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Mutations Found in the *pncA* Gene of *Mycobacterium tuberculosis* in Clinical Pyrazinamide-resistant Isolates from a Local Region of China

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This study was designed to investigate the presence of mutations in the *pncA* gene, minimum inhibitory concentrations and pyrazinamidase activity of pyrazinamide-resistant *Mycobacterium tuberculosis*. In total, 47 *M. tuberculosis* clinical isolates from a local region of China were assayed. Pyrazinamidase activity was measured by pyrazinamide deamination to pyrazinoic acid and ammonia, and a 721 bp region, including the entire *pncA* open-reading frame, 104 bp of the upstream sequence and 59 bp of the downstream sequence, was determined by DNA sequencing of purified polymerase chain reaction products. Of the 47 isolates resistant to pyrazinamide, 44 lost pyrazinamidase activity and had *pncA* mutations that occurred mainly near pyrazinamidase's active or metal ion binding sites; nine of them have not been reported previously. Three pyrazinamide-resistant isolates carried the wild-type *pncA* sequence and retained pyrazinamidase activity. These results show the molecular mechanism of pyrazinamide resistance in China and may also contribute towards the prevention of tuberculosis in China.

KEY WORDS: *Mycobacterium tuberculosis*; *pncA*; Pyrazinamidase; Pyrazinamide

Introduction

Tuberculosis (TB) still remains one of the leading causes of morbidity and mortality in the world, with China having the second highest rate worldwide. The World Health Organization reported that, in 2007, China had a TB incidence of 98 new cases per 100 000 population per year and a mortality rate of 15 cases per 100 000 population per year.[1] One of the major reasons for this high mortality rate is drug resistance, particularly the development of multi-drug resistance. Controlling TB has become more difficult with the emergence of drug-resistant *Mycobacterium tuberculosis*.[2]

Pyrazinamide is an important first-line drug used for the short-course treatment of TB in combination with isoniazid and

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rifampin. It is responsible for killing persistent tubercle bacilli during the initial intensive phase of chemotherapy, allowing the treatment time to be shortened from 9 months to 6 months.[3,4] Pyrazinamide is a prodrug that is converted to bactericidal pyrazinoic acid by pyrazinamidase produced by *M. tuberculosis*. Mutations in the *pncA* gene have been associated with pyrazinamide resistance *in vitro*.[5] Thus, the types and frequencies of mutations in the *pncA* gene of pyrazinamide-resistant clinical isolates from Hefei, Anhui Province, China, were characterized and are reported here.

Materials and methods

MYCOBACTERIAL ISOLATES

Isolates of pyrazinamide-resistant *M. tuberculosis* from Chinese patients with tuberculosis living in Hefei, Anhui Province in China were obtained by the Department of Bacteriology and Immunology, Beijing TB and Thoracic Tumour Research Institute, Beijing, China between May 2006 and December 2007. Each isolate was resistant to 100 µg/ml pyrazinamide when tested by the proportion method on Löwenstein–Jensen medium (pH 5.2). Isolates were stored frozen at –70 °C until required for the study.

PYRAZINAMIDE MICs

Pyrazinamide minimal inhibitory concentrations (MICs) were assayed by a modification of the proportion method on solid medium.[6,7] Pyrazinamide concentrations of 25, 50, 100, 200, 400 and 800 µg/ml were tested in triplicate assays for each concentration. All isolates were tested twice.

PYRAZINAMIDASE ASSAY

Pyrazinamidase activity was assayed by a modification of the method developed by Wayne.[7,8] Two tubes (10 ml/tube) of 7H10 medium containing 1 mg pyrazinamide and 20 mg sodium pyruvate were inoculated with each strain and incubated at 37 °C. One tube was examined after 7 days of incubation and the other was examined after 14 days. After incubation, 1.0 ml of 1% ferrous ammonium sulphate was added to each tube and the tubes were held at 4 °C for 4 h. A strain was considered positive for pyrazinamidase if a pink band was observed in the upper part of the agar butt. Each strain was compared with the positive (pyrazinamide-sensitive *M. tuberculosis* strain H37rv) and negative (*M. bovis* BCG Pasteur) controls. All the tubes were examined independently by three individuals.

GENOMIC DNA PREPARATION AND SEQUENCING

Chromosomal DNA was extracted from isolates cultured for 30 days at 37 °C in Löwenstein–Jensen medium as described by Morlock *et al.*[7] A 721 bp region, including the entire *pncA* open-reading frame, 104 bp of the upstream sequence and 59 bp of the downstream sequence, was amplified by polymerase chain reaction (PCR) with forward primer *pncA*-10 (5′-GCTGGTCATG TTCGCGATCG-3′) and reverse primer *pncA*-11 (5′-GCTTTGCGGCGAGCGCTCCA-3′).[7] Each 50 µl reaction mixture contained 1.0 ng of template DNA, 200 µM of each deoxynucleotide triphosphate, 0.1 µM of each PCR primer and 1 U of Pyrobest™ DNA polymerase (Takara Biotechnology, Dalian, China). The PCR amplifications were carried out in a MyCycler™ thermocycler (Bio-Rad Laboratories, Foster City, CA, USA) with the following parameters: 94 °C for 5 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 64 °C and 1.5 min at 72 °C, and a final extension for 10 min at 72 °C. The PCR products were purified with a gel extraction kit (E.Z.N.A.®, Omega Bio-Tek, GA, USA) according to the manufacturer's instructions.

The purified PCR products were sequenced using rhodamine DyeDeoxy Terminator chemistry according to the manufacturer's protocol (PerkinElmer, Foster City, CA, USA) with primer *pncA*-11. The sequence data were analysed using Sequence Navigator software, version 1.0.1 (PerkinElmer) and the results were compared with the *pncA* published sequence (GenBank Accession No. U59967).

Results

A total of 47 pyrazinamide-resistant *M. tuberculosis* clinical isolates (35 multi-drug-resistant isolates and 12 isolates resistant only to pyrazinamide) that were negative for pyrazinamidase activity and, therefore, resistant to pyrazinamide (MIC ≥ 200 µg/ml), were chosen for the sequence analysis. Of these, 44 (93.6%) exhibited 29 different changes in the *pncA* gene compared with the wild-type (H37rv) sequence and lacked pyrazinamidase activity (Table 1). The mutations in *pncA* included 22 different point mutations causing an amino acid

TABLE 1:
The *pncA* nucleotide and amino acid changes in pyrazinamide-resistant *Mycobacterium tuberculosis* clinical isolates from China

No. of isolates	Nucleotide change	Amino acid change
1	T➔C at position 11	Leu4➔Ser
2	A➔C at position 35	Asp12➔Ala
2	T➔C at position 40	Cys14➔Arg
1	G➔A at position 46	Gly16➔Ser[a]
1	T➔C at position 80	Leu27➔Pro
1	C➔G at position 108	Ala36➔Ala[a]
1	A➔G at position 142	Lys48➔Arg[a]
1	A➔C at position 152	His51➔Pro
1	C➔T at position 161	Pro54➔Leu
1	T➔C at position 173	Phe58➔Ser
1	C➔A at position 184	Pro62➔Thr
1	T➔C at position 199	Ser67➔Pro
2	T➔C at position 202	Trp68➔Arg
1	C➔A at position 216	Cys72➔stop
3	A➔C at position 226	Thr76➔Pro
1	T➔C at position 389	Val130➔Ala
1	A➔C at position 410	His137➔Pro
2	T➔C at position 416	Val139➔Ala
1	A➔C at position 422	Gln141➔Pro
1	A➔G at position 424	Thr142➔Ala
2	C➔T at position 458	Thr153➔Ile[a]
1	A➔C at position 499	Thr167➔Pro[a]
2	G➔T at position 538	Val180➔Phe
7	A➔G at position –11	Mutation in promoter
1	G deletion at position 61	Frameshift[a]
1	44-bp deletion at position 158–201	Frameshift[a]
1	10-bp deletion at position 378–387	Frameshift[a]
1	G insertion at position 391–392	Frameshift[a]
2	GG insertion at position 391–392	Frameshift

[a]New mutation not reported in previous studies.[3,5 – 25]

substitution among 33 isolates, one stop codon mutation found in one isolate, frameshift mutations caused by three deletions (158–201 deletion of 44 bp, 378–387 deletion of 10 bp, and 61 deletion G) in three isolates and by two insertions (391–392 insertion GG and 391 insertion G) in three isolates, and one promoter mutation (–11 A→G) found in seven isolates.

The frequency of mutations in regions along the *pncA* gene is shown in Table 2. In total, 60.8% of the mutations were located in the regions surrounding pyrazinamidase's active or metal ion binding sites, whilst 39.2% occurred in other regions.

Discussion

Since 93.6% of the *M. tuberculosis* clinical isolates exhibited 29 different changes in the *pncA* gene compared with the wild-type (H37rv) sequence and lacked pyrazinamidase activity, the results suggest that *pncA* mutation is a major contributor to pyrazinamide resistance in this region.

Nine of the mutations in *pncA* have not previously been reported, including five point mutations causing amino acid substitutions and four frameshift mutations causing premature terminations (except 391–392 insertion GG).[5,7 – 28] In one isolate with a negative pyrazinamidase test, the only change found was a synonymous mutation, alanine (Ala) 36→Ala, resulting in a GCC → GCG codon variation. As there is codon preference in bacterial protein synthesis, it may be that the codon prefers GCC to GCG in *M. tuberculosis* Ala synthesis, leading to termination or reduction of protein synthesis.

Although previous studies have detailed several mutations leading to pyrazinamide resistance scattered along the *pncA* gene, our group in a previous experiment discovered a concentration of *pncA* mutations, at amino acid residues 3 – 12, 46 – 62, 68 – 85, 94 – 103 and 132 – 142, that are areas near pyrazinamidase's active (C138, D8 and K96) and metal ion binding sites (D49, H51, H57 and H71) (Table 2).[29] Approximately 70% of mutations have previously been reported as located in the regions surrounding the pyrazinamidase's active and metal ion binding sites,[5,7 – 28] but only 60.8% of mutations occurred in these regions in the present study, while 39.2% occurred in other regions. The fact that these strains were isolated in distinct regions in China suggests that environmental factors may influence the distribution of mutations along the *pncA* gene.

Different mechanisms of pyrazinamide

TABLE 2:
Mutation frequency in regions along the *pncA* gene detected in previous studies and in the present study

AA sites	AA/total AA (%)	Previous studies mutation frequency (%)	Current study mutation frequency (%)
3 – 12	5.4	10.4[8 – 19,21]	8.7
46 – 62	9.1	14.4[5,8 – 24]	21.7
68 – 85	9.7	13.7[5,8 – 24]	13.0
94 – 103	5.4	10.4[5,8 – 23,25]	N/A
132 – 142	5.9	21.7[5,7 – 23,25,26]	17.4
Total	35.5	70.6	60.8

AA, amino acid; N/A, data not available.

resistance may exist. The *pncA* mutations in the 44 clinical *M. tuberculosis* isolates in the present study that lacked pyrazinamidase activity occurred mainly near pyrazinamidase's active or metal ion binding sites, which means they should be highly related to the loss of enzyme. Among the mutations, nine had not been reported previously. Three pyrazinamide-resistant strains that did not have any mutations in the *pncA* gene were identified in the present analysis, suggesting there are other *M. tuberculosis* genomic regions involved in resistance to pyrazinamide.

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Conflicts of interest

The authors had no conflicts of interest to declare in relation to this article.

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