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ORIGINAL RESEARCH

Molecular characterization of para-aminosalicylic acid resistant *Mycobacterium tuberculosis* clinical isolates in southwestern China

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Purpose: The aim of this study is to investigate para-aminosalicylic acid (PAS) resistance-related gene mutations in clinical *Mycobacterium tuberculosis* (MTB) isolates and analyze the associated risk factors in southwestern China.

Patients and methods: Total 122 PAS-resistant and 55 PAS-susceptible clinical isolates were obtained from Chongqing Public Health Medical Center between April 2014 and January 2018. Drug susceptibility test was performed, and the PAS resistance-related genes were sequenced.

Results: PAS-resistant strains were more likely to resist streptomycin (OR: 9.5, 95% CI: 3.87-23.3; $P<0.01$), isoniazid (OR: 5.98, 95% CI: 2.14–16.76; $P<0.01$), rifampin (OR: 5.01, 95% CI: 2.11–11.88; $P<0.01$), ethambutol (OR: 2.79, 95% CI: 1.44–5.4; $P<0.01$), levofloxacin (OR: 2.56, 95% CI: 1.33–4.93; $P<0.01$), and amikacin (OR: 4.29, 95% CI: 1.70–10.83; $P<0.01$). The sequencing results showed that 112 (91.8%) PAS-resistant strains harbored 30 different mutations in *folC, thyA*, and *ribD*. Mutations in *folC* were the most commonly observed in PAS-resistant isolates (54.5%, 61/112), followed by mutations in *thyA* and *ribD*. Residues I43 in *folC*, R235 in *thyA*, and $^{-11}$G in upstream of *ribD* were hotspots for mutation sites.

Conclusion: PAS drug resistance in MTB in southwestern China is mainly caused by mutations in *folC, thyA*, and *ribD*, among which *folC* was the most frequent mutation. Some mutation hotspots exist in the three genes, which accounts for about 80% of total mutations. These results highlight the possibility of developing molecular diagnostic methods for PAS-resistant tuberculosis in the future.

Keywords: *Mycobacterium tuberculosis*, para-aminosalicylic acid, resistance, molecular characteristic, southwestern China

Introduction

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (MTB) and is a major public health problem globally. The emergence and transmission of multidrug-resistant tuberculosis (MDR-TB), which is resistant to both rifampin and isoniazid, have aggravated this problem.[1] WHO estimated that there were 10.0 million new TB cases and 458,000 new MDR-TB cases in 2017.[2] China is one of the countries with a high burden of both TB and MDR-TB in the world. In 2017, there were 778,000 new TB cases in China, of which MDR-TB cases were 58,000.[2]

Para-aminosalicylic acid (PAS) is the second drug used for TB treatment after streptomycin and now is used as a second-line anti-TB drug of MDR-TB.[3–5]

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Although PAS has been used for more than 70 years for TB therapy, its mechanism of action is not fully understood. In recent years, studies have revealed that PAS is a prodrug that targets the folate synthesis in MTB.[6–8] PAS acts as an alternative substrate for dihydropteroate synthase (DHPS/FolP1). The product hydroxy dihydropteroate (H_2PtePAS), an analog of dihydropteroate (H_2Pte), can be glutaminated by dihydrofolate synthase (DHFS/FolC) to form hydroxy dihydrofolate (H_2PtePAS-Glu), which is an analog of dihydrofolate (H_2Pte-Glu).[6] H_2PtePAS-Glu inhibits dihydrofolate reductase (DfrA) and arrests the growth of *M. tuberculosis*.[7] Consistent with this model, mutations causing the overexpression of RibD, an alternate enzyme with dihydrofolate reductase activity, confer resistance to PAS.[7] Mutations in dihydrofolate synthase (FolC) trigger resistance to this drug.[7,9] In addition, loss-of-function mutations in thymidylate synthase ThyA trigger resistance to PAS through an undefined pathway.[8,10]

Chongqing is a municipal city located in southwestern China with 32 million residents and is a high-incidence area for both TB and MDR-TB in China.[11] Little is known about the molecular characteristics of PAS resistance in *M. tuberculosis* isolates in this region. In this study, we analyzed PAS resistance-related mutations including *ribD, folC*, and *thyA* in clinical isolates of *M. tuberculosis* in Chongqing, as well as the risk factors related to PAS resistance of *M. tuberculosis*.

Materials and methods

Bacteria strains

122 PAS-resistant and 55 PAS-susceptible clinical isolates were obtained from Chongqing Public Health Medical Center, a specialist hospital for TB and other infectious diseases in Chongqing, between April 2014 and January 2018. Patient's information was acquired from their medical records. For each patient, two sputum samples were collected for mycobacterium culture using Löwenstein–Jensen (L-J) medium and the BD Bactec MGIT 960. Positive cultures were examined by Zeihl–Nelson stain, and species identification was performed with a commercial MPB64 monoclonal antibody assay (Genesis, Hangzhou, China).[12] Positive cultures were recultured with L-J for further drug susceptibility testing (DST) and DNA extraction.

Drug susceptibility test

Drug susceptibility test was performed according to previous literature.[13] Concentrations of anti-TB drugs in L-J medium were as follows: streptomycin (STR), 4 μg/mL; rifampin (RIF), 40 μg/mL; isoniazid (INH), 0.2 μg/mL; ethambutol (EMB), 2 μg/mL; levofloxacin (LVX), 2 μg/mL; amikacin (AMK), 30 μg/mL; capromycin (CAP), 40 μg/mL; prothionamide (PTO), 40 μg/mL; and PAS, 1 μg/mL.

PCR amplification and sequencing

Genomic DNA was extracted from freshly cultured bacteria using Hipure mycobacterial DNA kit (Magen, Guangzhou, China) according to the manufacturer's instruction and used as the template for gene amplification. Each PCR mixture was prepared in a volume of 50 μL containing 5 μL PCR buffer, 2 mM $MgSO_4$, 200 μM of each deoxynucleoside triphosphate (dNTP), 2.5 μL of DMSO, 0.2 μM of each primer, 1 μL genomic DNA, and 1 μL KOD-plus DNA polymerase (TOYOBO, Osaka, Japan). The PCR program for amplification was initiated at 98°C for 10 mins followed by 5 cycles of 98°C for 1 min, 57°C for 1 min, and 72°C for 1 min. After that, there were 30 cycles of denaturation at 94°C for 30 s, annealing at different temperatures for 30 s for each gene, extension at 72°C for 30 s, and a final extension at 72°C for 5 mins. The annealing temperatures of the 30 cycles for each gene were as follows: 57°C was for 16 S DNA; 63.2°C was for *thyA*; 64.3°C was for *folC*; and 66.5°C was for *ribD*. 16S DNA was used to eliminate nontuberculosis mycobacteria (NTM) from the isolates. All amplicons were sent to Tsingke Company (Beiing, China) for sequencing. The sequence data were aligned with *thyA, folC,* and *ribD* of H37Rv (NC000962.3) by ClustalW2.[14] The primers used to amplify *thyA, folC, ribD*, and 16S DNA are listed in Table 1.

Statistical analysis

The Pearson Chi-square test was used to evaluate the associations among multiple categorical variables between PAS-susceptible and PAS-resistant groups. All statistical analysis

Table 1 The primer pairs used in this study

Gene	Primer pairs
thyA	F 5′-TGATCTCCCGGAAATGCGCCTGGT-3' R 5′-GGTTTTCGGCATGGCCTCCGTTGTA-3'
folC	F 5′-CGGTCAGCAGTATCAACAGCACGGC-3' R 5′-CGCCGCCTGGAAAAGGAGTTGG-3'
ribD	F 5′-CCGGCAAAAGTCCTGGCACGCCACG-3' R 5′-GTTCTTGGGTGCGGCGAGCGGTGGT-3'
16S DNA	F 5′-AGAGTTTGATYMTGGCTCAG-3' R 5′-TTACCGCGGCKGCTGGCAC-3'

was performed with SPSS 17.0 (IBM Corporation, Armonk, NY, USA). The results were summarized with ORs with 95% CIs. Differences were considered as statistically significant when the *P*-value was <0.05.

Ethics approval

Our study was conducted in accordance with the Declaration of Helsinki. The institutional review board of Chongqing Public Health Medical Center approved this study and waived the requirement for written informed consent. The institutional review board waived the need for informed consent because all patients' data were analyzed in anonymity and no additional informed consent was required.

Results

Demographic characteristics

Among 177 clinical isolates included in this study, 116 (65.5%) were from male patients and 61 (34.5%) were from female patients. The mean age of patients was 40.2 years (range 13–75 years). There were 143 MDR-TB strains including 47 extensively drug-resistant tuberculosis (XDR-TB) strains (defined as MDR-TB strains resistant to at least one of the quinolone and one of the second-line anti-TB injectable drugs).[15] A total of 30 strains were from new cases and 147 strains were from retreated cases. As shown in Table 2, of the 177 isolates, 147 (83.1%) were resistant to STR, 158 (89.3%) to INH, 150 (84.7%) to RIF, 95 (53.7%) to EMB, 111 (62.7%) to LVX, 48 (27.1%) to AMK, 49 (27.7%) to CAP, and 87 (49.2%) to PTO.

Factors associated with PAS resistance

There was no significant difference in the distribution of the PAS-resistant isolates according to gender and age ($P > 0.05$). However, the treatment history was clearly associated with the PAS resistance as the proportion of retreated patients in the PAS-resistant group was significantly higher (OR 95% CI: 2.68 [1.20–5.9], $P<0.05$). Further analysis of other drug-susceptibility data showed that PAS resistance was more likely to be related to the resistance of STR (OR: 9.5, 95% CI: 3.87-23.3; $P<0.01$), INH (OR: 5.98, 95% CI: 2.14–16.76; $P<0.01$), RIF (OR: 5.01, 95% CI: 2.11–11.88; $P<0.01$), EMB (OR: 2.79, 95% CI: 1.44–5.4; $P<0.01$), LVX (OR: 2.56, 95% CI: 1.33–4.93; $P<0.01$), and AMK (OR: 4.29, 95% CI: 1.70–10.83; $P<0.01$), but not correlated with CAP and PTO ($P > 0.05$). In addition, the percentages of MDR (OR: 5.18, 95% CI: 2.35–11.34; $P<0.01$) and XDR (OR: 4.13, 95% CI: 1.64–10.45; $P<0.01$) in PAS-resistant group were significantly higher than those of PAS-susceptible group.

Mutations in three PAS resistance-related genes

Three genes (*folC, thyA,* and *ribD*) from the 177 MTB strains were sequenced. Among the 122 PAS-resistant isolates, 112 (91.8%) strains harbored 30 different mutation types in the three genes and 10 strains had no mutation in these three genes. No mutation was found in those genes in PAS-susceptible strains. As shown in Table 3, 59 strains harbored mutations in *folC*; 39 strains had mutations in *thyA* and 11 strains in *ribD*; 2 strains had mutations in both *folC* and *thyA*; 1 strain had mutations in both *thyA* and *ribD*. Mutations in *folC* were the most commonly found in PAS-resistant isolates (54.5%, 61/112) and 11 mutation types were found at 7 condons in *folC*. The most common mutation sites are residues 40, 43, 49, and 150. We found 18 mutation types at 16 different sites throughout the entire *thyA* gene and residues, 147 and 235, which were the most frequent mutation sites. Three types of mutation were identified in *ribD* including a synonymous mutation, and the dominant mutation is a G/A transition in the 11th nucleotide upstream of the start codon. In general, the most frequent mutations in PAS-resistant strains were I43T in *folC* (35.7, 40/112), followed by R235P in *thyA* (14.3%, 16/112) and $^{-11}$G→A of *ribD* (8.0%, 9/112). The detailed information of all mutations is listed in Table S1.

Discussion

Although PAS has been used to treat TB for decades, its mechanisms of resistance are still not fully understood. Previously, mutations of three genes, *folC, thyA,* and *ribD*, were found to be related to PAS resistance in MTB clinical isolates.[7–10] However, there are few studies reporting on the distribution of mutations in these three genes in clinical PAS-resistant MTB isolates. Furthermore, the molecular characteristics of PAS-resistant MTB in southwestern China are still unclear.

In this study, we found that PAS resistance was significantly associated with resistance to most of the anti-TB drugs (STR, INH, RIF, EMB, LVX, and AMK), as well as the treatment history and clinical types of TB (MDR-TB and XDR-TB). These results are consistent with previous researches on PTO and pyrazinamide (PZA).[16,17] Previous studies have shown that the exposure to antimicrobial agents such as RIF, fluoroquinolone (FQ), and aminoglycosides induces the production of oxygen radicals, which would then cause high-frequency mutagenesis in *M. tuberculosis* resulting in cross-resistance between

Table 2 Risk factor associated with PAS resistance in Chongqing, China

Characteristics	Number (%) of isolates	Number (%) of isolates		OR (95% CI)	*P*-value
		PAS^R n=122	PAS^S n=55		
Gender					
Male	116 (65.5)	76 (62.3)	40 (72.7)	0.62 (0.32–1.24)	0.18
Female	61 (34.5)	46 (37.7)	15 (27.3)	1.0 (Ref)	
Age group					
< 30	49(27.7)	35 (28.7)	14 (25.5)	1.0 (Ref)	
30–59	107 (60.4)	71 (58.2)	36 (65.4)	0.79 (0.38–1.65)	0.91
≥60	21 (11.9)	16 (13.1)	5 (9.1)	1.28 (0.39–4.17)	0.68
Treatment history					
New cases	30 (18.1)	15 (12.3)	15 (27.3)	1.0 (Ref)	
Retreated cases	147 (83.1)	107 (87.7)	40 (72.7)	2.68 (1.20–5.97)	0.014
Resistance					
STR	147 (83.1)	114 (93.4)	33 (60.0)	9.5 (3.87-23.3)	< 0.001
INH	158 (89.3)	116 (95.1)	42 (76.4)	5.98 (2.14–16.76)	< 0.001
RIF	150 (84.7)	112 (91.8)	38 (69.1)	5.01 (2.11–11.88)	< 0.001
EMB	95 (53.7)	75 (61.5)	20(36.4)	2.79 (1.44–5.4)	< 0.01
LVX	111 (62.7)	85 (69.7)	26 (47.3)	2.56 (1.33–4.93)	< 0.01
AMK	48 (27.1)	42 (34.4)	6 (10.9)	4.29 (1.70–10.83)	< 0.01
CAP	49 (27.7)	33 (27.0)	16 (29.1)	0.9 (0.45–1.83)	0.78
PTO	87 (49.2)	62 (50.8)	25 (45.5)	1.24 (0.66–2.35)	0.509
MDR	143 (81.4)	109 (89.3)	34 (61.8)	5.18 (2.35–11.34)	< 0.001
XDR	47 (26.6)	41 (33.6)	6 (10.9)	4.13 (1.64–10.45)	< 0.01

Abbreviations: PAS^R, PAS resistant; PAS^S, PAS susceptible; STR, streptomycin; INH, isoniazid; RIF, rifampin; EMB, ethambutol; LVX, levofloxacin; AMK, amikacin; CAP, capromycin; PTO, prothionamide; MDR, multidrug-resistance; XDR, extensively drug-resistance.

PZA and other drugs.[17] Considering that retreated, MDR and XDR cases required longer durations of anti-TB therapy than new and non-MDR cases, we speculated the mechanism for this cross-resistance between PAS and other anti-TB agents should be the same as that of PZA cross-resistance.

Another finding of our study is that we observed a very high proportion (91.8%, 112/122) of mutations in PAS-resistant MTB isolates, and some mutation hotspots did exist in PAS-resistant TB isolates from Chongqing. So far, there has been limited data about the genotypic characteristics of PAS-resistant MTB. Recently, Zhang X et al examined gene mutations in PAS-resistant MTB from northern China.[18] Compared to the results from northern China (61.1%, 127/208), the mutation rate in PAS-resistant MTB isolates from Chongqing is much higher. In our study, mutations in *folC* can be found in half of the PAS-resistant isolates (50.0%, 61/122), which is much higher than that from northern China (29.3%, 61/208). We also found that mutation hotspots did exist, such as residues E40 (10 isolates), I43 (41 isolates), and S150 (5 isolates) in *folC and* H47 (6 isolates) and R235 (17 isolates) in *thyA*, $^{-11}$G (9 isolates) upstream of *ribD*. Mutations in these loci accounted for 79.1% (88/112) of total mutations. Although I43T is the dominant mutation in *folC* in PAS-resistant MTB from both Chongqing and northern China, it seems to be more common in isolates from Chongqing (64.5%, 40/62 from Chongqing; vs 23.0%, 14/61 from northern China). Previously, E153A was found to be the most frequently identified mutation type in *folC* in PAS-resistant isolates from Hongkong, which is also common in isolates from northern China. However, in this study, we did not find any E150 mutation in PAS-resistant isolates from Chongqing.[18,19]

Three new mutations were found in *folC*, including two single mutations (E40R and H100Y) and a double mutation

Table 3 Features of PAS-resistant isolates in Chongqing, China

	Mutation in drug resistance-related genes						Drug resistance[a]	
Number of isolates	***folC***		***thyA***		***ribD***		**MDR**	**Non-MDR**
1	GAG-GGA	E40G	TTC-TCC	F30S			1	
3	GAG-AAG	E40K					3	
1	GAG-CAG	E40Q					1	
5	GAG-CGG	E40R					4	1
1	ATC-GCT	I43A					1	
40	ATC-ACC	I43T					36	4
1	GAT-GGT, AGC-CGC	D44G, S46R					1	
1	CGG-CAG	R49Q					1	
2	CGG-TGG	R49W					2	
1	CAC-TAC	H100Y	CAA-CGA	Q97R			1	
5	AGC-GGC	S150G					5	
2			ACC-CCC	T22P			2	
1			TTC-TCC	F30S			1	
3			CCG-TCG	P43S			2	1
1			TTC-TCC	F52S				1
1			CAC-CAA	H75Q			1	
1			TGG-TAG	W83stop				1
1			253delA				1	
1			CAA-CGA	Q97R			1	
1			GAC-GGC	D110G			1	
1			TGG-TAG	W133stop			1	
1			TGG-TGA	W133stop			1	
6			CAT-CGT	H147R			4	2
1			GAC-AAC	D169N			1	
1			CAG-TAG	Q191stop			1	
1			CTC-CCC	L220P				1
1			CGG-CTG	R235L	GAG-GAA	E91E	1	
16			CGG-CCG	R235P			15	1
9					−11nt G-A		9	
2					GGC-AGC	G249S	2	
	Total (n=112)						100	12

Notes: [a]non-MDR, referring the strains which were not MDR-TB and susceptible to RIF or INH.

(E44G: S46R). To our best knowledge, this is the first report showing that double mutations exist in *folC* of PAS-resistant TB. Most mutations in *folC* such as E40, I43, R49, S150, and E153 are located in the substrate binding pocket, which leads to reduced efficiency of H_2PtePAS glutamination.[9,19,20] Since E44 and S46 were adjacent to I43 and R49, H100 was located in the Ω-loop just adjacent to the Ser-*cis*-Pro motif in the active site of FolC.[20] These results suggest that the mutations in these residues may also affect the glutamination process.

thyA was the first gene reported to be associated with PAS resistance.[8] Previous reports showed that about a quarter to one-third of the PAS-resistant strains contain *thyA* mutations, and our results (34.4%, 42/122) are consistent with those findings.[18,21] We identified 18 mutation types at 16 different sites throughout the entire *thyA*. Similar to the findings of northern China, most of the mutations are located in the active or dimeric domains.[18] Nevertheless, the most common mutation in *thyA* in our study was R235P, whereas the most frequent mutation in northern China, H75N, was found only in one strain in our study.[18] R235 is located on the outer surface of ThyA (PDB code 4FQS), and it is unreasonable to explain the impact of this mutation on thymidylate synthase activity by proximity of position. Therefore, how the mutation at this site affects thymidylate synthase activity needs further investigation. We observed that $^{-11}$G→A transition was the most common mutation in *ribD*, which is also consistent with the

previous finding in northern China.[18] This mutation leads to the overexpression of *ribD* and finally causes PAS resistance.[7] Interestingly, a G249S mutation was identified in *ribD*. Notwithstanding, further studies are warranted in order to verify the relationship between this mutation and PAS-resistance.

As far as we know, this is the first investigation of phenotypic and molecular characteristics of PAS-resistance in southwestern China. It is well understood that the accuracy of molecular diagnosis of drug-resistant TB depends on understanding the mechanisms of drug resistance and the prevalence of drug-resistant mutations in TB. Thus, our study will expand our understanding of the mechanisms of PAS-resistance, and benefit both the molecular diagnosis of PAS resistant MTB and clinical treatment of PAS-resistant TB patients in south-western China. Nevertheless, there were some obvious limitations in our research. For example, the sample size was small, which limits the representativeness of our results. What is more, all isolates in our study were from a specialist hospital for TB, which may lead to deviations in high TB drug resistance. Therefore, multicenter and larger sample studies are still needed.

Conclusion

PAS drug resistance in southwestern China is mainly caused by mutations in *folC, thyA*, and *ribD*, among which *folC* was the most frequent mutation. Some mutation hotspots exist in the three genes, which accounts for about 80% of total mutations. Our results highlight the possibility of developing molecular diagnostic methods for PAS-resistant TB in the future.

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Disclosure

The authors report no conflicts of interest in this work.

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