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Efficacy and safety of recombinant *Mycobacterium tuberculosis* ESAT-6 protein for diagnosis of pulmonary tuberculosis: A phase II trial

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Manuscript Preparation E
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Background: This study aimed to determine the efficacy and safety of recombinant *Mycobacterium tuberculosis* ESAT-6 protein for diagnosis of pulmonary tuberculosis (TB).

Material/Methods: A phase II trial was performed in 158 patients with pulmonary TB (145 initially-treated and 13 re-treated) and 133 healthy subjects. Skin testing was carried out by injecting purified protein derivative (PPD) (on left forearm) or recombinant ESAT-6 protein at a dosage of 2, 5, or 10 μg/mL (on the right forearm) in each subject. Reaction activity and adverse events were monitored at 24, 48, and 72 h following the injection. Receiver operating characteristic curves were plotted to determine the areas under the curves (AUCs) and the cut-off induration diameters for the optimal diagnostic performance.

Results: The reaction activity was significantly increased upon recombinant ESAT-6 injection in pulmonary TB patients compared with healthy subjects. In pulmonary TB patients, the reaction was dose-dependent, and at 48 h, 10 μg/mL recombinant ESAT-6 produced a reaction similar to that produced by PPD. The AUCs for a 10 μg/mL dosage were 0.9823, 0.9552, and 0.9266 for 24 h, 48 h, and 72 h, respectively, and the induration diameters of 4.5–5.5 mm were the optimal trade-off values between true positive rates and false positive rates. No serious adverse events occurred in any subjects.

Conclusions: Recombinant ESAT-6 protein is efficacious and safe for diagnosing pulmonary TB. Based on the reaction, performance, safety, and practicability, we recommend that 10 μg/mL at 48 h with an induration cut-off value of 5.0 mm be used.

Key words: pulmonary tuberculosis • recombinant ESAT-6 protein • skin testing • phase II trial

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Background

Tuberculosis (TB), an infectious disease caused by *Mycobacterium tuberculosis*, is one of the major morbidities and mortalities worldwide [1–3]. *M. tuberculosis* typically affects the lungs (so-called pulmonary TB) although it can also affect other sites (extrapulmonary TB). Pulmonary TB is a serious medical problem with social and economic burdens, especially in developing countries. Several factors may delay the diagnosis and treatment of pulmonary TB, such as health care system delays [4]. Many regimens have been established for the treatment of pulmonary TB over the last several decades, including isoniazid, rifampicin, pyrazinamide, ethambutol, and streptomycin. However, eradication of pulmonary TB has been proven elusive. In 2009, the treatment success rate among new cases of smear-positive pulmonary TB was 87% world-wide [5]; treatment fails in the remaining cases due to the resistance of *M. tuberculosis* to antimicrobials. The prevalence of pulmonary TB is increasing annually, and the incidence rate was reported to be 0.114% in Sichuan, China in 2005 [6]. Early detection of *M. tuberculosis* infection will enable early treatment of the infection, which will help prevent pulmonary TB and reduce incidence of the disease. In addition, accurate diagnosis of pulmonary TB, especially at the early stage, will ensure early treatment and thus increase the success rate in the treatment of pulmonary TB. Therefore, early and accurate detection of *M. tuberculosis* is critical.

A variety of technologies have been used to diagnose pulmonary TB, including medical imaging (e.g., chest radiography), microbiology tests (e.g., sputum smear microscopy), histopathology, and immune-based tests (eg, serologic, antibody detection tests, antigen detection tests, interferon-γ release assays, and skin tests) [7]. Among these, the skin test is the most widely used screening approach. The delayed-type hypersensitivity skin test reaction to tuberculin purified protein derivative (PPD) is widely used for the detection of *M. tuberculosis* infection [8]. However, the main drawback of this method is the lack of specificity, as it cannot distinguish *M. tuberculosis* infection from bacilli Calmette-Cuérin (BCG) vaccination or infection with non-tuberculous *Mycobacteria* [9,10]. Thus, screening and development of novel antigens is given high priority for the improvement of the current tools for pulmonary TB prevention, diagnosis, and disease management.

Region of difference 1 (RD1) is reported to be absent from strains of *M. bovis* BCG but is present in virulent strains of the tested *M. bovis* and *M. tuberculosis* [11–13]. RD1-encoded proteins, such as the early secreted antigenic target 6-kDa protein (ESAT-6) and culture filtrate protein 10 (CFP-10), have been developed as new specific diagnostic tests for *M. tuberculosis* infection [14]. However, the sensitivity, specificity, and safety of RD1-encoded proteins still need to be further evaluated. A recombinant 11 kD protein at the RD1 region of *M. tuberculosis*, which is equivalent to ESAT-6, has been successfully expressed [15]. It has been demonstrated that this protein can differentiate infection with live *M. tuberculosis* from immunization with killed *M. tuberculosis*, live BCG, or mycobacterioses other than TB in guinea pigs [16]. However, its promise for the use in the skin test has never been evaluated in humans. In a phase I study, we performed a preliminary assessment of the safety of a recombinant *M. tuberculosis* ESAT-6 protein in Chinese volunteers [17]. Then, we performed a phase II trial to examine the diagnostic performance and safety of the recombinant *M. tuberculosis* ESAT-6 protein used as a skin test for the diagnosis of pulmonary TB.

Material and Methods

Reagents

The recombinant ESAT-6 was produced in compliance with good manufacturing process conditions and provided by Beijing Xiangrui Biological Products Co., Ltd. (Beijing, China) at different concentrations.

Study subjects

A total of 158 patients with confirmed pulmonary TB, ranging from 18 to 65 years old, were selected in the Third Affiliated Hospital to Wenzhou Medical College and the Center for Disease Control and Prevention of Shanggao County, between January 1, 2009 and August 31, 2009. All these patients met pulmonary TB diagnostic criteria and received BCG vaccination therapy. None of the patients had vital organ failures, diabetes, autoimmune diseases, acute infectious disease, human immunodeficiency virus infection, nervous system diseases, long-term corticosteroid treatment, or any concurrent anti-tumor therapy. Patients who attended or were participating in other trials in the previous 3 months were excluded. Pregnant or breast-feeding women, and patients with allergic diathesis or drug or alcohol addiction were also excluded.

The selected patients consisted of 2 groups of patients, 145 treated initially and 13 re-treated. Initially-treated patients were defined as those with no previous anti-TB treatment or with previously irregular treatment or with less than 1 month of anti-TB chemotherapy. Re-treated patients were defined as those who failed initial treatment: pulmonary TB recurred after initial treatment for a complete course, continuous positivity in the sputum for 1–2 years without formal chemotherapy, or occurrence of new lesions or recurrence or deterioration of the lesions after surgical resection of the affected lung. In addition, 133 healthy volunteers aged 21–60 years who attended routine physical examinations were selected as a control cohort. Those with previous or current pulmonary and extrapulmonary

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tuberculosis, respiratory or other systemic symptoms, acute or chronic diseases, acute infectious disease, skin disease or skin allergy due to various causes, or close contact with individuals with TB were excluded from the healthy control cohort.

Written informed consent was obtained from every subject, and this study was approved by the Ethics Committee at Beijing Chest Hospital.

Detection of *M. tuberculosis* in the sputum and diagnosis of bacterium-positive and bacterium-negative pulmonary TB

Acid-fast smears were performed using a standard concentration method and the Ziehl-Neelsen acid-fast stain. Mycobacterial cultures were performed using Löwenstein-Jensen media. The sputum bacterium-positive status of *M. tuberculosis* was defined as when the sputum smear for acid-fast bacilli and/or culture was positive for *M. tuberculosis* within 3 months prior to the experiment. Sputum bacterium-negative status of *M. tuberculosis* was defined as when both of the above tests were continuously negative within 3 months before the experiment.

A subject was diagnosed as having pulmonary TB when 3 of the following items from 1–6 or 1 of items from 7 and 8 were met [18]: (1) typical clinical symptoms and chest X-ray results; (2) efficient anti-tuberculous chemotherapy; (3) exclusion of nontuberculous lung disease; (4) strongly positive for 5 IU PPD-T and positive for serum anti-TB antibody; (5) positive sputum as detected by PCR; (6) TB as determined by histopathology of extra-pulmonary organs; (7) positive for acid-fast bacilli as examined by BALF; and (8) TB as determined by histopathology of bronchi or lung tissues. Subjects who met the diagnostic criteria for pulmonary TB and were sputum-positive for *M. tuberculosis* were defined as having bacterium-positive pulmonary TB, whereas those who met the diagnostic criteria for pulmonary TB but were sputum-negative for *M. tuberculosis* were defined as having bacterium-negative pulmonary TB.

Skin testing

PPD skin testing was performed by injecting 5 international units (IU) (0.1 mL) of TB-PPD (TB-PPD; Beijing Xiangrui Biologicals Co., Ltd., Beijing, China) (n=291) into the skin of the volar aspect of the left forearm. Then, recombinant ESAT-6 skin testing was performed by injecting 2 (n=94), 5 (n=100), or 10 μg/mL (n=97) of recombinant ESAT-6 (Beijing Xiangrui Biologicals Co., Ltd., Beijing, China) into the skin of the volar aspect of the right forearm. Subjects were monitored 24, 48, and 72 h after the test. The diameter in millimeters (mm) of the induration at each skin test site was measured at each visit. Testing and reading were performed by highly experienced technicians using standardized procedures. At each visit, any adverse events, including skin reactions, were recorded. In addition, all subjects received follow-up 7 days after the test, and those who experienced adverse events at this time were treated appropriately if necessary and followed-up 7 days later.

Data collection and statistical analysis

The primary objective of this study was to determine the diagnostic performance of different doses of the recombinant ESAT-6 protein used in a skin test and identify the optimal dose. Thus, the primary endpoint was diagnostic performance, as reflected by the receiver operating characteristic (ROC) curve and subsequent sensitivity, specificity, positive (PPV) and negative (NPV) predictive values, and accuracy resulting from the ROC curve. In addition, the safety profiles (ie, the occurrence and extent of adverse events including skin reactions) of the recombinant ESAT-6 protein used in a skin test were also assessed.

The ROC curves were plotted in MATLAB R2010 (The MathWorks, Inc. Natick, USA). The best cut-off value for each dosage of recombinant ESAT-6 protein for clinical use was defined as the corresponding point in the ROC curve which was closest to the upper left corner, and thus with the highest overall accuracy of the test [19]. Then, the optimal dose was defined as the dose that produced the highest accuracy with satisfactory safety profiles. In addition, continuous data were presented as means ± standard error of the mean (SEM). ANOVA with a post hoc test was used to determine the difference in numerical variables among the different doses of recombinant ESAT-6 protein, whereas the paired t-test was used to determine the difference in numerical variables between the PPD and each of the different doses of recombinant ESAT-6 protein. The statistical analysis was performed with SPSS software (version 16.0, SPSS Inc. IL, USA). A P value of <0.05 was considered as statistically significant.

Results

Demographic characteristics of the study subjects

As shown in Table 1, a large portion of the study subjects in the healthy control group, the initially-treated patients and the re-treated patients were 20 to 39 year-old adults. More subjects over 40 years of age were found in re-treated patients as compared with other groups. There were more males than females in each group. The majority of the pulmonary TB patients underwent chest X-ray examination only once, whereas in the re-treated patient group, more subjects had received a chest X-ray examination at least twice. Moreover, an elevated incidence of pulmonary TB cavity was detected in re-treated patients as compared with the initially-treated patients. Most of the patients in the initially-treated and re-treated groups had a short course

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Table 1. Demographical and clinical characteristics of the study subjects.

	Healthy control (n=133)	Initially-treated patients (n=145)	Re-treated patients (n=13)
Age			
<20 yrs	1 (0.8)	4 (2.8)	0 (0)
20–39 yrs	73 (54.9)	101 (69.7)	9 (69.2)
≥40 yrs	59 (44.4)	40 (27.6)	4 (30.8)
Gender			
Male	76 (57.1)	100 (69.0)	11 (84.6)
Female	57 (42.9)	45 (31.0)	2 (15.4)
Chest radiography			
Less than once		1 (0.7)	0 (0)
Once		113 (77.9)	7 (53.8)
Twice		18 (12.4)	4 (30.8)
More than twice		13 (9.0)	2 (15.4)
With pulmonary TB cavity		21 (14.5)	6 (46.2)
Course of the treatment			
<1 wk		129 (89.0)	11 (84.6)
1–2 wks		13 (9.0)	0 (0)
>2 wks		3 (2.1)	2 (15.4)
Sputum bacterium status			
Positive		82 (56.6)	10 (76.9)
Negative		63 (43.4)	3 (23.1)

Data were presented as the number of cases and the percentages in parenthesis.

of treatment (less than 1 week). More patients with bacterium-positive results were observed in the re-treated group as compared with the initially-treated patients (76.9% *vs.* 56.6%).

Reactions to recombinant ESAT-6 at different doses and time points

Skin testing results in healthy controls (n=133), initially-treated patients (n=145), and re-treated patients (n=13) are presented as percentages of subjects with different ranges of induration diameters in each group (Figure 1). As shown in Table 2, significantly increased reaction activity was detected upon recombinant ESAT-6 or PPD injection in pulmonary TB patients as compared with healthy subjects. Moreover, the reaction activity was gradually enhanced with the increased dosage of recombinant ESAT-6 protein in healthy subjects as well as in pulmonary TB patients. Administration of 2 μg/mL recombinant ESAT-6 produced significantly smaller induration as compared with PPD injection at all time-points in patients with pulmonary TB. Administration of 5 μg/mL or 10 μg/mL recombinant ESAT-6 produced the skin reaction similar to that obtained by PPD at all time-points in initially-treated patients (Table 2). However, 5 μg/mL recombinant ESAT-6 produced a weaker reaction than PPD in re-treated patients at all time points. Whereas the skin reaction to 10 μg/mL recombinant ESAT-6 was similar to that to PPD at 48 h, it was stronger at 24 h, but weaker at 72 h than that to PPD in re-treated patients (Table 2). These results indicate that 10 μg/mL recombinant ESAT-6 administration at 48 h produced a reaction similar to the PPD test at the same time in initially-treated and re-treated patients.

Diagnostic performance of recombinant ESAT-6 at different doses and different time-points

To evaluate the specificity and sensitivity of skin testing with recombinant ESAT-6 in pulmonary TB patients, ROC curves

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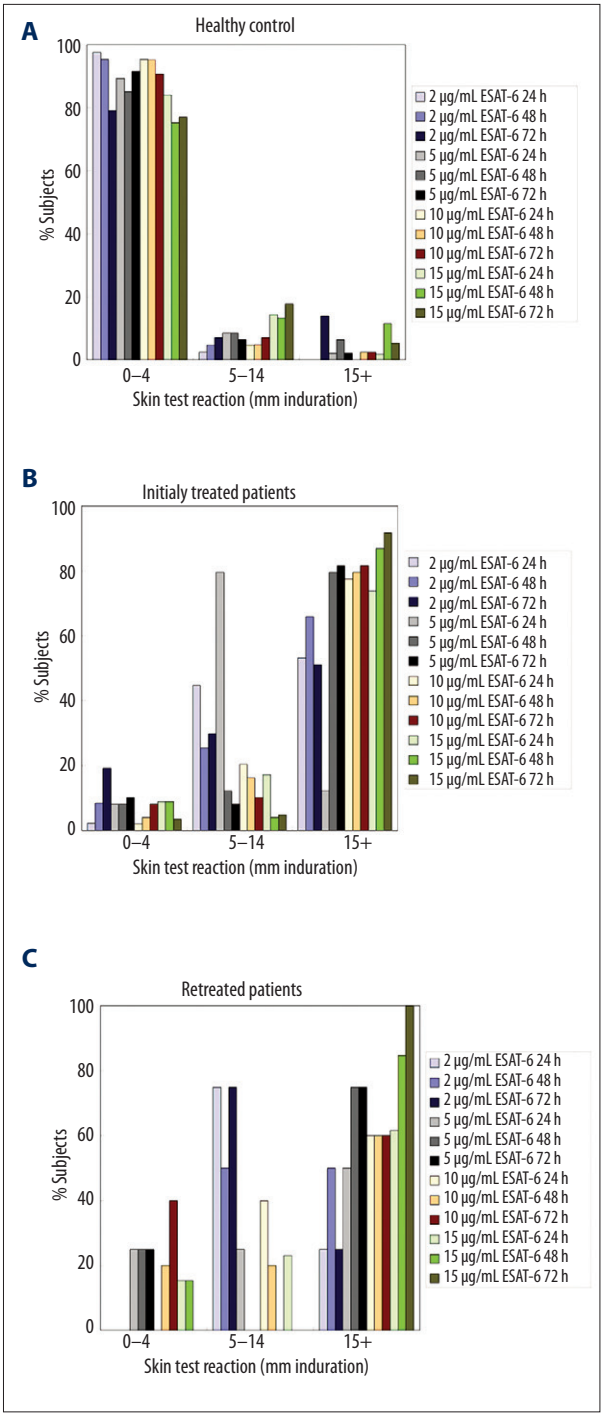

Figure 1. Skin testing results for healthy controls (n=133), initially-treated patients (n=145) and re-treated patients (n=13). Data were presented as percentage of the total subjects in each group.

were drawn (Figure 2). Due to the small number of re-treated patients (n=13), this cohort was combined with the initially-treated patients (n=145) for evaluation. The areas under the curves (AUCs) for a dose of 10 μg/mL were 0.9823, 0.9552, and 0.9266 for 24, 48, and 72 h, respectively, suggesting the high specificity and sensitivity of ESAT-6 skin testing (Figure 2). Based on the above ROC curves, a cut-off value can be set at between 4.5 and 5.5 mm (e.g., 5.0 mm) to obtain a good trade-off between true positive rates (TPRs) and false positive rates (FPRs). Accordingly, the sensitivity, specificity, PPV, NPV, and accuracy at the cut-off value of 5.0 mm were generated for 3 doses at different time-points (Table 3).

More initially-treated patients showed a strong positive (double circle) reaction 48 h after PPD than healthy subjects and re-treated patients (15.2%, 0.8%, and 0%, respectively, Table 4). However, approximately one-fourth of the initially-treated patients at doses 5 or 10 μg/mL ESAT-6 and one-third of re-treated patients at a dose of 10 μg/mL ESAT-6 presented a double circle, whereas none of the healthy control showed a double circle with the same treatment, suggesting the enhanced sensitivity of recombinant ESAT-6 protein as compared with PPD. All 3 re-treated patients with strong reaction for the ESAT-6 skin test were sputum bacterium-positive (Table 4). However, approximately half of the initially-treated patients with a strong reaction to the 5 or 10 μg/mL ESAT-6 skin test were sputum bacterium-positive.

Safety

To further examine the safety of recombinant ESAT-6 protein administration, all subjects were carefully monitored after injection. A slight itch at the site of injection was not recorded. One case in 5 μg/mL and 1 case in 10 μg/mL ESAT-6 protein administration had an obvious itch at the injection site after injection. No severe adverse events occurred in healthy subjects after skin testing with recombinant ESAT-6 protein at a dose of 2, 5, or 10 μg/mL, although 2 cases had skin blisters after 10 μg/mL ESAT-6 protein administration. However, 2 cases had itch and 5 cases had skin blisters after PPD injection. In addition, no adverse events occurred in initially-treated or re-treated patients after recombinant ESAT-6 protein administration, while 2 initially-treated patients showed increased alanine and aspartate aminotransferases (ALT and AST) levels on Day 7 after PPD skin test. Fever before and after skin test was present in 19, 24, and 23 subjects, and the skin test did not enhance fever status. Therefore, the incidence of fever may not be related to the skin test.

Recommendation of the cut-off of diameter of induration, the optimal dose and time-point

Based on the reaction, the diagnostic performance, safety profiles, and practicability, we recommend that administration of 10 μg/mL at 48 h with an induration cut-off value of 5.0 mm be used for recombinant ESAT-6 skin testing.

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Table 2. Skin testing results at indicated time points for healthy subjects, initially-treated patients and re-treated patients.

	Time point (h)	2 μg/mL ESAT-6 (n=94)		5 μg/mg ESAT-6 (n=100)		10 μg/mL ESAT-6 (n=97)		50 IU/mL PPD (n=291)
		Mean ±SEM	Ratio	Mean ±SEM	Ratio	Mean ±SEM	Ratio	Mean ±SEM
Healthy subjects (n=133)	24	0.2±0.2*	0.16	1.0±0.4	0.78	1.0±0.4	0.82	1.3±0.3
	48	0.5±0.3*	0.18	2.5±0.9	0.94	1.1±0.6	0.43	2.6±0.5
	72	0.7±0.4*	0.31	3.1±0.9	1.29	1.3±0.7	0.54	2.4±0.4
Initially-treated patients (n=145)	24	14.1±0.9*#	0.77	19.6±1.3#	1.08	20.7±1.2#	1.14	18.2±0.7#
	48	17.7±1.7*#	0.65	29.5±2.9#	1.08	29.8±2.9#	1.09	27.4±1.2#
	72	16.9±2.0*#	0.60	30.0±3.5#	1.07	30.9±3.1#	1.10	28.0±1.4#
Re-treated patients (n=13)	24	9.8±1.8#	0.61	14.1±5.0#	0.88	20.9±4.2#	1.30	16.1±2.6#
	48	13.5±3.8#	0.55	14.5±5.2#	0.58	23.3±7.2#	0.94	24.7±3.7#
	72	15.5±5.0#	0.55	14.1±4.8#	0.50	17.3±7.2*#	0.61	28.4±4.1#

Data are presented as mean ± SEM (mm in diameter) or ratio of ESAT-6 over PPD. * $P<0.05$ compared with PPD; # $P<0.05$ compared with healthy subjects.

Discussion

Diagnosis of TB is generally based on clinical suspicion and the response to anti-TB immune therapy. Although detection of acid-fast bacilli or mycobacterial cultures provides indicative values, it cannot serve as a definite diagnostic method due to its complicated procedures. The tuberculin skin test (i.e., the PPD test) has been the most widely-used approach for screening apparently healthy subjects for infection with *M. tuberculosis* since the early 1930s [10]. In addition, the PPD test is the most widely-used skin test for the detection of *M. tuberculosis* infection [8]. However, the specificity of the test is poor due to the cross-reactivity with proteins present in other mycobacteria, such as *M. bovis* BCG [9,10]. Therefore, there is an urgent need to develop novel proteins or antigens for the diagnosis of the diseases.

The recombinant ESAT-6 protein at the RD1 region has been successfully expressed and reported to be capable of distinguishing infection with *M. tuberculosis* from immunization with BCG or mycobacterioses other than TB in guinea pigs [15,16]. However, the efficacy and safety of its application in skin testing for pulmonary TB patients has not yet been fully illustrated. Therefore, we investigated the dosage, reaction and reaction time, performance, and safety for the use of recombinant *M. tuberculosis* ESAT-6 protein for the diagnosis of pulmonary TB. In the present study, 145 initially-treated and 13 re-treated patients with pulmonary TB were included. More pulmonary TB patients were males, which is consistent with other studies in the Chinese population, and is also in accordance with other populations in low-income countries [6,20,21].

Wu et al. reported for the first time the use of recombinant ESAT-6 protein in skin testing in human volunteers [22]. Their results showed that 1.0 μg (in 0.1 mL) of purified recombinant ESAT-6 antigen of *M. tuberculosis* produced a similar intensity of reaction in the skin test to that produced by the PPD antigens in humans [22]. In the same year, Arend et al. carried out a double-blind randomized phase I study to compare the efficacy of recombinant dimmer ESAT-6 synthesized in Lactococcus and tuberculin as a skin test reagent in the diagnosis of tuberculosis infection [23]. They showed that in treated TB patients, the responses to recombinant dimmer ESAT-6 were optimal at 0.1 μg (in 0.1 mL) [23]. Our phase I study showed that intradermal injection of 0.1 mL of 1 (15 cases), 5 (18 cases), and 10 μg/mL (10 cases) recombinant ESAT-6 protein is safe for human volunteers, and only a few local adverse events such as pain, itching, and blister occurred following ESAT-6 injection [17]. In the present study, administration of 0.1 mL of all doses, ranging from 2 to 10 μg/mL of ESAT-6, was well-tolerated by healthy volunteers and patients with pulmonary TB. Two healthy subjects had skin blisters after PPD injection and 2 initially-treated patients showed increased ALT and AST levels on Day 7 after the PPD skin test, whereas no serious adverse events were observed in subjects injected with recombinant ESAT-6 protein. These findings indicate the safety of recombinant ESAT-6 protein for clinical use of skin testing at doses from 2 to 10 μg/mL; however, it should be noted that the present study was not sufficiently powered to demonstrate complete safety profiles.

In patients with pulmonary TB, administration of 2 μg/mL recombinant ESAT-6 produced significantly smaller induration

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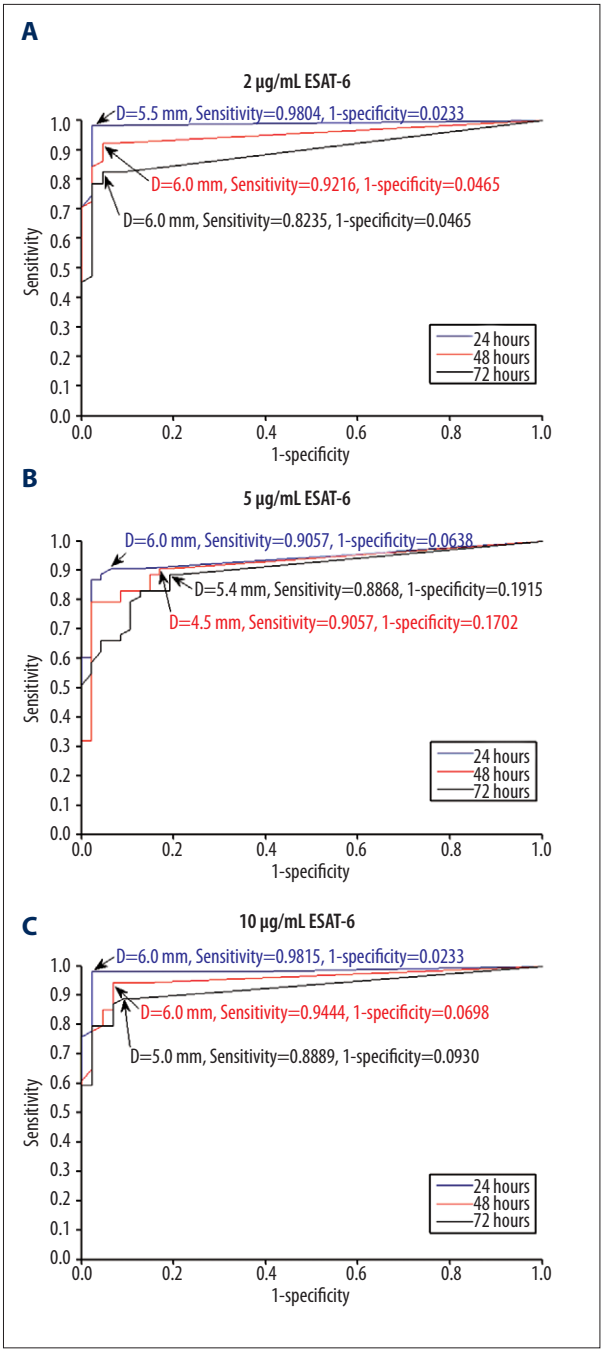

Figure 2. Receiver operating characteristic (ROC) curves for ESAT-6 injection at 2 μg/mL (**A**), 5 μg/mL (**B**) and 10 μg/mL (**C**). The turning point of each ROC curve is marked with an arrow, with the corresponding diameter of the induration, true positive rate, and false positive rate indicated.

compared with PPD injection at all time-points. Administration of 5 μg/mL or 10 μg/mL recombinant ESAT-6 produced the skin reaction similar to that obtained by PPD at all time-points in initially-treated patients, whereas 5 μg/mL recombinant ESAT-6 produced a weaker reaction than PPD in re-treated patients at all time-points. Notably, the skin reaction to 10 μg/mL recombinant ESAT-6 was similar to that to PPD at 48 h, and it was stronger at 24 h, but weaker at 72 h than that to PPD in re-treated patients. Therefore, the optimal reaction time was set as 48 h following the injection, which is also in accordance with the use of the recombinant ESAT-6 antigen [22]. It should be mentioned that, in guinea pigs, the strongest reaction was observed 24 h after recombinant ESAT-6 protein injection at doses of 5 to 20 μg/mL [16]. This discrepancy may be due to the differences in the maximum response times between the animal model and humans.

The optimal cut-off value for skin testing of ESAT-6 needs to be carefully considered. Although administration of 2 μg/mL ESAT-6 at 24 h produced the best accuracy (97.87%) at a cut-off of 5.0 mm, the induration size was smaller than PPD injection at the same time-points. Also, 10 μg/mL at 24 h gives gave a high accuracy (94.85%). However, considering that the skin reaction to 10 μg/mL recombinant ESAT-6 was similar to that to PPD only at 48 h, we recommend choosing a dose of 10 μg/mL and a reaction time of 48 h for recombinant ESAT-6 skin testing. In the present study, based on the ROCs, a cut-off induration diameter of 6.0 mm was generated. However, for practicability, we would recommend a cut-off value of 5.0 mm to keep it consistent with that for PPD. Thus, in the present study, a skin test with recombinant ESAT-6 protein at a dose of 10 μg/mL, for a reaction time of 48 h and with an induration cut-off of 5.0 mm, yielded an accuracy of 89.69%, which is higher than that of PPD (85.17%).

The interferon gamma assay is also commonly used to diagnose TB infection, showing the highly specificity. Considering the high cost and complex operation by conducting interferon gamma assay, it is not suitable for countries with high prevalence of TB infection, such as China. Therefore, the recombinant ESAT-6 protein is better because it is easy to use and is low-cost.

Moreover, this phase II study aimed to determine the efficacy and safety of recombinant ESAT-6 protein for diagnosis of pulmonary TB. In China, BCG vaccination is part of the national immunization program, and thus most (if not all) of the healthy subjects have already received BCG vaccination. Therefore, the BCG vaccination history was not specifically recorded, which is obviously a limitation of the present study, as we could not compare the effects of ESAT-6 recombinant protein between BCG-vaccinated or non-vaccinated subjects. In our phase III study, we will carefully choose the study cohort with a randomized controlled double-blind design. We will choose subjects with both ESAT-6 and PPD negative reactivity and give them BCG vaccination. Then, we will compare the different diagnostic effects of ESAT-6 and PPD.

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Table 3. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of different doses of recombinant ESAT-6 with a cut-off value of 5.0 mm at different time points.

	Time points (h)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
2 μg/mL ESAT-6	24	98.04	97.67	98.04	97.67	97.87
	48	92.16	95.35	95.92	91.11	93.62
	72	82.35	90.70	91.30	81.25	86.17
5 μg/mL ESAT-6	24	90.57	89.36	98.04	97.07	92.00
	48	88.68	82.98	95.92	91.11	88.00
	72	86.79	80.85	91.30	81.25	81.00
10 μg/mL ESAT-6	24	98.15	93.02	98.04	97.67	94.85
	48*	**94.44**	**90.70**	**94.00**	**90.91**	**89.69**
	72	88.89	90.70	91.30	81.25	83.51
50 IU/mL PPD	24	90.45	86.47	88.75	88.46	88.62
	48	90.45	78.95	83.53	87.50	85.17
	72	96.82	80.30	85.39	95.50	89.27

* Recommended for recombinant ESAT-6 skin testing, with consideration of reaction, diagnostic performance, safety profiles, and practicability.

Table 4. The number of subjects with strong (double circle) recombinant ESAT-6 protein or PPD positive reaction at 48 h, in relation to sputum bacterium status of *M. tuberculosis*.

Subjects	Number (%) of subjects with strong (double circle) reaction			
	2 μg/mL ESAT-6	5 μg/mL ESAT-6	10 μg/mL ESAT-6	50 IU/mL PPD
Healthy subjects (n=133)	0	0	0	1 (0.8)
Initially-treated patients (n=145)	2 (4.3)	13 (26.5)	12 (24.5)	22 (15.2)
Sputum bacterium-positive	0	6 (12.2)	5 (10.2)	9 (6.2)
Sputum bacterium-negative	2 (4.3)	7 (14.3)	7 (14.3)	13 (9.0)
Re-treated patients (n=13)	1 (25)	0	2 (33.3)	0
Sputum bacterium-positive	1 (25)	0	2 (33.3)	0
Sputum bacterium-negative	0	0	0	0

Conclusions

Recombinant ESAT-6 protein is efficacious and safe for diagnosing pulmonary TB. Based on the reaction, performance, safety, and practicability, a dose of 10 μg/mL at 48 h with an induration cut-off value of 5.0 mm is recommended for the skin testing of recombinant ESAT-6 injection. This phase II dose-finding trial provides proof of principle of a specific skin test for human use. Future investigation will be continued to investigate the association between the diagnostic value of skin testing with recombinant ESAT-6 protein and disease course of TB.

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