Analysis of mutational characteristics of the drug-resistant gene *katG* in multi-drug resistant *Mycobacterium tuberculosis* L-form among patients with pneumoconiosis complicated with tuberculosis

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Abstract. The aim of the present study was to investigate the mutational characteristics of drug-resistant genetic mutations in the *katG* gene to isoniazid (INH) in multi-drug resistant *Mycobacterium tuberculosis* (MDR-TB) L-form among patients with pneumoconiosis complicated with tuberculosis (TB), in order to reduce the occurrence of drug resistance in patients, and gain further insight into the mechanisms underlying drug resistance in MDR-TB L-form. A total of 114 clinically isolated strains of *Mycobacterium tuberculosis* (MTB)-forms were collected. The MDR-TB L-forms were identified using a conventional antimicrobial susceptibility test (AST). The DNA genomes were extracted, the target genes were amplified by polymerase chain reaction technology and the hotspot mutational regions in the *katG* gene were analyzed by direct sequencing. The results of AST analysis demonstrated that there were 31 strains of MDR-TB L-forms in 114 clinical isolates. The mutation rate of *katG* was 61.29% (19/31) in INH-resistant isolates, mainly concentrated in codon 315 (Ser315Thr, 48.39% and Ser315Asn, 9.68%) and 431 (Ala431Val, 3.23%). Base substitutions were identified, however, no multisite mutations were found. No mutations in *katG* were identified in 10 INH-sensitive strains that were randomly selected. INH-resistance was more severe in MDR-TB L-form isolates among patients with pneumoconiosis complicated with TB. The substitution of highly conserved amino acids encoded by the *katG* gene resulted in the molecular mechanisms responsible for INH resistance in MDR-TB L-form isolates. It was also verified that the *katG* gene was in diversiform. The *katG* Ser315Thr mutation is one of the main causes of resistance to INH in MDR-TB L-form isolates.

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Key words: pneumoconiosis, tuberculosis, multi-drug resistant *mycobacterium tuberculosis* L-form, drug resistance, *katG*, sequence analysis

Introduction

Tuberculosis (TB) is a chronic infectious disease caused by the bacterium *Mycobacterium tuberculosis* and it remains a significant public health risk worldwide. The pestilence of TB in the Cosmopolitan population has been eased with the introduction of anti-TB and anti-HIV drugs during the 1980's (1,2). Pneumoconiosis is an important occupational disease in employees of the Huainan coal mine. Once pneumoconiosis is associated with TB, not only is it accelerated, but it also worsens. The prognosis of patients with the disease is usually poor and the curative effect of current treatments is not as good as expected. At present, the therapeutic regimen includes curing pneumoconiosis and TB; however, anti-TB drugs are key for curing the disease since pneumoconiosis lacks a radically curative drug. As the first-elected anti-TB drug, the clinical therapeutic efficacy of isoniazid (INH) has markedly decreased due to the emergence of multi-drug-resistant and mycobacterial cell wall-deficient strains. At present, MDR-TB is a major drawback for the complete elimination of TB (3). According to the statistics, mutations in the *katG* gene in MDR-TB account for 40-90% of INH-resistant strains (4), however, no studies investigating the resistance of the L-form have been conducted. The present study applied a DNA sequence analysis technique for analyzing the mutational characteristics of the *katG* gene in MDR-TB L-form isolates in order to gain further information on the mechanisms underlying drug resistance in MDR-TB.

Materials and methods

Experimental subjects. The subjects included male patients aged 42-71 years old (mean age, 55.15±8.75 years) diagnosed with stage II and III pneumoconiosis complicated with TB in the active stage (n=114), treated in The Affiliated Hospital of Anhui University of Science and Technology (Huainan,

China) between May 2011 and May 2012. All the subjects were instructed to spit phlegm from the bottom of the trachea into a sterile wide-mouthed bottle following gargling several times. The strain used for quality control was $H_{37}Rv$, which was provided by the National Institute for the Control of Pharmaceutical and Biological products (Beijing, China). Patient consent was obtained from all patients. Approval was obtained from the Ethics Committee of the School of Medicine, Anhui University of Science and Technology (Huainan, China).

Experimental methods

MDR-TB L-form identification. In accordance with the book of the Chinese Medical Laboratory, an indirect absolute concentration method was adopted (1,5). INH and rifampin (RFP), used to detect drug sensitivity of MDR-TB L-forms, were purchased from Becton-Dickinson (Franklin Lakes, NJ, USA). Firstly, the medicine (INH and RFP) was added to 92-3 TB-L liquid culture medium (Bengbu Medical College, Bengbu, China), respectively, to prepare the antimicrobial susceptibility medium. The concentrations used were 10 μg/ml (high concentration) and 1 μg/ml (low concentration) for INH, and 250 μg/ml (high concentration) and 50 μg/ml (low concentration) for RFP. Under bacteria-free conditions, 0.1 ml of specimen was removed and inoculated to the medium. Simultaneously, the blank control used medium without medicine and 0.1 ml of specimen was added. Subsequently, the medium was mixed thoroughly using a dropper and placed at 37˚C for 3 weeks. The medium was observed three days later and three times every week for 4 weeks to determine whether or not there was growth on the surface or bottom of the medium. If there was growth, a deposit was obtained to produce a smear, and was identified by intensified Kinyoun acid fast staining. All the specimens were resistant to INH and RFP at low concentrations, and the specimens resistant to high concentrations were identified as the MDR-TB L-form.

DNA extraction. The sputum specimens of patients were inactivated with autoclave. Genomic DNA was extracted using DNA extraction kits (Takara Biotech, Dalian, China). DNA lysate (200 μl) was added to the inactivated specimen, following a water bath at 55˚C for 1-3 h and at 95˚C for 5 min. The lysate was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1). DNA was precipitated by adding ammonium acetate. Two volumes of pure ethanol were added, followed by incubation at -20˚C overnight. DNA was pelleted by centrifugation at 12,000 x g for 15 min, washed with 70% ethanol, air-dried and dissolved in the TE buffer (6).

Primer synthesis. The primers were synthesized by Sangon Biotech Co. (Shanghai, China) according to the MDR-TB conserved genomic DNA sequence (NM_X68081). The primer sequences for *katG*, used in the present study were as follows: Forward: 5'-CGCGATGAGCGTTACAG-3' and reverse: 5'-CGTCCTTGGCGGTGTATTG-3, with a product size of 458 bp.

Polymerase chain reaction (PCR) and sequence analysis. The total PCR reaction volume was 50 μl and included 5.0 μl of 10X buffer (15 mmol/l $MgCl_2$), 4.0 μl dNTP (200 μmol/l), 1.0 μl of each primer (25 pmol/μl), 0.5 μl Taq DNA polymerase (5 U/μl) and 4 μl of the DNA template. Deionized water was added to produce a total volume of 50 μl. The reaction conditions were: 95˚C for 5 min of denaturation, 94˚C for 30 sec, 55˚C for 90 sec and 72˚C for 30 sec, for 33 cycles in total, followed by maintenance at 72˚C for 10 min. The amplified product (5 μl) was separated by electrophoresis with 2% agarose gel electrophoresis (containing 0.5 mg/ml ethidium bromide), then images were captured and analyzed using the Image Master Totallab software (Nonlinear Dynamics Ltd., Durham, NC, USA). A 458 bp specific band indicated that a mutation in *katG* existed, otherwise it was negative. The DNA sequences of the PCR products were detected using an ABI PRISM 7700 sequencer (Takara Biotech).

Statistical analysis. Statistical analyses were performed using SPSS 12.0 software (SPSS, Inc., Chicago, IL, USA).

Results

Antimicrobial susceptibility test (AST) analysis. In 114 cases of MTB L-form isolated from sputum samples, 31 cases of MDR-TB L-form isolates were detected, and the resistance rate was 27.19% (31/114). In total, 42 strains were detected to tolerate INH, including 20 strains with high-level resistance and 42 strains with low-level resistance. In addition, 101 strains were detected to tolerate RFP, including 53 strains with high-level resistance and 101 strains with low-level resistance (Table I).

PCR analysis. In 31 cases of MDR-TB L-form strains isolated from sputum samples, 19 INH-resistant strains and 12 INH-sensitive strains were identified (Fig. 1).

Sequence analysis of DNA of the MDR-TB L-form. In total, 19 mutational strains of the *katG* gene were identified in 31 INH-resistant strains. The mutation rate was 61.29% (19/31; Table II) and mutations were mainly concentrated in codon 315 (58.06%; 18/31) and 431 (3.23%; 1/31). Base substitutions were detected, however, no multisite mutations were identified (Figs. 2-4). The former included 15 strains of Ser→Thr (AGC→ACC; 48.39%) and 3 strains of Ser→Asn (AGC→AAC; 9.68%), and the latter was chiefly Ala→Val (GCG→GTG). No mutation in *katG* was identified in 10 randomly selected INH-sensitive strains.

Discussion

The World Health Organization and the International Union Against Tuberculosis and Lung Disease identified that MDR-TB refers to at least two types of drug resistance, including resistance to RFP and INH (2). The emergence of resistant *Mycobacterium tuberculosis*, particularly the generation and propagation of MDR-TB, is an important cause of the high global TB incidence. Compared with other types of TB, MDR-TB is more severe and leads to a higher incidence of mortality (7). Due to a deficiency in the cell wall of bacteria, the biological characteristics and drug sensitivity of the MDR-TB L-form is able to be altered. Several previous studies

Table I. Results of drug resistance using the antimicrobial susceptibility test analysis in 114 cases of *Mycobacterium tuberculosis* L-forms.

	Drug concentration (μg/ml) and results				
	INH		RFP		
Group	10	1	250	50	Total (no. of cases)
1	+	+	-	-	4
2	-	-	-	+	39
3	-	-	+	+	31
4	+	+	+	+	10
5	-	+	+	+	12
6	-	+	-	-	7
7	-	+	-	+	3
8	+	+	-	+	6
9	-	-	-	-	2
Total	20	42	53	101	114

+, resistant; -, susceptible. INH, isoniazid; RFP, rifampin.

Table II. Mutational characteristics of the *katG* gene in 31 clinical drug-resistant strains of multi-drug resistant *Mycobacterium tuberculosis* L-form.

Strain (no. of cases)	Location of amino acid	Codon change	Amino acid change	Percentage (%)
15	315	AGC→ACC	Ser→Thr	48.39
3	315	AGC→AAC	Ser→Asn	9.68
1	431	GCG→GTG	Ala→Val	3.23
12	-	-	-	38.17

-, no mutation in *katG*.

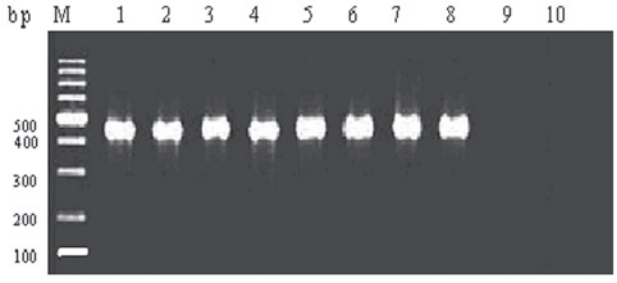

Figure 1. Detection of amplification products of *katG* by polymerase chain reaction in clinical sputum specimens. Lane M, DL500 marker (500, 400, 300, 200 and 100 bp); lane 1, quality control strain ($H_{37}Rv$); lanes 2-8, clinical isoniazid-resistant strains with a mutation in *katG*; lane 9, clinical isoniazid-sensitive strain to *katG*; lane 10, negative control.

have demonstrated that common clinical therapeutic concentrations of RFP, INH and streptomycin, used at the same time in order to inhibit or kill MDR-TB, are also able to induce the formation of MTB L-form bacteria, causing difficulty in the diagnosis and treatment of TB (8,9). Therefore, there is an urgent requirement to investigate the drug resistance mechanisms underlying the MDR-TB L-form to aid in the development of anti-TB drugs and novel diagnostic methods.

As the main anti-TB drug, INH is the basis for a variety of drug and chemotherapy combined treatments for TB. The molecular mechanisms underlying the resistance of MDR-TB to INH was associated with the *katG* gene mutation that encodes catalase-peroxidase. INH is a hydrazine chemical synthetic drug, which is able to be oxidized to isonicotinic acid by the catalase-peroxidase encoded by the *katG* gene that participates in the synthesis of coenzyme I (NAD) to inhibit the biosynthesis of mycolic acid of the cell wall in *Mycobacterium tuberculosis,* so as to damage the MDR-TB's barricade of resisting antioxygen and invasion. Due to deletion or mutation in the *katG* gene, resistance is able to be generated as the enzymatic activity is lost or degraded, thus, inhibiting the activation of INH (10-13).

The Mycobacterium tuberculosis L-form was also termed the mycobacterium cell wall-deficient form. In 1960, Mattmand (14) examined the biological characteristics in detail and revealed that alterations in the biological characteristics,

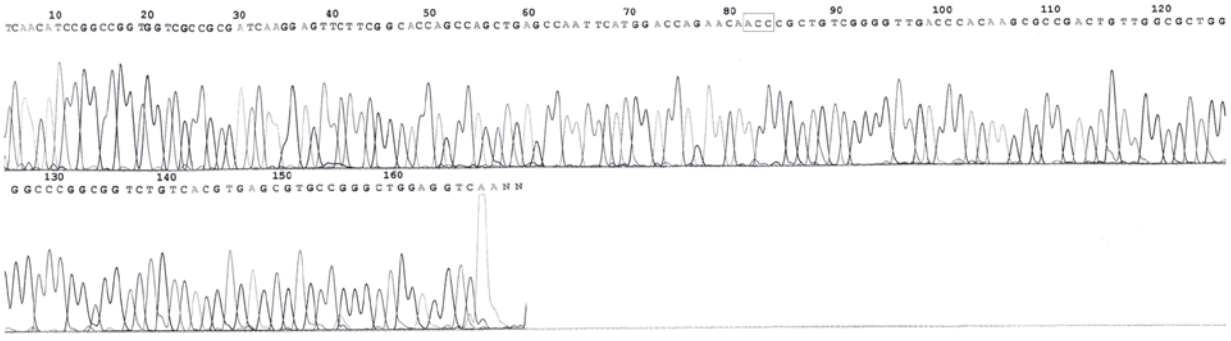

Figure 2. Sequence analysis of clinically isolated DNA of the *katG* gene in multi-drug resistant *Mycobacterium tuberculosis* L-form: codon 315 AGC→ACC.

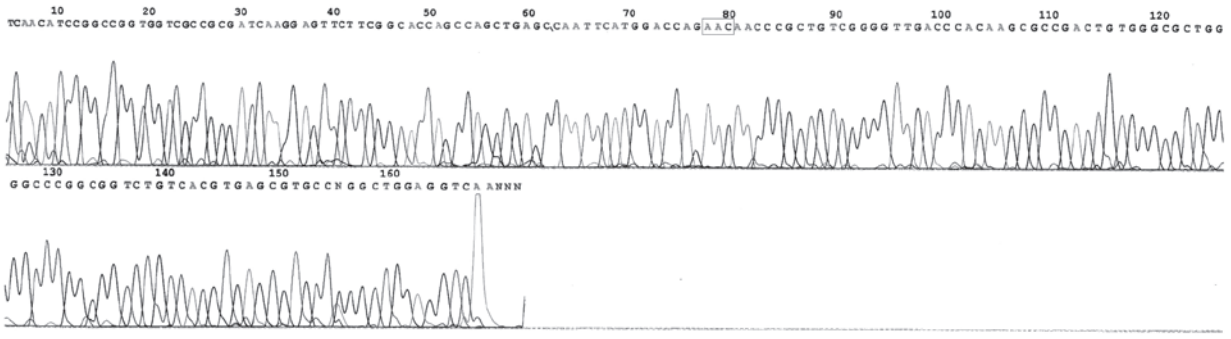

Figure 3. Sequence analysis of clinically isolated DNA of the *katG* gene in multi-drug resistant *Mycobacterium tuberculosis* L-form: codon 315 AGC→AAC.

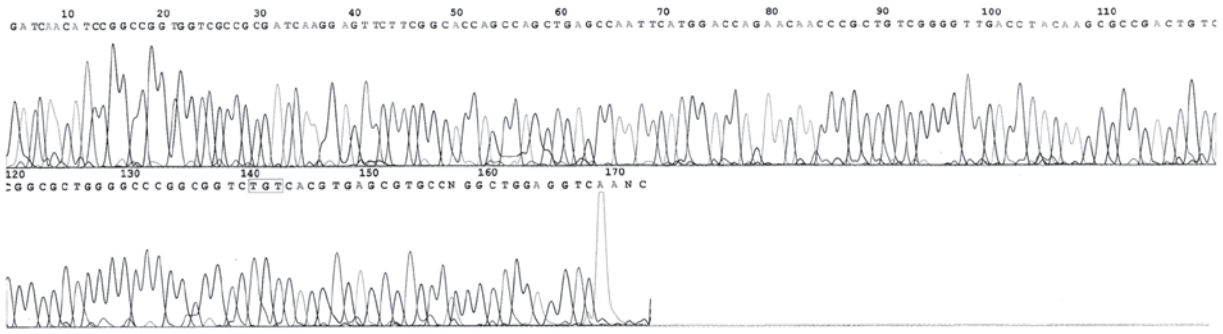

Figure 4. Sequence analysis of clinically isolated DNA of the *katG* gene in multi-drug resistant *Mycobacterium tuberculosis* L-form: codon 431 GCG→GTG.

drug sensitivity and DNA of the L-form were owed to the partial or complete absence of the cell wall. The L-form is a type of mutation. Several mechanisms are able to induce the occurrence of this phenomenon, for instance, chemotherapeutics, lysozymes and bacteriophages. The *Mycobacterium tuberculosis* L-form possesses pathogenicity and previously caused chronic transformation of the disease process, a worse prognosis and lacked typical tubercles. This has led to difficulty in diagnosing and treating TB (15).

In the present study, 31 cases of MDR-TB L-form isolates were detected by AST analysis in 114 cases of MTB L-forms isolated from sputum samples, and the resistance rate was 27.19% (31/114). The present study demonstrated that the situation of multidrug resistance is currently severe in MDR-TB L-form isolates among patients with pneumoconiosis complicated with TB in the Huainan coal mine, and that the commonly used concentrations of anti-TB drugs in the clinic are less effective.

The results of PCR and gene sequence analysis demonstrated that there were 19 mutational strains of *katG* in 31 INH-resistant strains, the mutation rate was 61.29% (19/31), mainly concentrated in codon 315 (Ser315Thr, AGC→ACC, 48.39%; Ser315Asn, AGC→AAC, 9.68%) and 431 (Ala431Val, GCG→GTG, 3.23%), and involved base substitutions. The results indicated that point mutations in *katG* of MDR-TB L-forms concentrated in codon 315 (94.74%; 18/19), were greater than the mutation rate of bacteric MDR-TB, which was reported to be 50-60% in previous studies (16,17).

Furthermore, the present study also demonstrated that *katG* mutations in 12 INH-resistant isolates (38.17%; 12/31) were not detected. This verified that other mechanisms leading to INH resistance in the MDR-TB L-form exist. In

addition, no point mutations, multisite mutations, large fragment deletions and insertion mutations were identified at codons 327, 144 and 143 (4). The probable cause may be associated with novel characteristics of mutations of the MDR-TB L-form and the effects of sampling error, thus, it must be verified using larger sample sizes.

In conclusion, sequence analysis of DNA is the most reliable method for detecting gene mutations, not only can it be used for mutational screening, but it is also able to determine the mutation site. The present study investigated the mutational characteristics of the drug-resistant gene of *katG* in MDR-TB L-form among patients with pneumoconiosis complicated with TB, and provided an experimental basis for the clinical diagnosis and treatment of the disease.

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