpoC* mutations. Furthermore, the MDR isolates with both the *rpoB* S531L and *rpoC* mutations exhibited a higher clustering rate than those with the *rpoB* S531L mutation alone (49.3% versus 29.9%; $P = 0.011$).

As noted above, two RIF-susceptible strains harbored the *rpoC* T721C mutation. Upon 24-locus VNTR typing, they were found to be orphans and were located in distant parts of the minimum-spanning tree (not shown).

DISCUSSION

Although *M. tuberculosis* strains of the Beijing family are, overall, widespread in China, they have always been overwhelmingly predominant in the northern part of the country (19, 20). The present study found no exception in this regard. The Beijing genotype was identified in 95.3% of the isolates in the collection studied, which led us to focus on a detailed comparative analysis of the Beijing family isolates. Recent studies suggested that many MDR TB cases result from patient-to-patient transmission rather than from the *de novo* acquisition of resistance during treatment (2, 21, 22). A population-based study in five provinces in China found that 43.7% of MDR isolates from individuals with clinical disease were in genetic clusters (3). This is similar to the findings of our study, where 38.3% of MDR strains and 40.8% of XDR strains were in clusters, indicating that circulating drug-resistant *M. tuberculosis* strains have the ability to efficiently spread in the population.

Whereas a causative role of *rpoB* mutations in RIF resistance in *M. tuberculosis* has been well-known for more than 20 years, the next valuable step in elucidating the genetic background of such isolates that are in circulation was made only relatively recently. Comas et al. (15) proposed that secondary mutations in the *rpoA* or *rpoC* gene could alleviate the fitness cost incurred by *rpoB* mutations, especially those in the RRDR. Subsequent studies found that mutations in the *rpoA* or *rpoC* gene were common in RIF-resistant clinical isolates from different countries, including South Africa, Russia, Ghana, Abkhazia/Georgia, Kazakhstan, and Uzbekistan, and about 90% of the mutations were located in the *rpoC* gene (4, 15, 23). In this study, mutations occurred more frequently in *rpoC* than *rpoA* (93.3%, 83/89 isolates). At the same time, the *rpoC* mutations showed a high level of diversity, but those in

codons 483 and 491 were the most frequent (Fig. 1) which is concordant with the findings of the previous reports (4, 15, 23).

Inspired by the first publication of Comas et al. (15), this study did not analyze the entire *rpoC* gene but analyzed its most meaningful portion, which is the large portion involved in the RpoA-RpoC interaction. However, most recent research has described variations in *rpoC* beyond this core region (4, 24). In particular, in one geographically delimited setting in central Russia where TB was epidemic, 8% of isolates harbored such mutations in *rpoC* (4). The lack of analysis of the entire *rpoC* gene could be considered a limitation of our study. Accordingly, the prevalence rate of *rpoC* mutants in our setting could be somewhat underestimated, and an additional study is warranted in the future.

It is noteworthy that not all variations in the *rpoA* or *rpoC* gene represented the evolution of compensatory mutations. Instead, some single nucleotide polymorphisms in the *rpoC* gene were lineage-specific markers; e.g., *rpoC* A542A and *rpoC* G594E are markers of the Latin American Mediterranean (LAM) lineage and the Erdman strain (Haarlem lineage), respectively (15). With regard to the present study, two isolates with the *rpoC* T721C mutation were both RIF sensitive and genotypically distant (according to the 24-locus VNTR). Accordingly, this *rpoC* mutation is neither a lineage marker nor an RIF resistance marker and likely occurred independently in those two isolates; its function would best be assessed experimentally.

In this study, the RIF-resistant strains with *rpoC* mutations were prevalent in new cases, and the MDR strains with *rpoC* mutations were significantly associated with clustering. A similar study of 286 drug-resistant and 54 drug-susceptible *M. tuberculosis* isolates in the Western Cape Province of South Africa also found a higher prevalence of *rpoC* mutants among isolates clustered by IS*6110*-RFLP than nonclustered isolates (30.8% versus 9.4%) (23). de Vos et al. proposed that mutations in the *rpoC* gene (codons 245 to 560) contributed to the transmission of MDR TB (23), although they did not detail a putative mechanism for this contribution to transmission. Our results also demonstrate that there is some association between *rpoC* mutations and the spread of MDR strains, but the mechanism remains to be elucidated.

Here, the *rpoC* mutations were significantly associated with the *rpoB* S531L mutation, which is in accordance with the findings described in the previous publication (23) and is explained by the lower fitness cost of the *rpoB* S531L mutation (13). Mutations in *rpoC* can act by restoring structural interactions between the RNA polymerase β′, β, and α subunits (14). Furthermore, the *rpoC* mutants were more frequently found, albeit marginally, in pre-XDR and XDR groups of isolates than in MDR and RIF-monoresistant groups. These findings appear to support the hypothesis that epistatic interactions of different drug resistance and compensatory mutations are involved in the survival and evolution of drug-resistant *M. tuberculosis* (25–27).

In conclusion, mutations occurred frequently in the *rpoC* genes of *M. tuberculosis* clinical strains isolated from the northern region of China and were seen more frequently in clustered than nonclustered isolates. We believe that the findings of this study indirectly suggest an epistatic interaction of particular mutations related to RIF resistance (i.e., *rpoB* versus *rpoC*) and strain fitness and, consequently, highlight the role of these mutations in the transmission of MDR *M. tuberculosis*.

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