180

ELSEVIER

Available online at www.sciencedirect.com

SCIENCE DIRECT

JNMU

Journal of Nanjing Medical University, 2007, 21(3): 180-184

www.elsevier.com/locate/jnmu

Research Paper

Detecting drug resistant genetic mutation among pneumoconiosis patients complicated with tuberculosis in *Mycobacterium tuberculosis* L-forms application of PCR-SSCP technique in Huainan mining district☆

Jun Lu[a,*], Shan Jiang[b], Song Ye[a], Chaopin Li[c]

[a]*Medical College, Anhui University of Science & Technology, Huainan 232001, China*

[b]*Department of Mining Engineering, Huainan Vocational & Technical College, Huainan 232001, China*

[c]*Wannan Medical College, Wuhu 241001, China*

Received 23 January 207

Abstract

Objective: To study the relationship between drug resistant genetic mutation and drug resistance in *Mycobacterium tuberculosis* L-form, discuss the internal relationship between drug resistances and drug-resistant related genes and explore the value of PCR-SSCP to clinical application. **Methods:** A total of 52 clinically isolated strains of tuberculosis L-form were collected among 97 pneumoconiosis patients complicated with tuberculosis. The gene mutations of katG, rpoB and rpsL were detected by PCR-SSCP, and the results were compared with those analyzed by traditional antimicrobial susceptibility test(AST). **Results:** The gene mutation rates of katG, rpoB and rpsL by PCR-SSCP were respectively 57.70%(30/52), 65.38%(32/52) and 40.38%(21/52). The rate of reversion was 78.85%(41/52) and the result of drug-resistant genes was invariable. The results of AST showed that there were 40 (76.92%) multi-drug resistant strains in 52 clinically isolated strains. The number for three-drug resistant strain was 21 (40.38%) and that of two-drug resistant was 19(36.54%), but only 12(23.08%) strains were one drug resistant. The rate of total drug-resistance was 100%, but there were 15 strains of allied mutation of three genes, 16 of two mutations and 6 of only one by PCR-SSCP. The coincidences were respectively 71.43%, 84.12% and 50.00%. Then there was no significant difference between the allied mutations of multi-drug resistant gene and the mutations of only one drug resistant gene ($P > 0.05$). **Conclusion:** PCR-SSCP technique has a higher sensibility and specificity to detect the genes of katG, rpoB and rpsL in tuberculosis L-form among pneumoconiosis complicated with tuberculosis, and the detecting rate of two drug resistant strains and three drug resistant strains was higher. The combined application of PCR-SSCP and AST has advantages at earlier diagnosis and guidance of clinical medications.

Keywords: tuberculosis; *Mycobacterium tuberculosis* L-form; drug-resistance; katG; rpoB; rpsL; polymerase chain reaction and single-strand conformation polymorphism (PCR-SSCP)

INTRODUCTION

Tuberculosis is a chronic infectious disease that had previously been considered under control. Cosmopolitan tuberculosis pestilence has quickly turned back with misuse of antituberculosis drugs, and infection of HIV since the 1980s[1]. As first-elected antituberculosis drugs, the clinical therapeutic efficacies of Isoniazid (INH), Rifampicin (RFP), and Streptomycin (SM), have severely degraded with appearances of drug resistant strains and L-form mutation of *Mycobacterium tuberculosis*. Nowadays, drug resistance has become a drawback to eliminate tuberculosis for mankind [2]. In this study, we compared PCR-SSCP technique with AST method, and explored the value of clinical application of PCR-SSCP in rapid diagnosis of the gene mutations of katG, rpoB and rpsL in *tuberculosis* L-forms.

MATERIALS AND METHODS

Sputum specimen

☆This work was supported by the Youth Natural Science Foundation of Anhui University of Science & Technology(200537)

*Corresponder anthor.

E-mail address: cfdxlujun@126.com

52 specimens of tuberculosis L-forms were selected from pneumoconiosis patients complicated with tuberculosis, treated in a coal miner sanitarium from the Huainan mining industry group in Huainan, Anhui, between 2004 and 2005. All the patients were male only and aged 55 to 72 years, with the average age of 61.5. All subjects were instructed to spit phlegm coming from the bottom of trachea into sterile wide mouthed bottle after their gargling for several times. The strain of quality control is H37Rv provided by the Center of Biological Product of Department of Health.

Reagents

katG, rpoB and rpsL used to detect drug sensitivity of tuberculosis L-forms were purchased from BD in USA. Liquid mediums including tuberculosis L-form(92-3 TB-L) and general bacteric tuberculosis (92-3 TB) were provided by the Institute of Microorganism of Bengbu Medical College.

Contrivance of primer

Primers were designed by Zhongya Biological Genes institute in Shanghai according to *Mycobacterium tuberculosis* conserved genomic DNA sequence (Genbank IS6110). The primer sequences of katG were: upstream 5′-CGC GAT GAG CGT TAC AG-3′, downstream 5′-CGT CCT TGG CGG TGT ATT G-3′. The size of amplification fragment was 458 bp. The primer sequences of rpoB were: up stream 5′-GAT CAA GAG TCA GAC GGT TTC-3′, downstream 5′-ACG GTG TTG TCC TTC TCC AG-3′. The size of amplification fragment was 365 bp. The primer sequences of rpsL were: upstream 5′-ACA CCA CCA CTC CGA AGA AG-3′, downstream 5′-TGC GTA TCC AGC GAA CCG-3′. The size of amplification fragment was 201 bp.

Antimicrobial susceptibility test (AST)

Absolute concentration indirect AST method [3,4] was adopted in the experiment. Briefly, INH, RFP and SM were respectively added to liquid culture medium(92-3 TB-L and 92-3 TB) to prepare antimicrobial susceptibility medium. The concentration of INH was 10 μg/ml or 1 μg/ml,RFP was 250 μg/ml or 50 μg/ml, and SM was 100 μg/ml or 10 μg/ml. Under bacteria-free circumstances,0.1 ml of specimen was taken out, inoculated to the medium; the blank medium was used as control. After being thoroughly mixed with bacteria by using dropper, the medium was placed at 37℃ for 1~3 weeks. Deposition was prepared to make smear on day 3 and 3 times a week for 4 weeks and identified by IK (intensified Kinyoun) acid fast staining.

Reversion test[5]

According to the book of detecting method of *Mycobacterium tuberculosis* L-form, a 0.1 ml specimen of *Mycobacterium tuberculosis* L-form was taken out to 92-3 TB liquid culture medium, then thoroughly mixed and placed 1-3 weeks at 37℃. After 3 days, we observed the growth phenomenon 3 times every week for 4 weeks.

Polymerase chain reaction and single-strand conformation polymorphism (PCR-SSCP)

10 μl of amplified PCR products of clinical separated strains and type strain, was added to 10 μl formamide to make 1∶1 diluted solution, and then denatured for 5 min at 95℃, ice bath for 2 min, and added to 100 g/L un-denaturing polyacrylamide gel. At 15℃, electrophoresis was done for 10 min on 400 V,and 12 h on 130 V. Lastly gelatin of Wharton was taken out to dye by argentation staining,and the results were representative photos, and interpretations taken from them[6].

Result judgment

After degeneration and electrophoresis, it was judged as positive if the amplified products formed 2 single strands and showed differential migration on gel compared to standard H37Rv strain.

Statistical analysis

The data were analyzed by Chi-square test. $P < 0.05$ was considered as significant difference.

RESULTS

Results of AST

In 52 cases of *Mycobacterium tuberculosis* L-forms isolated from sputum samples, the positive rates of katG, rpoB and rpsL were respectively 76.92%(40/52), 90.38%(47/52) and 50.00% (26/52). There were 40 (76.92%) multi-drug resistant strains including 21 of three-drug resistant strains (40.38%), 19 of two-drug resistant(36.54%) and 12 of only one-rug resistant (23.08%). The rate of total drug-resistance was 100%. (***Tab 1***)

Results of PCR-SSCP

The gene mutation rate of katG, rpoB and rpsL by PCR-SSCP were respectively 57.70% (30/52), 65.38% (32/52) and 40.38% (21/52). The rate of reversion was 78.85% (41/52) and the result of drug-resistant genes was invariable. There were 15 strains of allied mutation of three genes, 16 of two mutations and 6 of only one by PCR-SSCP. (***Tab 2***)

Tab 1 **Results of the drug resistant by AST in 52 cases of *Mycobacterium tuberculosis* L-forms**

Group	Drug concentration (μg /ml) and results						Total (Cases)
	INH		RFP		SM		
	10	1	250	50	250	50	
1	+	+	−	−	−	−	4
2	−	−	−	+	−	−	1
3	−	−	+	+	−	−	7
4	+	+	+	+	−	−	5
5	−	+	+	+	−	−	9
6	−	−	+	+	+	+	1
7	−	+	−	−	−	+	1
8	−	−	−	+	−	+	3
9	−	+	−	+	−	+	6
10	+	+	+	+	−	+	11
11	+	+	−	+	−	+	4
Total	24	40	33	47	1	26	52

+ resistant; − susceptible.

Tab 2 **Compared AST with PCR-SSCP analysis results of the drug resistant genes in 52 cases of *Mycobacterium tuberculosis* L-forms**

AST	Drug-resistant strain (Cases)	Mutation gene(strains)							Non-mutation gene (strains)
		G	B	L	G+B	G+L	B+L	G+B+L	
INH	4	3	0	0	0	0	0	0	1
RFP	8	0	3	0	0	0	0	0	5
SM	0	0	0	0	0	0	0	0	0
INH+RFP	14	1	1	0	8	0	0	0	4
INH+SM	1	0	0	1	0	0	0	0	0
RFP+SM	4	0	1	0	0	0	1	0	2
INH+RFP+SM	21	1	0	1	1	1	2	15	0
Sensitive strains	0	0	0	0	0	0	0	0	0
合 计	52	5	5	2	9	1	3	15	12

G: katG; B: rpoB; L: rpsL.

Relationship between the mutation gene and drug resistance

From the 52 there were 15(28.84%)strains of allied mutation of three genes, 16 (30.77%) of two mutations and 6 (11.54%) of only one by PCR-SSCP. The coincidences were respectively 71.43%, 84.12% and 50.00%, then there was no significant difference between the allied mutations of multi-drug resistant gene and the mutations of only one drug resistant gene($P > 0.05$).

DISCUSSION

Domestic and overseas scholars generally consider that generation of drug resistance in *Mycobacterium tuberculosis* is related to the mutations of drug-resistant related genes [7-9]. The molecular mechanism of *Mycobacterium tuberculosis* to anti-INH was related with katG mutation that encodes for catalase-peroxidase. The gene product can oxidize INH to isonicotinic acid (INA). The participated synthesis of NAD serves to inhibit the biosynthesis of mycolic acid of cell wall, so as to damage the *Mycobacterium tuberculosis's* barricade of resisting antioxygen and invasion. With deletion or mutation of katG, the resistance can generate because the enzymatic activity is lost or degraded to hamper activation of INH [7,10,11]. Molecular mechanism of *Mycobacterium tuberculosis* resistance against RFP was related to the gene mutation of rpoB, which was encoded by β subunit of RNA polymerase. RFP inhibits transcription course of the polymerase and causes the death of infected cells [8]. There are several theoretical hypotheses of the reason of drug resistance of *M. tuberculosis.* One is mutation of β subunit of RNA polymerase of target molecule by drug action; another is that the ingesting capability of infected cells decreased because of change of osmosis across cell wall [8]. SM mainly affected the ribosome of *Mycobacterium tuberculosis* to induce miscoding of genetic code and inhibit protein synthesis. The resistance of SM resulted from the mutations of rpsL, mainly in the No. 43 codon. Especially, the mutation of No.512 or No.513 site led to higher drug-resistance[9].

Many scholars [12-16] have studied drug-resistant re-

lated genes by PCR-SSCP, and Yang LIU found that the gene mutation rates of katG, rpoB and rpsL were respectively 40%, 45% and 38%. It indicated that the creation of drug resistance of *Mycobacterium tuberculosis* L-form complied with the genetic mutations. Other studies [9,17] manifested that 50% of multi-drug resistant strains of *Mycobacterium tuberculosis* L-forms isolated from clinical samples simultaneously carried allied mutations of three genes, while 30% carried allied mutation of two genes. The results above-mentioned hinted that coding genes of target molecule were gradually mutated by various kinds of medicine[18].

In this study, in 52 cases of *Mycobacterium tuberculosis* L-forms isolated from sputum samples, the gene mutation rates of katG, rpoB and rpsL by PCR-SSCP were respectively 57.70% (30/52), 65.38%(32/52) and 40.38%(21/52). There were 15 strains of allied mutation of three genes, 16 of two mutations and 6 of only one by PCR-SSCP, which was slightly less than the detection rate of bacteric *Mycobacterium tuberculosis* reported in previous literature [18,19]. The probable cause may be related to the geographical diversity, effects of pneumoconiosis, and technical localization that leads to low detection rate. Then there was no significant difference between the allied mutations of multi-drug resistant genes and the mutations of only one drug resistant gene($P > 0.05$).

At the same time, the positive rates of katG, rpoB and rpsL by AST were respectively 76.92% (40/52), 90.38%(47/52) and 50.00%(26/52). 40 strains were detected to tolerance INH, including 24 strains of high-level resistance to RFP, 16 strains of low-level resistance, and anti-RFP and anti-SM were respectively 47,33,14 and 26,1,25. In the meantime, there were 40 (76.92%) multi-drug resistant strains including 21 of three-drug resistant strains (40.38%), 19 of two-drug resistant(36.54%) and 12 of only one-rug resistant (23.08%). It showed the presence of serious drug resistance of *Mycobacterium tuberculosis* L-forms in Huainan mining district, and clinical common doses didn't have any effect in the patients. In addition, we detected 10 cases of negative katG, 15 cases of negative rpoB and 5 ones of negative rpsL in persisters. The phenomenon implied that it still exists other resistant mechanisms [21,22].

During the course of the test, we found that the operative procedure of PCR-SSCP technique was easily affected by environmental factors, especially gelatinous temperature. For this reason, we must make up specification operation and the optimization reaction condition to decrease the interference of nonspecific product.

In conclusion, PCR-SSCP technique is a convenient and dependable screening technique of gene mutation. It has higher sensibility and specificity in detecting gene polymorphism of katG, rpoB and rpsL, and plays a vital role in earlier diagnosis and guidance of clinical medications.

References

[1] Li YL. The book of Chinese medical laboratory science. *Beijing: People's Medical Publishing House*, 2000;1119-20.

[2] Telenti A, Lseman M. Drug-resistant tuberculosis. *Drug* 2003; 59:171-9.

[3] Li YL. The book of Chinese medical laboratory science. *Beijing: People's Medical Publishing House*, 2000;1262-4.

[4] Zhu ML, Xia PY, Zhang YH. Detection mycobacteria and their L-forms in peripheral blood from pulmonary tuberculosis patients by cultivation with hemolyzed-centrifugated blood in liquid medium. *Chin J Tuberc Respir Di* 2000;23:556-8.

[5] Ye HP, Huang BB, Mao L. Liquid culture of *Mycobacterium tuberculosis* and L-forms. *Chin J of Zoonoses* 2002;18:128-9.

[6] Cheng XD, Yu WB, Su MQ, Bie LF, Duan L, Hao XK, *et al.* Study on *M.tuberculosis* isoniazid-resistant isolates by multi-PCR. *J Fourth Mil Med Univ* 2003;24:849-51.

[7] Dribnieweski FA. The rapid diagnosis of isoniazid and rifampicin resistance in *Mycobacterium tuberculosis* a molecular story. *J Med Microbial* 1998;47:189.

[8] Musser JM. Antunimicrobial agent resistance in *M. tuberculosis*: molecular genetic insights, *Clin Microbial Rev USA* 1995;8: 496-514.

[9] Morris S, Bai GH, Stffys P. Molecular mechanism of multiple drug resistance in clinical isolates of M.tuberculosis. *J Infect Dis* 1995;171:954-5.

[10] Shin TS, Yolo CG, Han SK, Dong JR, Fors L. Isoniazid resistance and the point mutation of the colon 463 of katG gene of *Mycobacterium tuberculosis*. *J Korean Med Sci* 1997;13:92-4.

[11] Lee H, Cho SN, Bang HE, Neri BP. Exclusive mutations related to isoniazid and elihionamide resistance among mycobacterium isolates from Korea. *Int Lung Dis* 2000;4:441.

[12] Yang L, Su MQ, Cheng XD, Hao XK, Yue QH, Yu WB. Clinical application of PCR-SSCP in detecting *Mycobacterium tuberculosis* drug resistance. *J Fourth Mil Med Univ* 2003;24: 2142-6.

[13] Tang SJ, Xiao HP, Mei Q, Zhang YR, Wu XZ, Wu FR, *et al.* The detection of *Mycobacterium tuberculosis* L-forms in from pulmonary tuberculosis patients and clinical sense. *Shanghai J of Med Sci* 2002;25:535-8.

[14] Sakhelashvili MI, Platonova IL, Lapovets' NI. Laboratory diagnostic methods in primary pulmonary tuberculosis in adults. *Lik Sprava* 1998;1:95-9.

[15] Zhuang YH, Li YH, Zhang XG. Study of isolated culture method of Aciduric Bacterial L-forms. *Microbiology* 1991;18: 348-9.

[16] Zhu CM, Xie SM, Zhang JH. *Mycobacterium tuberculosis* L forms in patients with pulmonary silicotuberculosis and silicosis. *Chin J Tuberc Respir Dis* 2001;24:236-8.

[17] Wu XQ, Zhuang YH, Zhang JX, Zhang XG, He XY, Li GL, *et al.* Study on the molecularmechan ism of multi-drug resis-

tance in clinical isolates of *Mycobacterium tuberculosis*. *Chin J Tuberc Respir Dis* 1997;20:332-5.

[18] Li HM, Wu XQ, Wang W, Chao XL, Zheng ZG, Yan LL, *et al.* Identification of five drug resistant genes in *Mycobacterium tuberculosis*. *Chin J Microbiol Immunol* 2002;22:644-6.

[19] Cheng DJ, Zhang XZ. Comparison of *M. tuberculosis* with 4 detection methods. *Clin J Med Lab Sci* 1996;19:229-30.

[20] Musser JM. Antunimicrobial agent resistance in *M. tuberculosis*: molecular genetic insights. *Clin Microbial Rev USA* 1995;8:496-9.

[21] Dribnieweski FA. The rapid diagnosis of isoniazid and rifampicin resistance in *M.tuberculosis* a molecular story. *J Med Microbial* 1998;47:189-91.