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Enzyme-linked immunospot assay response to recombinant CFP-10/ESAT-6 fusion protein among patients with spinal tuberculosis: implications for diagnosis and monitoring of surgical therapy

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SUMMARY

Objective: This study aimed to assess the performance of a laboratory-developed recombinant CFP-10/ESAT-6 fusion protein (rCFP-10/ESAT-6)-based enzyme-linked immunospot (ELISPOT) assay for the diagnosis of spinal tuberculosis (TB) in China, and to evaluate the value of the ELISPOT assay for monitoring the efficacy of surgical treatment.
Methods: In the first part of the study, a total of 78 participants were consecutively recruited for ELISPOT using rCFP-10/ESAT-6 as a stimulus. The cutoff value for ELISPOT positivity was based on the results of receiver operating characteristic curve analysis. In the second part, this approach was evaluated in a prospective study including 102 patients with suspected spinal TB. Data on clinical characteristics of the patients and conventional laboratory results were collected, and blood samples were obtained for ELISPOT using rCFP-10/ESAT-6 as a stimulus.
Results: Among the 102 patients with suspected spinal TB, 11 were excluded from the study. Twenty-three patients (25.2%) had culture-confirmed TB and 29 (31.9%) patients had probable TB. Among the spinal TB patients, the ELISPOT had a sensitivity of 82.7%, compared to a sensitivity of 61.5% for the purified protein derivative (PPD) skin test. The specificity was 87.2% for ELISPOT and 46.2% for the PPD skin test among 39 subjects with non-TB disease. The number of spot-forming cells and/or the positive rate of the ELISPOT assay were associated with aging, emaciation, and paravertebral abscess. The number of subjects with responses to rCFP-10/ESAT-6 slightly decreased after surgical treatment in spinal TB patients.
Conclusions: A laboratory-developed rCFP-10/ESAT-6 ELISPOT assay is a useful adjunct to current tests for the diagnosis of spinal TB.

1. Introduction

Spinal tuberculosis (TB) is found in 1–3% of all TB cases and in 50–60% of cases of musculoskeletal TB.[1] However, the diagnosis of spinal TB is difficult to set, not only because of its nonspecific clinical presentation, but also because of the lack of useful diagnostic tests. Diagnostic methods used for spinal TB are tuberculin skin testing, radiographic image examination, culture, and PCR assays. Tuberculin skin testing is a conventional test with a sensitivity ranging from 67% to 72%, but it cannot differentiate between active and past infections and can also be positive due to bacille Calmette–Guérin (BCG) vaccination.[2] Most importantly, confirmation testing by mycobacterial culture is time-consuming and often takes weeks to complete. Thus, there is a need for a rapid and accurate diagnostic test.

An in vitro T-SPOT-TB assay using pools of early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) peptides, has recently been manufactured and commercialized (Oxford Immunotec, Oxford, UK).[3] It is based on the detection of interferon-gamma (IFN-γ) released by activated T lymphocytes. The stimuli used, ESAT-6 and CFP-10 peptides, are located within region of difference 1 (RD1) of the *Mycobacterium tuberculosis* and *Mycobacterium bovis* genomes, but is absent from all strains of *M. bovis* BCG, as well as from most non-tuberculous mycobacteria (NTM).[4–6] This assay has been demonstrated to provide useful support in the diagnosis of skeletal TB.[7,8] However, the use of peptides increases the cost of the kit, making it unaffordable in developing countries. A fusion protein as the stimulus would offer

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a cheaper and more realistic alternative for large-scale production and clinical use in developing countries. A laboratory-developed recombinant CFP-10/ESAT-6 fusion protein (rCFP-10/ESAT-6)-based enzyme-linked immunospot (ELISPOT) assay has been demonstrated to be useful for the diagnosis of active pulmonary TB in China.[9] The aim of this study was to assess the diagnostic value of a laboratory-developed rCFP-10/ESAT-6 ELISPOT assay in clinical cases of spinal TB in China. In subgroups of subjects, we repeated the assay before and after surgical treatment to assess the evolution of responses. To our knowledge, this is the first clinical evaluation of rCFP-10/ESAT-6 as the stimulus in an ELISPOT assay for the diagnosis of spinal TB and for monitoring the efficacy of surgical treatment.

2. Materials and methods

2.1. Participants and study design

Participants were consecutively recruited at two hospitals (Nanfang Hospital, Southern Medical University, an 1800-bed medical center in southern China; Guangzhou Thoracic Hospital, a 600-bed medical center in southern China) from May 2011 to September 2012. Ethical approval for the study was granted by the ethics committee of Nanfang Hospital.

In phase 1, 78 participants were consecutively enrolled into the study. Patients were untreated or had received <2 weeks of anti-TB therapy at the time of venipuncture for the ELISPOT assay. Fifty cases had a final diagnosis determined according to the following criteria: patients classified as having confirmed TB were those with clinical specimens positive for *M. tuberculosis* on culture; patients were classified as having probable TB if histological findings of a biopsy specimen were consistent with a diagnosis of TB infection (granulomatous inflammation and/or caseating necrosis) and if they responded clinically and radiologically to a full course of anti-TB treatment according to the criteria described in previous studies;[10,11] patients were classified as not having TB if another diagnosis was made or if there was clinical improvement without anti-TB therapy. Enrolled participants included 30 spinal TB patients, 20 non-TB patients, and 28 healthy volunteers. Based on the rCFP-10/ESAT-6 ELISPOT results of these participants, receiver operating characteristic curve analysis was performed to determine the optimal cutoff value of the ELISPOT assay.

In phase 2, a total of 102 patients with suspected spinal TB were prospectively recruited during the study period. Data on clinical characteristics of the patients and conventional laboratory results were collected. The specific data collected were age, sex, underlying diseases, radiographic image examination, lymphocyte count, pathology, microbiology results, and follow-up observations. All cases were independently classified by two of the study investigators (QZ, JC) who were blinded to the ELISPOT assay results. Diagnostic criteria were based on the above standard. After a follow-up to September 2012, 11 patients were excluded from the study, among whom five did not complete the ELISPOT assay and six had no final diagnosis. The remaining 91 patients were ultimately included for ELISPOT analyses. The spinal TB group consisted of 52 cases (23 confirmed cases and 29 probable cases), with a mean age of 42.3 ± 18.4 years (range 2–86 years). The non-TB disease group consisted of 39 subjects, with a mean age of 46.5 ± 13.9 years (range 18–69 years). All patients enrolled in the present study were tested for HIV by serology and all had negative results. Details of the 91 patients with suspected spinal TB are shown in Table 1.[12]

Table 1
Epidemiological and demographic characteristics of 91 patients with suspected spinal TB

Variables	Definite TB (n = 52)	Non-TB disease (n = 39)[a]
Age, years, mean ± SD (range)	42.3 ± 18.4 (2–86)	46.5 ± 13.9 (18–69)
Male to female sex ratio	31:21	17:22
Underlying condition or illness (%)		
Hypertension	3 (6)	7 (18)
Pneumonia	1 (2)	2 (5)
Hepatitis B	5 (10)	0
Syphilis	2 (4)	1 (3)
Diabetes mellitus	3 (6)	2 (5)
Immunocompromised condition[b] (%)	10 (19)	2 (5)
Suspected sites of infection (%)		
Cervical vertebra	0	6 (15)
Thoracic vertebra	22 (42)	5 (13)
Thoraco-lumbar	5 (10)	0
Lumbar vertebra	23 (44)	28 (72)
Sacral vertebrae	2 (4)	0
Combined pulmonary TB (%)	7 (13)	2 (5)

TB, tuberculosis; SD, standard deviation. Data are presented as number (%).
[a] Non-TB disease: sixteen patients with lumbar disc herniation; six patients with cervical spondylosis; eight patients with spinal canal tumor; five patients with spinal tumor; four patients with suppurative spondylitis.
[b] Immunocompromised patients were defined as those with underlying diseases such as malignancy, liver cirrhosis, and chronic renal failure, or those receiving immunosuppressive treatment.[12]

2.2. Laboratory procedures and histopathology

Microbiological and pathological specimens for diagnosing spinal TB were processed by standard techniques and procedures. In brief, mycobacteria were cultured on solid culture medium, and the *M. tuberculosis* complex was identified with a commercial DNA probe (AccuProbe Mycobacterium TB complex culture identification kit; GenProbe, San Diego, CA, USA). If the AccuProbe assay was negative, cultures were identified with a commercially available PCR test for NTM (kit for the identification and drug sensitivity testing of non-tuberculous Mycobacterium; Gaoteng, Nanchang, China). For histopathological examination, formalin-fixed and paraffin-embedded tissue blocks of biopsied specimens were stained with hematoxylin–eosin stain. Smears of the decontaminated specimens were stained with the Ziehl–Neelsen stain and examined for acid-fast bacilli (AFB).

2.3. Preparation of rCFP-10/ESAT-6 fusion protein

The fusion protein of CFP-10 (Rv3874) and ESAT-6 (Rv3875) was engineered as described previously.[13–15] In brief, the individual genes were amplified from *M. tuberculosis* H37Rv genomic DNA by PCR. During the amplification steps the genes were fused with a linker encoding glycine–glycine–glycine–glycine–serine–glycine–glycine–glycine–glycine–serine–glycine–glycine–glycine–glycine–serine. The product was subsequently cloned into plasmid vector pET-28a (Novagen, San Diego, CA, USA) containing a C-terminal hexa-histidine tag. Sequencing was performed to confirm the identity of the cloned DNA fragment. The recombinant fusion protein was over-expressed in *Escherichia coli* BL21 (DE3) (Invitrogen, Carlsbad, CA, USA) and was purified by metal chelate column chromatography using nickel–nitrilotriacetic acid (Ni–NTA) resin in accordance with the manufacturer's protocol (Qiagen). Recombinant protein batches were analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie brilliant blue staining and Western blotting with a murine anti-His tag monoclonal antibody (Novagen, San Diego, CA, USA), to confirm the size and purity of the protein.

2.4. ELISPOT assay

A laboratory-developed rCFP-10/ESAT-6 ELISPOT assay for IFN-γ was performed as described previously.[9] Four to five milliliters of venous blood samples from the volunteers were taken in heparinized glass tubes, and peripheral blood mononuclear cells (PBMC) were isolated and quantified the second day after the patients went into hospital. PBMC, 250 000 cells/well, were seeded in 96-well plates (Millipore, USA) pre-coated with anti-IFN-γ capture monoclonal antibody (Mabtech AB, Sweden) and stimulated with the different antigens for 20 h at 37 °C in air plus 5% CO_2. rCFP-10/ESAT-6 fusion protein, 40 μg/ml, was used as the specific antigenic stimulus. PBMC in medium alone and phytohemagglutinin (PHA, Sigma, USA) at 10 μg/ml were used as negative and positive controls, respectively. Biotinylated anti-IFN-γ detection monoclonal antibody (Mabtech AB, Sweden) was added for 1 h at 37 °C, followed by the addition of streptavidin–alkaline phosphatase conjugate (Mabtech AB, Sweden) for 30 min at room temperature. After a washing step, the nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP; Mabtech AB, Sweden) chromogenic substrate were added. The number of spot-forming cells (SFC) in each well was automatically counted with a CTL-ImmunoSpot S5 Versa Analyzer (Cellular Technology Ltd, USA). The responses were scored as positive if the test well contained at least 15 more SFC than the negative control well or had at least twice as many SFC as the negative control well. PHA-positive control wells were set to at least 100 SFC/well/250 000 cells. Negative control wells were required to have <15 SFC.

2.5. Purified protein derivative (PPD) skin test and serological (antibody detection) test

PPD produced from *M. tuberculosis* (50 IU/ml) was purchased from Guangzhou Longcheng Technology Inc., China. All patients with suspected spinal TB were injected intradermally in the left forearm with 0.1 ml of 5 IU PPD (Mantoux technique) after extraction of blood for the ELISPOT. The diameters of both axes of the skin induration were measured and recorded by two certified doctors (QZ, JC) at 72 h after antigen injection. Results were expressed as the mean of the diameter of induration in millimeters. A positive result was defined as an induration ≥5 mm in diameter. For the serological (antibody detection) test, spinal TB was identified with a commercial antibody (IgG) detection kit (diagnostic kit for IgG antibody to *M. tuberculosis*; Yaji Inc., Shanghai, China).

2.6. Data management and statistical analysis

All data were entered into a Microsoft Office Excel file. Diagnostic performance was expressed in terms of sensitivity, specificity, positive predictive value, and negative predictive value. Analyses were performed using the commercial statistical software SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA). Contingency analysis was by Chi-square test or Fisher's exact test. The Mann–Whitney *U*-test was used to compare nonparametric distribution of the SFC among different groups. All of the significance tests were two-sided, and a *p*-value of <0.05 was considered to be statistically significant.

3. Results

3.1. Determination of the cutoff value for positivity

To determine the sensitivity and specificity of the rCFP-10/ESAT-6 ELISPOT assay in the diagnosis of spinal TB, we compared the ELISPOT responses of 30 patients with spinal TB to those from 48 controls (20 non-TB disease patients and 28 healthy controls). Receiver operating characteristic curve analysis was performed to determine the optimal cutoff value of the ELISPOT assay, balanced with the highest possible positive rate for patients with spinal TB and the lowest positive rate for controls (data not shown). Based on this concept, the cutoff value for the rCFP-10/ESAT-6 ELISPOT assay was 15 SFC per 250 000 cells. Therefore, the ELISPOT assay was scored as positive when the number of SFC in the stimulated wells minus the number in the negative control wells was ≥15 or when there were at least twice as many SFC as in the negative control well, and scored as negative when the number of SFC in the stimulated wells minus the number in the negative control wells was <15.

3.2. Clinical diagnostic value of the rCFP-10/ESAT-6 ELISPOT assay for spinal TB

Of 91 patients with suspected spinal TB with valid ELISPOT results, 52 were categorized into the spinal TB group and 39 into the non-TB disease group. The ELISPOT results and the number of SFC in the two groups are shown in Figures 1 and 2. The results of conventional diagnostic tests and ELISPOT assays are shown in Table 2. We found that 35.7% and 57.5% of spinal TB patients were positive for AFB staining and cultures for *M. tuberculosis*, respectively. Overall, 41 (87.2%) of 47 specimens from these patients had histopathological features consistent with a diagnosis of TB infection. Among the 23 patients with culture-confirmed spinal TB, pulmonary TB involvement was found in two (8.7%). All of the patients with culture-confirmed spinal TB had positive ELISPOT results.

Among the spinal TB patients and non-TB disease patients, the overall sensitivity, specificity, positive predictive value, and negative predictive value for spinal TB diagnosis by the PPD skin

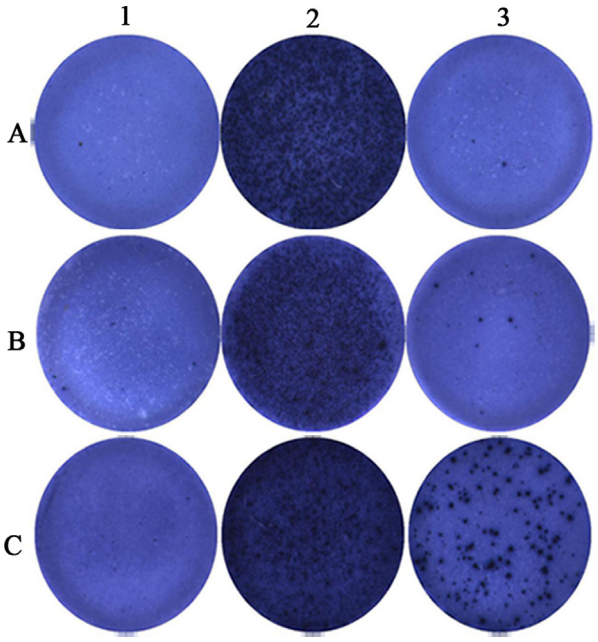

Figure 1. Representative results of the laboratory-developed rCFP-10/ESAT-6 ELISPOT assay from three patients. (A) Negative result from a non-spinal TB disease patient. (B) Negative result from a spinal TB patient. (C) Positive result from a spinal TB patient. Lane 1: negative control, in which 2.5×10^5 PBMCs were unstimulated; lane 2: positive control, in which 2.5×10^5 PBMCs were stimulated by PHA; lane 3: test well, in which PBMCs were stimulated by rCFP-10/ESAT-6 antigen.

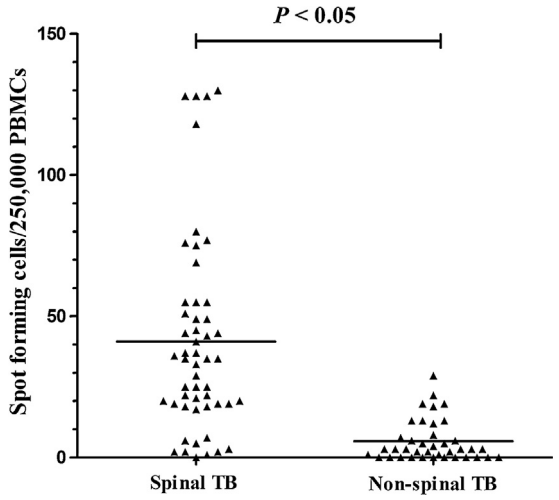

Figure 2. Spread of spot-forming cells (SFCs) above the negative control/2.5×10^5 peripheral blood mononuclear cells (PBMCs) in response to recombinant CFP-10/ESAT-6 fusion protein in 52 spinal TB patients and 39 non-spinal TB patients. The bold lines indicate the median values for the respective groups.

test were 61.5%, 46.2%, 60.4%, and 47.4%, respectively. By comparison, the sensitivity, specificity, positive predictive value, and negative predictive value for spinal TB diagnosis by the ELISPOT assay were 82.7%, 87.2%, 89.6%, and 79.1%, respectively. The overall sensitivity of the ELISPOT assay was higher than that of the PPD skin test and antibody detection test ($p < 0.05$).

3.3. Clinical characteristics associated with the rCFP-10/ESAT-6 ELISPOT results in spinal TB patients

Table 3 shows the clinical factors associated with the number of SFC and positive rate of the ELISPOT assay among the 52 patients with spinal TB. The mean number of SFC in spinal TB cases aged <60 years was higher than that of the spinal TB cases aged ≥60 years. The diagnostic sensitivity of the ELISPOT assay in TB cases aged <60 years was significantly higher (89.7%) than that of the TB cases aged ≥60 years (61.5%) ($p < 0.05$). Likewise, the mean number of SFC in spinal TB patients with mild malnutrition and normal nutrition (BMI 16–25 kg/m^2) was significantly higher than that of spinal TB patients with severe malnutrition (BMI <16 kg/m^2). However, the overall diagnostic sensitivity of the ELISPOT assay was not significantly different ($p > 0.05$). For these patients, the mean number of SFC in spinal TB patients with abscesses was 47.6 ± 35.8, in contrast to 27.6 ± 19.9 in spinal TB patients without abscesses ($p < 0.05$).

Table 2
Comparison of 52 patients with spinal tuberculosis detected by PPD skin tests, antibody detection, histopathology detection, and ELISPOT

	PPD skin tests[a]			Antibody detection[b]			Histopathology detection[c]		
	Pos	Neg	Total	Pos	Neg	Total	Pos	Neg	Total
ELISPOT-positive	29	14	43	26	17	43	37	2	39
ELISPOT-negative	3	6	9	3	6	9	4	4	8
Total	32	20	52	29	23	52	41	6	47

PPD, purified protein derivative; ELISPOT, enzyme-linked immunospot assay. Data are number of subjects.
[a] ELISPOT vs. PPD skin test (Chi-square test): $p = 0.013$.
[b] ELISPOT vs. antibody test (Chi-square test): $p = 0.003$.
[c] ELISPOT vs. histopathology detection (Chi-square test): $p = 0.688$.

Table 3
Factors associated with number of SFC and positive rate of ELISPOT among the 52 patients with spinal tuberculosis

		ELISPOT assay			ELISPOT assay		
	Number of cases	SFC	*U*	*p* [a]	No. of positive (positive rate)	Chi-square	*p* [b]
Sex							
Male	31	38.4 ± 32.4	302.5	0.688	26 (83.9%)	0.075	0.785
Female	21	44.9 ± 39.1			17 (80.9%)		
Age, years							
<60	39	43.8 ± 35.0	191.5	0.190	35 (89.7%)	5.419	0.020
≥60	13	32.7 ± 35.0			8 (61.5%)		
Medical history, months							
<6	19	48.2 ± 45.8	306.5	0.894	15 (78.9%)	0.293	0.588
>6	33	36.9 ± 26.9			28 (84.4%)		
BMI, kg/m^2							
<16	10	19.4 ± 12.5	102.5	0.013	7 (73.9%)	1.394	0.238
17–25	42	46.2 ± 36.8			36 (85.7%)		
>25	0						
Coexisting diseases							
No	12	33.2 ± 33.4	191.0	0.287	9 (75.0%)	0.645	0.422
Yes	40	43.4 ± 35.6			34 (85.0%)		
Lymphocyte count, cells/mm^3							
<1000	9	29.7 ± 22.6	156.5	0.371	7 (77.8%)	0.184	0.688
≥1000	43	43.4 ± 36.9			36 (83.7%)		
Paravertebral abscess							
No	23	27.6 ± 19.9	225.5	0.046	17 (73.9%)	2.221	0.136
Yes	29	47.6 ± 35.8			26 (89.7%)		

SFC, spot-forming cells; ELISPOT, enzyme-linked immunospot assay; BMI, body mass index. Data are number of subjects.
[a] Mann–Whitney *U*-test was used to compare nonparametric distribution of the SFC (mean ± standard deviation).
[b] Pearson Chi-square test was used to compare the positive rate of two groups.

3.4. Response to rCFP-10/ESAT-6 fusion protein before and after surgery

Additional responses were evaluated in a consecutive subset of 15 spinal TB patients at 2 days before and after surgery. Six spinal TB patients only underwent focal cleaning. The remaining nine spinal TB patients underwent one-stage anterior debridement and bone graft plus anterior or posterior instrumentation. The median rCFP-10/ESAT-6 (43.0 ± 23.6) response was slightly lower after surgery than before surgery (48.5 ± 29.9), but this failed to reach statistical significance ($p > 0.05$). There were three spinal TB patients with an atypical clinical presentation in this study. These patients presented with a paravertebral giant cold abscess without obvious bony lesion. The median rCFP-10/ESAT-6 fusion protein response was noticeably lower after surgery (40, 20, and 77 spots per 250 000 PBMCs) than before surgery (72, 53, and 130 spots per 250 000 PBMCs).

4. Discussion

Despite the availability of an inexpensive and effective therapy, TB continues to be a major global public health problem. Early diagnosis plays a vital role in controlling TB. Delayed diagnosis results in more extensive disease, complications, and increased mortality.[16] Spinal TB usually presents in a slowly indolent manner with nonspecific clinical presentations, making the diagnosis a great challenge for clinicians. A definite diagnosis of spinal TB depends on the demonstration of *M. tuberculosis* by culture from biopsy specimens. It is reported that PPD positivity is 90% in immunocompetent patients with skeletal TB, but this test is much less sensitive in immunosuppressed patients.[17] Among the 52 patients with spinal TB in this study, an immunocompromised condition was found in 10 patients. Only two of these patients had positive PPD skin test results. This factor might be responsible for the somewhat disappointing sensitivity of the PPD skin test in our study. Conventional methods for the diagnosis of spinal TB, however, are often inefficient. Thus, rapid, sensitive, and specific diagnostic tests for spinal TB are required. Previous studies have shown that, with blood samples, the ELISPOT assay has a high sensitivity for diagnosing extrapulmonary TB,[18,19] including osteoarticular TB, cutaneous TB, TB lymphadenitis, and TB pleurisy.[7,8,20–22] We therefore conducted an observational study to assess the diagnostic value of a laboratory-developed rCFP-10/ESAT-6 ELISPOT assay in spinal TB.

In this study, we used the rCFP-10/ESAT-6 fusion protein as the stimulating antigen. It is less costly to prepare and detect than bulk peptides. The advantage of this construction is that the rCFP-10/ESAT-6 fusion protein has improved molecular flexibility with decreased interaction between the two component proteins during protein folding, thus ensuring that the spatial conformation of the fusion protein is consistent with that of native CFP-10 and ESAT-6.[14] The sensitivity of a laboratory-developed rCFP-10/ESAT-6 ELISPOT assay for patients with active pulmonary TB was recently reported to be 67.7%.[9] In this study, we found that the sensitivity of the ELISPOT assay for diagnosing spinal TB was 82.7%, which was higher than that of the PPD skin test, AFB smear, and *M. tuberculosis* culture from a biopsy sample. The differences in sensitivity may be due to various degrees of immune response at different sites of TB.[11] A previous study showed that the ELISPOT assay had a sensitivity of 86.7% and a specificity of 61.9% for diagnosing skeletal TB. What's more, the ELISPOT assay had a sensitivity of 100% for diagnosing spinal TB.[8] The results suggest that our ELISPOT assay has potential utility in the diagnosis of spinal TB.

Histopathological findings, including granulomatous inflammation, caseating necrosis, and AFB, usually indicate mycobacterial infection and are helpful in diagnosing TB. In this study, 47 patients with spinal TB underwent an invasive procedure to obtain a tissue specimen for the purpose of diagnosis, and specimens from 41 (87.2%) of these patients had positive histopathological features. Thirty-seven of 41 patients with positive pathologic findings had positive ELISPOT assays. Therefore, our findings suggest that, as a relatively less invasive tool than tissue biopsy, the ELISPOT assay would be a useful adjunct to current tests for the diagnosis of spinal TB.

In this study, we found that age, emaciation, and paravertebral abscess were associated with the number of SFC or the positive rate of ELISPOT in spinal TB patients. A previous study showed that aging is a risk factor for false-negative results.[23] In another study conducted by Liao et al.[24] using an ELISPOT assay, increasing age was associated with false-negative results. In our study, the diagnostic sensitivity of the ELISPOT assay in TB cases aged <60 years was higher (89.7%) than that in TB cases aged ≥ 60 years (61.5%). Likewise, the low BMI in our study population was associated with a weak response to rCFP-10/ESAT-6 in the ELISPOT assay. Severe wasting disease or malnutrition causes unhealthy emaciation with an extremely low BMI, debilitating the patients and also suppressing the systemic immune response.[25] Hang et al., also found emaciation to be a risk factor for false-negative results in the ELISPOT assay.[26] These risk factors might responsible for the somewhat disappointing sensitivity of our ELISPOT assay. Paravertebral abscess was one of the most common clinical symptoms in spinal TB.[27] In this study, there was a trend for the rCFP-10/ESAT-6 fusion protein response to be greater in spinal TB patients with abscesses than in spinal TB patients without abscesses. A potential mechanism could underlie this finding. Spinal TB with abscess usually has an insidious onset and slow progression. Chronic forms of TB have been associated with intermediate to high antigenic load and a robust host immune response. A previous study showed that this mechanism in extrapulmonary TB varies between different sites of disease.[28] Thus, the immune response of spinal TB with abscesses may be more efficient than that of spinal TB without abscesses at eluting IFN-γ-producing T-cells. However, the overall diagnostic sensitivity of the ELISPOT assay was similar in the two groups.

Sequential monitoring of ELISPOT responses may also be useful in monitoring the response to anti-TB chemotherapy.[29] The role of rCFP-10/ESAT-6 ELISPOT in monitoring the efficacy of the surgical treatment of spinal TB has not been reported. In our study, the median rCFP-10/ESAT-6 fusion protein responder T cells after surgery decreased slightly in comparison to that before surgery, but the difference was not significant ($p > 0.05$). Three spinal TB patients with an atypical clinical presentation were recruited into our study. These patients presented with a giant cold abscess without obvious bony lesion. To our surprise, the response to rCFP-10/ESAT-6 in these three patients was obviously lower after surgery than before surgery. The aim of the operation was the debridement of the cold abscess. Thus, we analyzed the impact that the surgical treatment might have in reducing the response to the specific antigens in these atypical spinal TB patients. However, the specific mechanism is unknown. So, further studies are needed to assess the clinical value of the ELISPOT assay for monitoring the efficacy of surgical treatment in cases of atypical spinal TB.

Our study had several limitations. First, the drawbacks of the ELISPOT assay are the relatively high labor intensity in performing the test and the inability of the test to differentiate between active and latent TB infection (LTBI). Therefore, a positive ELISPOT assay result might be due to LTBI or a previous history of TB. The inability of this test to discriminate active TB from LTBI might limit its application in the detection of active TB, especially in countries with a high prevalence of TB, such as China. Second, the

performance of a laboratory-developed ELISPOT assay in the diagnosis of spinal TB was not evaluated by parallel comparison with the commercial QuantiFERON-TB Gold In-Tube (QFT-G) and T-SPOT.TB (Oxford Immunotec). In contrast to the wide use of IFN-γ assays for the diagnosis of *M. tuberculosis* infection in Europe and America, the utilization of IFN-γ assays in China is scarce because of the high cost of these kits. We plan to evaluate the performance of our laboratory-developed rCFP-10/ESAT-6 ELISPOT assay in spinal TB by parallel comparison with the commercial T-SPOT.TB assay (Oxford Immunotec). Third, we did not perform a subgroup analysis in immunocompromised patients with suspected spinal TB because only limited numbers of immunocompromised patients were enrolled in this study. Thus, further studies are needed on the diagnostic performance of our laboratory-developed ELISPOT assay in immunocompromised patients.

In conclusion, the rCFP-10/ESAT-6 ELISPOT assay appears to be useful as a complementary method in the diagnosis of spinal TB. Although the difference in rCFP-10/ESAT-6 fusion protein responder T cells before and after the surgery for spinal TB was not significant, this assay might be useful for monitoring the efficacy of surgical treatment of atypical spinal TB with giant cold abscess.

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Ethical approval: This study protocol was approved by the Ethics Committee on Human Experimentation of Nanfang Hospital, Southern Medical University. Written informed consent was obtained from all patients.

Conflict of interest: The authors state that there are no conflicts of interest regarding the publication of this article.

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