Differences in the expression of LSP and S100A9 in peripheral blood of patients with tuberculosis of different pathogens combined with pulmonary infection and their clinical significance

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Introduction. To analyze the the clinical significance of endotoxin (LSP) and calcium-binding protein (S100A9) detection in patients with pulmonary tuberculosis co-infection.

Methods. 132 patients with pulmonary tuberculosis admitted to our hospital were divided into infected and uninfected groups according to the presence or absence of pulmonary infections. Compared the levels of LSP, S100A9 protein between the infected and uninfected groups, and analyzed the risk factors leading to pulmonary infections with pulmonary tuberculosis and the diagnostic value of LSP and S100A9 protein tests on pulmonary infections with pulmonary tuberculosis.

Results. LSP and S100A9 are significantly overexpressed in the sera of patients co-infected with pulmonary tuberculosis. LSP and S100A9 were positively correlated with the level of M2 type macrophages in the peripheral blood of patients. Both LSP and S100A9 had good sensitivity and specificity for dinidazole resistance.

Conclusion. The detection of LSP and S100A9 Protein Levels in patients is conducive to the early diagnosis of patients with pulmonary tuberculosis and pulmonary infection, and has important reference value for evaluating disease progress and selecting treatment options.

Keywords. pulmonary tuberculosis; co-infection of the lung; pathogenic bacteria; endotoxin;

calcium-binding protein

Iranian Journal of Kidney Diseases / Volume 18 / Number 02 / 2024 (DOI: 10.53547/ijkd.8314)

INTRODUCTION

Tuberculosis and bacterial pneumonia are both common infectious diseases of the lungs in respiratory medicine, and there are many clinical similarities between these two diseases, both of which can present with symptoms such as fever, cough, cough, chest tightness, chest pain, and hemoptysis. Patients with pulmonary tuberculosis often suffer from delayed diagnosis or improper treatment resulting in destruction of bronchial and pulmonary structures, and even drug resistance and systemic dissemination of tuberculosis bacteria. Because Mycobacterium tuberculosis can cause destruction of bronchopulmonary tissue anatomy and damage to the body's immune system, patients with TB are prone to co-infection with other bacteria in the lungs [2]. Tuberculosis itself is a pulmonary infection caused by Mycobacterium tuberculosis, and compared with pulmonary infections caused by other pathogens, there are no characteristic symptoms, signs and imaging, so without a reliable etiologic basis, the diagnosis of tuberculosis combined with pulmonary bacterial infection is difficult to establish. Therefore, the diagnosis of tuberculosis combined with pulmonary bacterial infection is very difficult. At present, the differential diagnosis is mainly made by PPD test, Y-interferon release test, serum TB antibody, blood sedimentation, antacid staining of sputum concentrates, and culture of sputum Mycobacterium tuberculosis.

However, the sensitivity of blood TB antibody, PPD test and interferon release test is poor; the specificity of blood sedimentation is poor; the positive rate of antacid staining of sputum smear is low, the time required for sputum Mycobacterium tuberculosis culture is long and some patients have no sputum. However, due to the misuse of antibiotics in recent years, the imaging features of bacterial pneumonia have changed, showing a reduction in the extent of solid lesions, uneven absorption of exudative lesions when they are well absorbed, which may appear as patches or striated shadows on imaging, and certain foci of pneumonia may occur at the site of tuberculosis predilection (e.g., the posterior segment of the upper lobe and the dorsal segment of the lower lobe), which is very similar to This is very similar to the imaging changes of pulmonary tuberculosis. On the other hand, many pulmonary tuberculosis manifest as solid shadows in the lower lung fields, which resemble pneumonia on imaging and are difficult to distinguish from tuberculosis [3].

Clinically, we often make the final diagnosis by anti-infective treatment, or experimental anti-tuberculosis and anti-infective treatment in parallel, with short-term review of chest X-ray and chest CT, and according to the absorption of lesions. This inevitably results in the irrational use of antibiotics and anti-tuberculosis drugs, or delays in the disease as a result. s100A9 protein is a

member of the calcium-binding protein S100 protein family with a low molecular weight (13,000) and belongs to the model molecule of injury-related molecules in innate immunity [3]. s100A9 protein has both intracellular and extracellular roles, and the intracellular role is mainly through calcium sensing, Gopal et al. found that $100A9 protein plays an important role in promoting immune response and inflammatory repair by inducing the production of inflammatory chemokines and cytokines through regulating neutrophil aggregation proteins and inflammation associated with tuberculosis, promoting neutrophil aggregation and leading to increased lung injury in a mouse model of tuberculosis, the S100A9 protein has the potential to be a target site for tuberculosis diagnosis and treatment, and molecular treatments targeting the S100A9 protein have the potential to reduce lung tissue injury without compromising protective immunity against tuberculosis treatment [4-8]. This protein may play an important role in the development and progression of tuberculosis by promoting the aggregation of neutrophils that cause tuberculosis tissue damage.

LSP is a component of the cell wall of gram-negative bacteria, also known as lipopolysaccharide. The horseshoe crab test method was discovered and established by Levin and Bang in 1968 as an effective method for monitoring the presence of Gram-negative bacterial infections, and is widely used in clinical practice [9]. When a small fraction of LSP arrives in the organism, the self-defense system can degrade and phagocytose it, and it disappears rapidly in the blood [9]. However, if the immunity of the organism decreases and serious infections are not controlled in time, LSP will not be cleared, and thus a large amount of aggregation will occur, which eventually leads to endotoxemia. In both domestic and international literature, it has been shown that LSP can be used as a useful reference indicator for the assessment and prognosis of Gram-negative bacterial infections, and as a basis for the selection of clinical therapeutic drugs [10]. It has also been shown that LSP is the real cause of sepsis in patients and that elevated LSP levels can be detected in the early stages of bacterial infection [11]. In the present study, LSP and S100A9 protein were

significantly higher in patients with TB combined with pulmonary infection than in those without infection, and abnormal LSP and S100A9 protein were independent risk factors for the development of pulmonary infection in patients, suggesting that abnormal LSP and S100A9 protein are closely related to TB combined with pulmonary infection and play a role in the development of TB. The clinical assessment of LSP and S100A9 protein changes is important for the early assessment of infection, but the value and difference of each index are not yet known.

MATERIALS AND METHODS

Sample Collection

Peripheral blood specimens were collected from 132 patients diagnosed with pulmonary tuberculosis in our hospital from 2019 to 2020, among which 62 patients with pulmonary tuberculosis combined with pulmonary bacterial infection (bacterial infection group) and 70 patients with pulmonary tuberculosis without pulmonary bacterial infection (simple tuberculosis group). The differences in age, sex, and body mass index (BMI) between the two groups were statistically significant ($r>0.05$) and comparable. The study was approved by the medical ethics committee of our hospital, and all study subjects signed an informed consent form.

Inclusion exclusion criteria

Inclusion criteria: (1) complete clinical data; (2) the subjects were aware of the study and signed the

informed consent form; (3) All the candidates met the diagnostic criteria in the guidelines for the diagnosis and treatment of tuberculosis.(4) age ＞18year.Exclusion criteria: (i) exclusion of viral, fungal, mycoplasma and chlamydia infections; (ii) patients with malignant tumors, immune system diseases or those receiving immunotherapy; (iii) those who are in the period of baby vibration or nursing.

Tuberculosis diagnosis was confirmed based on.
1) Positive sputum or pleural fluid for Mycobacterium tuberculosis, or characteristic lesions of tuberculosis on biopsy.
2) characteristic foci of tuberculosis on x-ray chest radiographs of the lungs and other parts of the body.
3) Obvious clinical symptoms of tuberculosis intoxication and other tests consistent with tuberculous features.
4) Fast absorption of lesions and pleural fluid after anti-tuberculosis treatment.

The diagnosis is confirmed if three or more of the above criteria are present.

Criteria for determining the progressive phase of tuberculosis activity, the period of improvement in absorption, and the stable phase.

1) Active progressive stage: Anyone with one of the following is in the active progressive stage: newly discovered active TB lesions.

2) New cavity; enlargement of cavity; worsening of lesion; positive sputum for Mycobacterium tuberculosis.

3) Improvement period of absorption: Any one of the following is considered as improvement period: better absorption of TB lesions than before; closure of cavities and smaller cavities; negative
sputum Bacillus tuberculosis.

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4) Stable stage: no active lesions; cavity closure; sputum Bacillus tuberculosis continuously negative (at least one examination every month for more than six months; if cavity exists, sputum
Bacillus tuberculosis must be continuously negative for more than one year).

Peripheral blood collection

About 3 ml of venous blood was drawn from the elbow vein in the early morning on an empty stomach and placed in a vacuum blood collection tube containing a procoagulant. The blood was gently shaken and then centrifuged for 10 minutes at 3000 rpm. The serum specimens were separated and placed in 2 ml EP tubes, numbered and labeled, and stored in a refrigerator at -20
degrees Celsius.

Enzyme-linked immunosorbant assay(ELISA)

S100A9 was detected by ELISA, and the experimental operation was carried out in strict

accordance with the instructions of the reagent.

Sample preparation: EDTA-anticoagulated plasma was removed from the -80 ℃ refrigerator, thawed at room temperature, and centrifuged at 2500 rpm for 10 min at 4 ℃ to remove flocculent precipitates formed during freezing.

1) Dilution and spiking of standards: set up 10 wells of standards on the enzyme plate, add 50 μL

of standard diluent in the first well, then add 100 μL of standards in the first well, mix gently and aspirate 100 μL from the first well to the second well, then add 50 μL of standard diluent in the second well and mix gently; then aspirate 50 μL in the second well and discard it, then aspirate 50 μL to the third well. In the third well, 50 μL of standard diluent was added, gently mixed, and then

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50 μL was pipetted from the third well to the fourth well; in the fourth well, 50 μL of standard diluent was added, gently mixed, and then 50 μL was pipetted from the fourth well to the fifth well; in the fifth well, 50 μL of standard diluent was added, gently mixed, and then 50 μL was pipetted from the fifth well and discarded. The concentrations of EMAb standards were 6000 pg/mL, 4000 pg/mL, 2000 pg/mL, 1000 pg/mL and 500 pg/mL for each well, and the concentrations of AsAb standards were 60 pg/mL, 40 pg/mL, 20 pg/mL, 10 pg/mL and 5 pg/mL for each well, respectively. The concentrations of TAb standards were 150 ng/L, 100 ng/L, 50 ng/L, 25 ng/L, and 12.5 ng/L, and the concentrations of ACA-IgG standards were 120 U/mL, 80 U/mL, 40 U/mL, 20 U/mL, and 10 U/mL, respectively. CurveExpert 1.4 was used to plot the standard curves of each antibody
respectively.

Sample addition: Add 40 μL of sample dilution first, then aspirate 10 μL of the plasma to be tested and add it to the wells of the enzyme-coated plate without touching the bottom of the wells. At the same time, set up a blank control well without sample and enzyme reagents, and do the same for the
rest of the procedure. The test concentration is 1/5 of the final concentration.

2) Incubation: After sample addition, the plate was sealed with sealing film and incubated at 37 ℃ for 30 min.

3) Liquid preparation: Dilute 20 times the concentrated washing solution with distilled water 20 times and prepare for use.

4) Washing: Remove the sealing membrane from the edge, shake off the liquid inside the plate, pay attention to avoid cross-contamination between wells, add 200 μL of the prepared washing solution to each well with a row gun, let it stand for 30 s and then shake it off, repeat this 5 times and pat dry on filter paper.

5) Add enzyme standard reagent: 50 μL of enzyme standard reagent was added to each well, and blank wells were not added.

6) Incubation: After sample addition, the plate was sealed with sealing film and incubated at 37 ℃ for 30 min.

7) Washing: Remove the sealing membrane from the edge, shake off the liquid inside the plate, pay attention to avoid cross-contamination between wells, add 200 μL of the prepared washing solution to each well with a row gun, let it stand for 30 s and then shake it off, repeat this 5 times
and pat dry on filter paper.

8) Color development: add 50 μL of A chromogenic solution and then 50 μL of B chromogenic solution to each well, mix gently, and place the wells at 37 ℃ for 15 min to avoid light
development, and the chromogenic solution will turn blue.

9) Termination: Add 50 μL of termination solution to each well to terminate the reaction, at which time the blue color turns yellow. The absorbance (OD) of each well was measured sequentially at
450 nm wavelength.

Statistical analysis Methods

SPSS 24.0 was applied to statistically analyze the data obtained, and the mean ±standard deviation was used for measurement data and the chi-square test was used for comparison between

groups. Draw the receiver operating characteristic (ROC) curve, analyze the predictive value of LSP and S100A9 Protein Detection for pulmonary tuberculosis complicated with pulmonary infection, and calculate the area under the curve (AUC).The difference was considered statistically significant at
r<0.05.

RESULTS

LSP and S100A9 are significantly overexpressed in the sera of patients co-infected with pulmonary tuberculosis

We first analyzed the expression levels of LSP and S100A9 in the serum of patients with simple pulmonary tuberculosis and patients with pulmonary bacterial infection combined with pulmonary tuberculosis, and we found that the expression levels of S100A9 and LSP were significantly increased in patients with co-infected pulmonary tuberculosis (Fig 1A~B). Moreover, we further used Pearson correlation analysis to find no significant correlation between gender, age, and body mass index and the elevated expression of S100A9 and LSP (Fig 1C~E).

LSP and S100A9 were positively correlated with the level of M2 type macrophages in the peripheral blood of patients

According to available literature, tuberculosis infection leads to increased levels of PD-L1 expression, which reduces the inhibitory effect of the in vivo immune response against Mycobacterium tuberculosis. Thus, we further analyzed the proportion of different types of macrophages in the sera of patients. In the results, we found a significant increase in the proportion of M2 macrophages in the serum of patients with tuberculosis and a further increase in the proportion of M2 macrophages in the serum of patients further co-infected with pulmonary bacteria (Fig 2A), but we found that the proportion of M0 and pro-inflammatory M1 macrophages was significantly opposite to the proportion of M2 macrophages (Fig 2B~C). Furthermore, we further

analyzed the correlation between the serum levels of LSP and S100A9 and the proportions of M1 or M2 macrophages in the patients and showed that LSP and S100A9 were significantly positively correlated with the content of M2 macrophages and negatively correlated with the proportions of M1 macrophages (Fig 2D~E).

Significant correlation between LSP and S100A9 levels and resistance in tuberculosis patients

Subsequently, our analysis revealed that 25.8% of patients with co-infection had dinicinozid resistance, but only 11% of patients with TB infection alone had dinicinozid resistance (Fig 3A). Subsequently, we analyzed the predictive ability of LSP and S100A9 levels with respect to dinidazole resistance in TB patients using ROC curves, and we found that both LSP and S100A9 had good sensitivity and specificity for dinidazole resistance (Fig 3B~C).

Correlation of LSP and S100A9 levels with secondary respiratory failure in patients with pulmonary tuberculosis

Subsequently, our analysis revealed that 29% of patients with co-infection had secondary respiratory failure, but only 12% of patients with TB infection alone had dinicinozid resistance (Fig 4A). Subsequently, we used ROC curves to analyze the predictive ability of LSP and S100A9 levels with secondary respiratory failure in TB patients, and we found that both LSP and S100A9 had good sensitivity and specificity for secondary respiratory failure (Fig 4B~C).

DISCUSSION

Tuberculosis is a common and frequent chronic infectious disease caused by Mycobacterium tuberculosis. In recent years, due to the widespread use of fiberoptic bronchoscopy, it has been found that patients with pulmonary tuberculosis often have tuberculous lesions of the tracheal and bronchial mucosa: damage to the bronchial mucosa and submucosa, increased airway reactivity, and consequently decreased purification of the respiratory system. In addition, various

lesions of pulmonary tuberculosis impair the structural integrity of the lung tissue to varying degrees, such as

exudative lesions leading to tissue edema with subsequent infiltration of inflammatory cells, proliferative lesions leading to diffuse proliferative lesions, and caseous necrosis starting with tissue swelling followed by fatty degeneration to necrosis and liquefaction. These three basic pathological types are intertwined in patients with pulmonary tuberculosis, which provides very favorable conditions for fungal infection to colonize and grow. The incidence of fungal infections in hospitals is on the rise with the improvement of people's standard of living and the increasing elderly population, the increasing incidence of many serious clinical diseases that lead to immune deficiency such as acquired immunodeficiency syndrome, diabetes mellitus, chronic renal insufficiency, malignant tumors, and other medical factors such as the development of many invasive operating techniques and surgeries [9-10].

Long-term use of powerful broad-spectrum antibiotics and glucocorticoids is one of the common causes of fungal infections in the lungs. There are many kinds of normal flora in the human body, which are mutually exclusive and maintain a dynamic balance in the normal human body, but once this balance is disturbed, it may cause disease. In clinical practice, patients with tuberculosis are treated not only with anti-tuberculosis but also with antibiotics, because they are often accompanied by other bacterial infections. However, the long-term and repeated application of broad-spectrum antibiotics can lead to imbalance in the dynamic balance between normal flora, killing of sensitive bacteria and proliferation of conditionally pathogenic bacteria, leading to secondary infections.

The calcium-binding protein S100A9 is a protein closely associated with the inflammatory response.The S100A9 protein molecule belongs to a specific ligand structure [13] and forms a heterodimeric S100A8/A9 protein complex with S100A8 often in a calcium-dependent manner [14].S100A9 is widely distributed in human organs and tissues, including the normal human oral mucosa, tongue, esophagus , uterus, etc. It is now found to be involved in nerve impulse generation and transmission, muscle contraction, cell migration and growth differentiation, cell activation, regulation of enzyme activity, cell regulation, necrosis, and inflammation, trauma, and tumors.S100A9 protein can be specifically secreted to inflammatory foci, which can cause

chemotaxis and neutrophil adhesion, and is abundantly expressed in neutrophil-infiltrated lesions.S100A9 In vitro, it can inhibit mast cell degranulation and mast cell activation mediated by the IgE high-affinity receptor Fc εRI by scavenging reactiveoxygenspecies (ROS) [15, 16], which is

important for suppressing acute asthma attacks. In addition, S100A9 regulates tumor cell growth, proliferation, metabolism and invasion, and S100A9 produced by cancer cells converges to myeloid cells expressing macrophage antigen 1 to the lung [17, 18].

Endotoxin is a highly toxic pathogenic substance. It can cause a series of pathophysiological reactions in animals and humans, such as fever, shock, diffuse intravascular coagulation, B-lymphocyte division, and substantial damage to internal organs, which can be life-threatening in severe cases [19,20]. Among the substantial injuries to internal organs, lung injury often occurs first [21,22]. In the present study, the LSP and S100A9 protein were significantly higher in patients with TB combined with pulmonary infection than in those without infection, and the abnormal LSP and S100A9 protein were independent risk factors for the development of pulmonary infection in patients, suggesting that the abnormal LSP and S100A9 protein are closely related to TB combined with pulmonary infection and have a role in the development of TB. S100A9 protein changes can be used as a guide for early assessment of infection, but the value of each index and its difference are not yet known. Further ROC curve analysis showed that the diagnostic AUC of LSP + S100A9 was 0.832 (95% CI: 0.731-0.934), indicating that LSP and S100A9 have good diagnostic value for pulmonary tuberculosis combined with pulmonary infection, and the combination of the two can

improve the diagnostic sensitivity and specificity.

LIMITATION

The study was subject to a number of limitations. First, the sample size of the study is relatively small.Second, the measured value(LSP and S100A9) is single, and there are no multiple samples, so

the dynamic changes of parameters cannot be studied.

CONCLUSION

The detection of LSP and S100A9 protein levels in patients is beneficial for the early diagnosis of pulmonary tuberculosis combined with pulmonary infection, which is important for the assessment of disease progression and treatment plan selection.

DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available from the corresponding author upon request.

FUNDING STATEMENT

We haven't received any funding.

CONFLICT OF INTEREST

Authors have no conflict of interest to declare.

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FIGURE LEGENDS

Fig 1. LSP and S100A9 were significantly overexpressed in the serum of patients co-infected with pulmonary tuberculosis. a~b, ELISA to detect the levels of S100A9 and LSP in the serum of patients with pulmonary tuberculosis; c~e, Pearson correlation analysis of the correlation between gender, age, body mass index and the levels of S100A9 and LSP in the serum of patients.

Fig 2. LSP and S100A9 were positively correlated with the content of M2 macrophages in patients' peripheral blood. a-C, Flow cytometry analysis of the proportion of M0, M1, and M2 macrophages in the serum of TB patients; D-E, Pearson correlation analysis of the correlation between the content of LSP and S100A9 in the serum of patients and the proportion of M1 or M2 macrophages.

Fig 3. The levels of LSP and S100A9 were significantly correlated with drug resistance in TB patients. a, Analysis of levels in patients with dinicinozid resistance; ROC curves analyzed the predictive ability of the levels of LSP and S100A9 with dinicinozid resistance in TB patients.

Fig 4. Correlation of LSP and S100A9 levels with secondary respiratory failure in patients with pulmonary tuberculosis. a, Analysis of levels in patients with dinicinozid resistance; ROC curves analyzed the predictive power of LSP and S100A9 levels with dinicinozid resistance in patients with pulmonary tuberculosis.

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