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Association of *Mycobacterium tuberculosis* L-form *mpb64* gene and lung cancer

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Abstract. – OBJECTIVE: Tuberculosis is one of the most infectious diseases worldwide and lung cancer is one of the leading causes of death. The major contagious agent for tuberculosis, *Mycobacterium tuberculosis (M. tuberculosis)*, has been well studied for its pathogenicity. Even though there are studies showing that patients with a history of tuberculosis are more likely to develop lung cancer, the association between *M. tuberculosis* and lung cancer largely remains unknown. In this study, we used in situ hybridization to analyze lung tissues from patients who underwent surgical resection or bronchoscopy for the expression of M. tuberculosis-specific gene, *mpb64*, to provide evidence for the association of *M. tuberculosis* L-form to the occurrence of lung cancer.

PATIENTS AND METHODS: Experiments were conducted in the lung cancer group (80 cases), pulmonary tuberculosis group (80 cases) and pulmonary tuberculosis plus lung cancer group (77 cases). For each group of tissue samples, *in situ* hybridization was used to detect the expression of *mpb64* gene fragment in cell nuclei.

RESULTS: *Mpb64* gene was positively expressed in 45% (CI: 38.63-51.37) of the cancerous cell nuclei. When compared to the expression level of 66.25% (CI: 59.88-72.62) in the pulmonary tuberculosis cells, the difference was statistically significant (p=0.007). However, when compared to the expression of 49.35% (CI: 42.98-55.72) in pulmonary tuberculosis plus lung cancer cells, the difference was not statistically significant (p=0.585). *Mpb64* gene expression level was independent from the different tissue types, pathological stages, or metastasis situations in the lung cancer group (p>0.05).

CONCLUSIONS: The *mpb64* gene fragment is highly expressed in the nucleus of pulmonary tuberculosis tissues. Its expression in the nucleus of pulmonary tuberculosis plus lung cancer tissue is significant and the expression in the nucleus of lung cancer tissue is also high. The expression of *mpb64* is independent from the various pathological features of the cancerous tissues. Taken all together, we provided evidence for the correlation of *M. tuberculosis* L form and the occurrence of lung cancer. Thus, patients with a history of tuberculosis may be more likely to develop lung cancer than those without a history of tuberculosis.

Key Words
Pulmonary tuberculosis, Lung cancer, Pulmonary tuberculosis plus lung cancer, Mycobacterium tuberculosis L-form, mpb64 gene fragment.

Abbreviations

TB: Tuberculosis, MTB-L: *Mycobacterium tuberculosis* L-form, ISH: in situ hybridization, DIG: Digoxigenin, FHIT: Fragile histidine triad protein, WHO: World Health Organization, UICC: Union for International Cancer Control.

Introduction

Lung cancer is one of the leading causes of death for a malignant tumor with an incidence and mortality rate continuingly increasing for the last decades[1]. China has the highest incidence for malignant tumors and lung cancer has the highest incidence rate among them with approximately 781,000 deaths each year[2]. In Xinjiang, the incidence of lung cancer is 17.70 per 100,000 (13.88%), which is as high as the national trend[3]. According to the World Health Organization (WHO) report, the incidence of tuberculosis worldwide was 10.4 million in 2016 and the mortality rate was 1.67 million, the multidrug-resis-

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tant TB was 600,000[4]. The incidence of TB in China was 68 per 100,000 with a mortality rate of 38,000 and the multidrug-resistant TB was 6.6%. Xinjiang has the highest incidence of TB in China with an annual estimate of 181.42 per 100,000. The incidence of tuberculosis complicated with lung cancer is 2-8%[5]. A large number of reports[6] reported that there is more pulmonary tuberculosis with lung cancer occurring in the ipsilateral lobe than in contralateral lung lobes. Also, inactive tuberculosis is more common than active tuberculosis in these patients. The infectious agent for tuberculosis is *Mycobacterium tuberculosis* (*M. tuberculosis*) and it includes human *M. tuberculosis*, *M. bovis*, *M. Africanum*, and *M. vaccae* [7]. Among these, the first three are pathogenic to humans with human *M. tuberculosis* being the most infectious one. Based on the morphology, *M. tuberculosis* is further sub-categorized into bacillus (basic), granular, cocciform (L-form), and filtration forms. Like many other bacteria, *M. tuberculosis* can transform into L-form under unfavorable physical, chemical, and immunological conditions. Isolates of cell wall deficient *M. tuberculosis* L-form (MTB-L) often exhibits altered morphology, stain variability, and antigenicity[8]. The pathogenicity of MTB-L has been well investigated over the years. However, the association between MTB-L and lung cancer remains largely unknown. Wang et al[9] detected MTB-L in different types of lung cancer tissues. The same group was also able to detect MTB-L in the sputum and effusion specimen samples of patients with lung cancer[10]. Yang et al[11] identified a large amount of MTB-L in breast cancer tissues and expression of *mpb*64 gene in the nuclei of breast cancer cells. In this study, we used *in situ* hybridization to detect the presence of MTB-L and mpb64 gene expression in the lung tissues of lung cancer patients. We provided evidence for the correlation of *M. tuberculosis* and lung cancer from an etiological perspective.

Patients and Methods

Patients

Inclusion criteria: Pulmonary tuberculosis combined with lung cancer group: 1- Middle-aged and elderly patients that meet the diagnostic criteria for lung cancer and the diagnostic criteria for pulmonary tuberculosis. 2- Lung cancer patients with previous tuberculosis (most tuberculosis are old lesions). 3- Lung cancer patients with different histological types, histological grades, and pathological stages. Exclusion criteria: 1- Patients with recent myocardial infarction and unstable angina; 2- Patients with severe heart, liver, kidney, or other complications, with acute organ failure; 3- Alzheimer's disease or medically proven spiritual instincts; 4- Patients with bronchoscopy or surgery. 5- There have been clear or suspected HIV infections in the past.

Lung Cancer Group

We collected 80 pathologically confirmed lung cancer tissues from patients who underwent surgical resection or bronchoscopy from 2014 to 2017 at the Chest Hospital of Xinjiang Autonomous Region. The inclusion criteria are: 1) Middle-aged and elderly patients meeting the diagnostic criteria for lung cancer; 2) Patients with lung cancer who have no previous history of TB; 3) Lung cancer patients with different histological types, histological grades, and pathological stages. We included 58 males and 22 females with the mean age of 42.8 years (range: 28-79 years). According to the 2015 World Health Organization (WHO) Classification of Lung Cancer[12], we classified 25 cases to be squamous cell carcinoma, 40 cases to be adenocarcinoma, and 15 cases to be small cell carcinoma. Based on the lung cancer staging guidelines[13] from the 8th edition of the Union for International Cancer Control (UICC) (2017) we sorted 19 stage I+II cases and 61 stage III+IV cases. There were 27 cases with their tumor diameter $\leq$ 3 cm and 53 cases with their tumor diameter $\geq$ 3 cm.

Pulmonary Tuberculosis Group

We collected 80 pathologically confirmed pulmonary tuberculosis tissues from patients who underwent surgical resection or bronchoscopy from 2014 to 2017 at the Chest Hospital of Xinjiang Autonomous Region. The inclusion criteria are middle-aged and elderly patients meeting the diagnostic criteria for pulmonary tuberculosis. We included 37 males and 43 females with the mean age of 37 years (range: 18-79 years). Among these, 37 were new TB cases and 43 were previously treated cases. 15 cases had positive acid-fast smear test result while the other 65 were negative.

Tuberculosis Plus Lung Cancer Group

We collected 77 pathologically confirmed tissues that had both pulmonary tuberculosis and lung cancer from patients who underwent surgical resection or bronchoscopy from 2014 to 2017

at the Chest Hospital of Xinjiang Autonomous Region. The inclusion criteria are: 1) Middle-aged and elderly patients that meet the diagnostic criteria for lung cancer and the diagnostic criteria for pulmonary tuberculosis; 2) Lung cancer patients with previous history of tuberculosis (majority are old lesions); 3) Lung cancer patients with different histological types, histological grades, and pathological stages. We included 54 males and 23 females with mean age of 58.2 years old (range: 31-82 years). 71 cases had both tuberculosis and tumor on the same side of their lungs and 9 cases had tuberculosis and tumor on the opposite lungs. Exclusion criteria: 1) Patients with recent myocardial infarction and unstable angina; 2) Patients with severe heart, liver, kidney, or other complications, those with severe organ failures; 3) Patients with Alzheimer's disease or medically proven mental disorders; 4) Patients who could not support bronchoscopy or surgery; 5) Patients carry or suspected HIV infections in the past.

Reagents

My250-224 and My310-283 fragments were selected from the *mpb64* gene (X75361 687bp mycobacteria mpb64 Mycobacterium tuberculosis H37Rv) of *Mycobacterium tuberculosis* H37Rv standard strain. 5' labeled Digoxigenin (DIG) probes were generated by Sangon Biotech (Shanghai, China, Order No.: 100320974). The MPB64 probe sequences are: 1) 5'-CGGTA TCGGT GCCTT TCAAC TCCTC GC-3', 2) 5'-GGGCA GGCTG ATGTT GATGT TGTAG GC-3'. These probes were mixed when used in this study.

In Situ Hybridization

Enhanced sensitive ISH detection kit was purchased from Boster Biological Technology (Wuhan, China, Catalog No. MK1030). Detection of mpb64 gene by *in situ* hybridization was performed according to the manufacturer's instructions. Briefly, the slides were dewaxed after baking at 60°C for 1 hour. 3% H_2O_2 was used to inactivate endogenous peroxidase. Slides were rinsed with distilled water and digested with 0.2 mol/L HCl and followed by a rinse with distilled water. 3% pepsin diluted with citric acid was used to digest the slides, which were then rinsed with phosphate-buffered saline (PBS). Next, they were fixed with 4% paraformaldehyde at 4°C and dried at room temperature for 5 min. 20 ul pre-hybridization solution were added to each slide to pre-hybridize at 42°C for 30 min. 20 ul 4 ug/ml hybridization solution were added and heated at 95°C for 10 min and *in situ* hybridization apparatus followed by 2 min ice incubation. Hybridization was placed at 42°C overnight. On the next day, slides were washed with different concentrations of SSC, blocked with blocking buffer at 37°C for 30 min, and then biotinylated mouse anti-digoxigenin was added dropwise for 60 min at 37°C. It was followed by washing with phosphate-buffered saline (PBS) and adding SABC dropwise and incubated at 37°C for 20 min. Slides were rinsed with PBS and biotinylated peroxidase was added dropwise and incubated at 37°C for 20 min. Finally, DAB was developed for 30 min and counterstained with hematoxylin. Positive tuberculous tissue sections were used as positive control. Pulmonary tuberculosis sections without probe hybridization were used as negative controls. Positive result is determined when there are yellowish brown granules detected in cancer cell nucleus. Five random fields were observed for each slide, excluding the areas of necrosis. For each area of the slide, the percentage of positive expression cells was calculated. The average positive expression of $\geq 5\%$ for the given slide is considered positive whereas the positive expression of $<5\%$ is considered negative. For the tuberculosis tissues, the positive is defined as yellowish brown cytoplasm and interstitial fluid.

Statistical Analysis

SPSS 17.0 (SPSS Inc., Chicago, IL, USA) statistical software was used for statistical analysis. The positive rate of each group was compared using the χ^2-test, $p<0.05$ was considered statistically significant.

Results

Mpb64 Gene Expression in Tuberculosis, Lung Cancer, and TB + Lung Cancer

MPB64 is a bacterial antigen specifically secreted by *M. tuberculosis* and *M. bovis*[14]. It is encoded in their genome and used by many laboratories to identify the bacteria. In this study, using *in situ* hybridization, we have positively detected *mpb64* gene fragment in the cell nuclei of tuberculosis (Figure 1), lung cancer (Figure 2), and tuberculosis plus lung cancer tissues (Figure 3). More specifically, 66.25% (53/80) of the tuberculosis tissues, 45% (36/80) of the cancer tissues, and 49.35% (38/77) of the tuberculosis plus cancer tissues stained positive for the presence of *mpb64* (Table I-III).

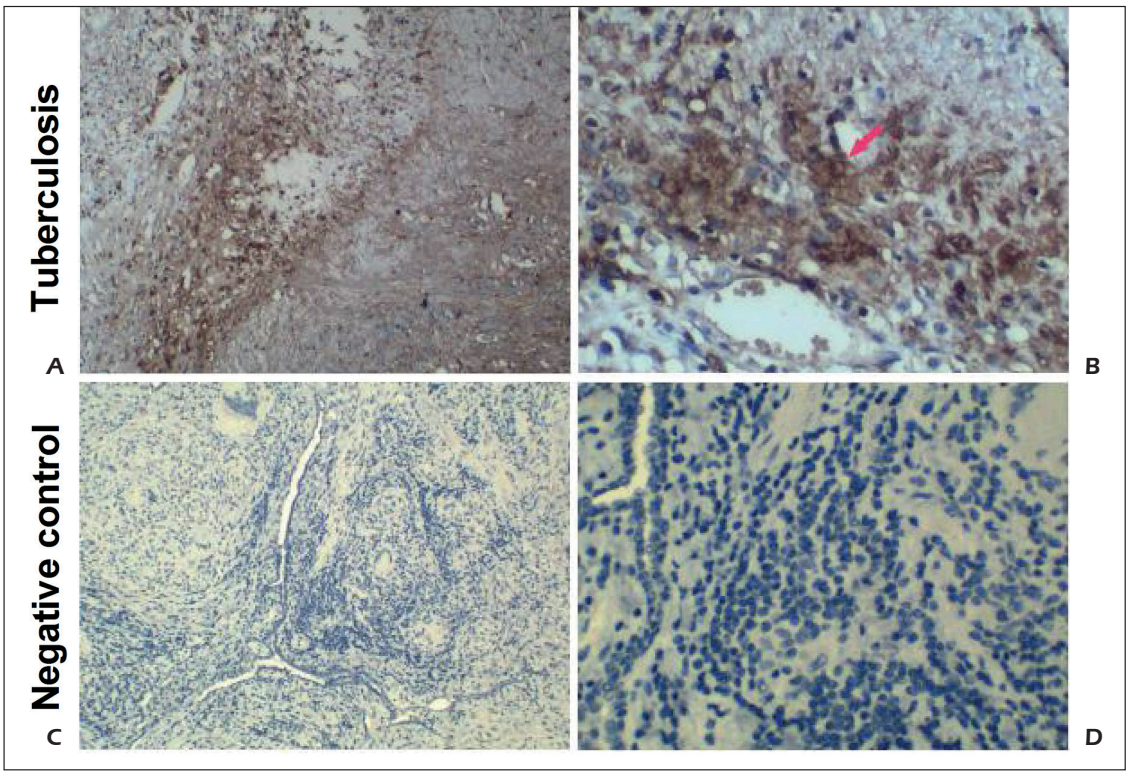

Figure 1. Mpb64 staining in the pulmonary tuberculosis group. **A-B**, Microscopic view of mpb64 staining in the tuberculosis slides under 100X and 400X magnification. **C-D**, Microscopic view of mpb64 staining in the tuberculosis negative control under 100X and 400X magnification.

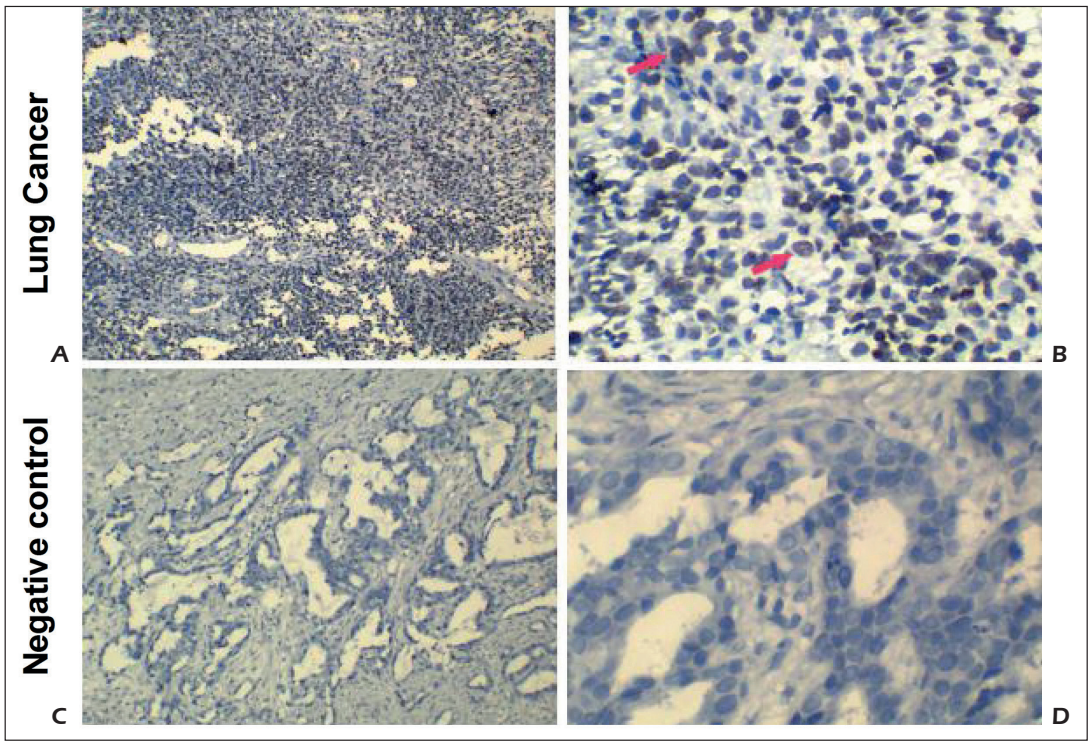

Figure 2. Mpb64 staining in the lung cancer group. **A-B**, Microscopic view of mpb64 staining in the lung cancer slides under 100X and 400X magnification. **C-D**, Microscopic view of mpb64 staining in the lung cancer negative control under 100X and 400X magnification.

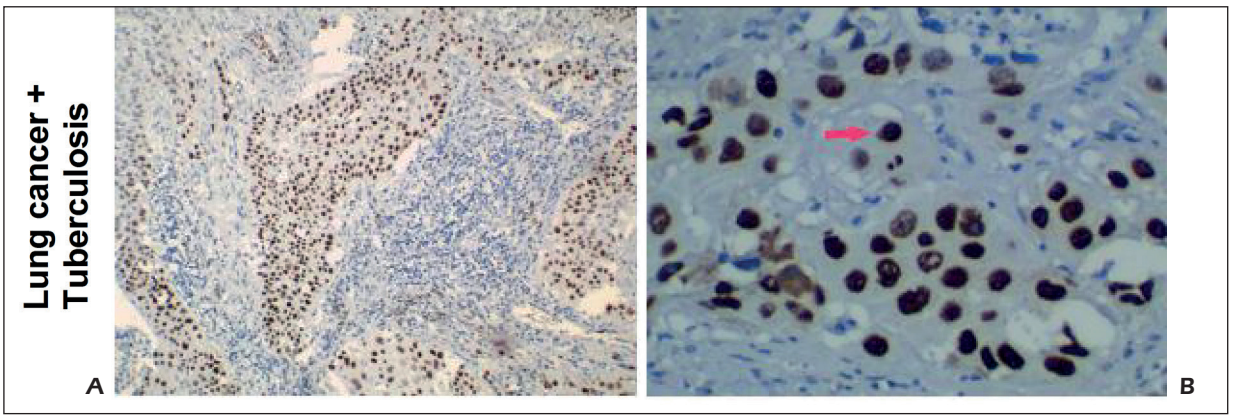

Figure 3. Mpb64 staining in the lung cancer plus tuberculosis group. **A-B**, Microscopic view of mpb64 staining in the lung cancer plus tuberculosis slides under 100X and 400X magnification.

The expression of *mpb64* in the TB group was significantly higher than that of either cancer group (p=0.032) or TB plus cancer group (p=0.007). However, the expression of *mpb64* between cancer group and TB plus cancer group is not significant (p=0.585). These results indicate that mpb64 gene is highly expressed in tuberculosis and it is present in lung cancer and patients with both tuberculosis and lung cancer. Together, we provided evidence that tuberculosis is relevant to the incidence of lung cancer.

Mpb64 Expression in Lung Cancer

We also analyzed the expression of mpb64 in various histo-pathologically different lung cancer tissues. The results are summarized in Table IV. We found that the expression of mpb64 does not significantly differ among squamous cell carcinoma (37/62, 59.6%), adenocarcinoma (31/74, 41.9%) and small cell carcinoma (7/21, 33.3%) (p=0.043). There were no significant differences in the expression levels between stage I, II (18/33, 54.5%) and stage III, IV (55/124, 44.3%) cancer tissues

Table I. Lung cancer group vs. lung cancer plus tuberculosis.

	Lung cancer (n=80)	Lung cancer + tuberculosis (n=77)	χ^2	*p*
Positive	36 (45.00%)	38 (49.35%)	0.298	0.585
Negative	44 (55.00%)	39 (50.65%)		

Table II. Lung cancer group vs. tuberculosis group.

	Lung cancer (n=80)	Tuberculosis (n=80)	χ^2	*p*
Positive	36 (45.00%)	53 (66.25%)	7.318	0.007
Negative	44 (55.00%)	27 (33.75%)		

Table III. Lung cancer plus tuberculosis vs. tuberculosis.

	Lung cancer + tuberculosis (n=77)	Tuberculosis (n=80)	χ^2	*p*
Positive	38 (49.35%)	53 (66.25%)	4.599	0.032
Negative	39 (50.65%)	27 (33.75%)		

Table IV. Relationship between *mpb64* gene expression level and clinicopathological parameters of lung cancer.

Clinicopathological parameters		Total (n)	Mpb64		χ^2	*p*-value
			Positive	Negative		
Histological type	Adenocarcinoma	74	31	43	6.302	0.043
	Small cell	21	7	14		
	Squamous	62	37	25		
Differentiation	Low	115	52	63	2.345	0.309
	Moderate	32	16	16		
	high	10	7	3		
Clinical stage	I +II	33	18	15	1.088	0.296
	III +IV	124	55	69		
Tumor size	> 3 cm	114	56	58	0.305	0.580
	≤ 3 cm	43	19	24		
Lymphatic metastasis	Yes	116	53	63	0.771	0.379
	No	41	22	19		
TB History	Yes	77	38	39	0.224	0.63
	No	80	37	43		

(p=0.296). mpb64 was positively expressed in 45.2% (52/115) of the poorly differentiated lung cancer tissues, 50% (16/32) of the moderately differentiated tissues and 70% (7/10) of the highly differentiated ones. However, the differences of the expression levels among differently differentiated lung cancer tissues are not statistically significant (p=0.309). Its expression was also independent of the tumor sizes (p=0.580) or lymphatic metastasis (p=0.379). In this regard, it was positively detected in 49.1% (56/114) of the tissues with tumor size greater than 3cm and 44.2% (19/43) of the tissues with tumor size smaller/equal to 3 cm. 45.7% (53/116) of the tissues with lymphatic metastasis and 53.6% (22/41) with no metastasis had positive expression of mpb64. Comparing lung cancer patients and non-cancerous patients, mpb64 was positively detected in 47.1% of cancer patients and 66.3% of non-cancerous patients expressed mpb64 (Table V). The differences were significant (p=0.005). In the squamous cell carcinoma group, the positive detection of mpb64 gene was 59.6%, which indicated that MTB was mainly in L-form in lung cancer tissues. In MTB-L-positive specimens, *mpb64* gene was positively expressed. MTB-L is related to the lung cancer histological, suggesting that MTB-L infection may be one of the pathogenic factors of squamous cell carcinogenesis. Many more future studies are needed to prove this hypothesis.

Discussion

A tumor is a genetic disease resulting from changes in genetic information[15]. It is now recognized that the activation of proto-oncogenes with or without the inactivation of tumor suppressor genes is an important mechanism that promotes malignant transformation of cells[15,16]. Whether or not MTB-L infection is associated to the incidence of the lung cancer remains controversial. Some scholars[8] speculate that MTB-L may play an important role in the pathogenesis of lung cancer. Song et al[1] reported that *M. tuberculosis*-infected lung cancer tissues showed slightly higher expression of fragile histidine triad protein (FHIT) than non-infected lung cancer tissue, suggesting that *M. tuberculosis* infection may participate in developing lung cancer by modifying cancer related gene expressions. This current study is to investigate and document the relevance of MTB-L infection and lung cancer

Table V. Relationship between lung cancer and *mpb64* gene positive expression.

	Mpb64		χ^2	*p*-value
	Positive	Negative		
Positive	38 (49.35%)	53 (66.25%)	4.599	0.032
Negative	39 (50.65%)	27 (33.75%)		

by analyzing the expression of *mpb64*, the *M. tuberculosis*-specific gene, in the cancer cell nucleus. Due to paraffin-embedded nature of the samples, we chose ISH to detect the expression of *mpb64* in lung cancer, pulmonary tuberculosis, and pulmonary tuberculosis plus lung cancer tissues. ISH detection of the *mpb64* gene expression was enhanced by increasing the permeability of the tissue and improving the penetrance of the probe. As the result, we have detected *mpb64* fragment expression in pulmonary tuberculosis, lung cancer and pulmonary tuberculosis plus lung cancer tissues. The expression of *mpb64* is independent from other pathological features such as cancer types, cancer stages, tumor sizes, metastasis status, or whether or not the tumor was on the same side of the lung with tuberculosis. *Mycobacterium tuberculosis* is the infectious agent for tuberculosis, and tuberculosis is a risk factor for lung cancer[17]. Workman et al[18] found that about 5% of pulmonary tuberculosis has lung cancer; the risk of developing lung cancer of tuberculosis patients is 2 to 5 times higher than that of the general population. As the incidence of lung cancer and tuberculosis increase every year, so does the concurrent tuberculosis-associated lung cancer[19-21]. Researchers[10,22] observed that cancer cells produce immunosuppressant that lower patients' immune system and contribute to the acquirement of tuberculosis. Researches showed that the pulmonary tuberculosis predisposition to lung cancer is due to the direct stimulation of lymph nodes in the vicinity of bronchial tuberculosis. Others believe that the epithelial of hollow tuberculosis changes into squamous cells and contributes to the development of cancer. Some speculate that local bronchiectasis during tuberculosis causes retention of the nicotine and in the long term contributed to carcinogenicity. Engels et al[23] and Lienhardt et al [24] speculate that it is the scarring post-tuberculosis the cause of lung cancer. Researchers[25] suggest that there are significant differences in gene expression in different pathological types of lung cancer. In this study, mpb64 gene fragment was positively expressed in 36 (45%) lung cancer tissues, in 38 (49.35%) pulmonary tuberculosis plus lung cancer tissues, and in 53 (66.25%) pulmonary tuberculosis tissues. Although the expression of mpb64 gene in the above three groups was lower than that reported by other scholars in China, it suggests that the infection of *M. tuberculosis* L-form is relevant to the occurrence of lung cancer. Thus, subjects with a history of tuberculosis may be more likely to develop lung cancer than those who have never been infected with *M. tuberculosis*. The detection of *M. tuberculosis* L-type mpb64 gene did not show difference between lung cancer tissue types, pathological stages, tumor sizes, lymph node metastasis, etc. It may be related to the small sample sizes. Further study in larger sample volume is needed.

Conclusions

We found that *M. tuberculosis*-specific *mpb64* gene was positively detected in tuberculosis, lung cancer, and tuberculosis plus lung cancer tissue samples. However, its presence was independent from various cancer types, tumor sizes, cancer progression status, etc. In this study, we show that patients with a history of tuberculosis have higher chance of developing lung cancer.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Chest Hospital of Xinjiang Uygur Autonomous Region (Urumqi, China). The methods used in this study were performed in accordance with relevant guidelines and regulations. Written consent was obtained from the participants or guardians of participants under 16 years old.

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Conflict of Interests

The Authors declare that they have no conflict of interests.

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