Diagnostic value of CXCR3 and its ligands in spinal tuberculosis

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Abstract. The present study aimed to investigate whether C-X-C motif chemokine receptor 3 (CXCR3) and its ligands may aid in diagnosing spinal tuberculosis (ST). A total of 36 patients with ST and 20 healthy controls were enrolled in the present study. The morphology of tuberculous granuloma in spinal tissue was observed by hematoxylin and eosin staining. The presence and distribution of acid-fast bacilli (AFB) were observed by Ziehl-Neelsen (ZN) staining. The protein expression of Ag85B, IFN-γ, and CXCR3 and its ligands (CXCL9 and CXCL10) were detected by immunohistochemistry. The levels of IFN-γ, CXCR3, CXCL9 and CXCL10 in peripheral blood of patients with ST and healthy controls were detected by reverse transcription-quantitative polymerase chain reaction and ELISA. Typical tuberculous granuloma was observed in the ST close tissue. AFB was observed by ZN staining. Positive expression of Ag85B was found in the surrounding caseous necrotic tissue of the tuberculous granuloma. IFN-γ, CXCR3, CXCL9 and CXCL10 were expressed in the tissue surrounding the tuberculous granuloma and their expression levels were markedly higher than those in the distant tissues. The levels of IFN-γ, CXCR3, CXCL9 and CXCL10 in peripheral blood of patients with ST were significantly higher than those in the healthy controls. Receiver operating characteristic curve analysis demonstrated that IFN-γ, CXCR3 and CXCL10 were more reliable diagnostic markers in terms of sensitivity and specificity. IFN-γ, CXCR3, CXCL9 and CXCL10 were highly expressed in the lesion tissue and peripheral blood samples of patients with ST, and IFN-γ, CXCR3 and its ligands aided in diagnosing ST.

Introduction

Tuberculosis (TB), caused by Mycobacterium tuberculosis (M.tb), is one of the major infectious diseases that threaten human life and health. The number of patients with M.tb infection accounts for ~1/3 of the world's total population (1,2). TB is mainly transmitted through the respiratory tract, and following infection, it may reach numerous systems of the body via the blood, including the skeletal and digestive systems. Extrapulmonary TB accounts for ~50% of patients with TB. Spinal tuberculosis (ST) is a common type of extrapulmonary TB (3). The diagnosis of ST is more challenging than pulmonary TB for two main reasons. To begin with, patients with ST do not have enough secretions for positive M.tb culture. Furthermore, although diagnosis may be made by pathological results and imaging methods, including magnetic resonance imaging (MRI), the process of collecting puncture tissue samples is invasive and the specificity of MRI results is low (4).

T-lymphocyte-mediated immune responses serve a crucial role in the pathogenesis of TB and may be an important source of diagnostic biomarkers. For example, the tuberculin skin test (TST) is used to facilitate TB diagnosis (5). However, the TST has limited accuracy, and latent TB infections may lead to false negatives. The recent interferon-gamma (IFN-γ) release assays (6) have shown superior accuracy compared with the TST, but continue to be affected by similar limitations.

When the body defends and removes invading pathogens, it causes leukocytes to gather. Certain substances, which are known as chemokines, may cause this function. Chemokines are cytokines that can induce chemotaxis in leukocytes. Chemokines exert a biological role through binding with their ligands and are mainly involved in the immune response of leukocytes, as well as various forms of pathological inflammation. Chemokines not only have a chemotactic function, but also may promote the activation of T cells, co-stimulate...
cytotoxic T lymphocytes and natural killer cells, and promote the differentiation, maturation and antigen presentation of dendritic cells (7,8). The C-X-C motif chemokine receptor 3 (CXCR3) is a G protein-coupled receptor, whose ligand is induced by IFN-γ (9,10), and it may aggregate immune cells associated with the pathogenesis of TB. CXCR3 ligands include monokines induced by IFN-γ (CXCL9), IFN-γ-inducible 10-kd protein (CXCL10) and interferon-inducible T cell alpha chemotactant (CXCL11). CXCL11, as a biomarker of TB, has been widely studied. However, for patients with ST, the expression levels of CXCL9 and CXCL10 are poorly understood (11).

The present study focused on the levels of IFN-γ, and CXCR3 and its ligands CXCL9 and CXCL10 in patients with ST. Levels of IFN-γ, CXCR3, CXCL9 and CXCL10 in ST tissues and peripheral blood were detected to evaluate their diagnostic value in ST. Their expression levels suggested that they may be markers that aid in diagnosing ST and in the monitoring of treatment responses.

Materials and methods

Subjects. A total of 36 patients were enrolled, with a mean age of 43.14 years (range, 18-77 years), consisting of 18 males and 18 females, who had been operated on for ST at the Spine Surgery Department of the First Affiliated Hospital of Xinjiang Medical University (Urumqi, China) between January 2017 and December 2018. Samples from TB lesions and distal paravertebral cartilage tissue and connective tissue were collected patients with from ST, and peripheral blood was collected at the same time. The distant tissue was collected 2 cm away from the area of caseous necrosis (12). Meanwhile, healthy subjects (n=20) who underwent physical examination between January 2017 and December 2018 were enrolled as the control group, and their peripheral blood was collected.

The inclusion criteria for patients with ST were as follows: i) Patients with typical symptoms of tuberculous infection, including low fever, night sweats, weight loss and fatigue; ii) patients with symptoms of spinal cord compression, including pain, myodynamia, muscle tension, tendon reflexes, limited activity and spinal deformities; iii) patients with positive M.tb antibody; iv) patients with tuberculous granuloma; and v) patients with the typical features of ST on MRI, including bone marrow edema, endplate erosion, vertebral destruction and spinal compression. Patients with other immune diseases, neoplastic diseases, HIV infection or metabolic disorders were excluded.

All participants provided written informed consent and the present study was approved by the Ethics Committee of Xinjiang Medical University.

Pretreatment of tissue specimens. The tissue samples collected in the present study were bone tissues and connective tissues at the lesion site. After the patients' tissue specimens were rinsed with normal saline, they were fixed in 4% paraformaldehyde for 24-48 h at 20-23°C. Next, the tissues were decalcified in 10% EDTA decalcification solution for 21 days. Paraffin sections (4 μm) were obtained following ethanol gradient dehydration and embedding.

Ziehl-Neelsen (ZN) staining. ZN staining was performed using a modified acid-fast staining kit from Baso Diagnostics, Inc. The sections (4 μm), were deparaffinized and rinsed several times with ethanol. Slides were incubated with Carbol Fuchsin solution for 7 min, decolorized for 10 sec with hydrochloric acid and then counterstained for 3 min with methylene blue at 20-23°C. The presence of AFB was confirmed under a X100 oil immersion lens. In every experiment, one positive control was included to confirm the efficiency of the ZN staining.

Hematoxylin and eosin (H&E) staining. H&E staining was performed according to routine procedure, including Hematoxylin staining for 2.5 min, Eosin staining for 3 min, 1% hydrochloric acid differentiation, ethanol gradient dehydration, neutral gum seal, were all performed at 20-23°C, prior to light microscope (magnification, x20; cat. no. BX43F, Olympus Corporation) observation of histopathological changes.

Immunohistochemical (IHC) staining. In brief, 4-μm thick sections, were heated at 56°C for 1 h, deparaffinized with xylene and alcohol (95, 80 and 75%). Sections were blocked using 3% Hydrogen Peroxide (cat. no. P0100A; Beyotime Institute of Biotechnology) for 25 min at 37°C. Following antigen retrieval, sections were treated with primary antibodies (all BIOS) against Ag85B (cat. no. 9268R), CXCR3 (cat. no. 0341R), CXCL9 (cat. no. 2551R), CXCL10 (cat. no. 1502R) and IFN-γ (cat. no. 0481R) at antibody diluent (dilutions: Ag85B, 1:300; CXCR3, 1:200; CXCL9, 1:200; CXCL10, 1:200; and IFN-γ, 1:50) (CO1-04001, BIOS) at 4°C overnight. Sections were incubated with a ready-to-use secondary antibody (1:200; cat. no. PV9000; OriGene Technologies, Inc.) for 1.5 h at 20-23°C. Visualization was performed using 3,3′-diaminobenzidine as the substrate, which was applied for 1.5 min. Sections were counterstained (20-23°C) with hematoxylin (0.025%; 1 min). Antigen expression was analyzed under a X40 high power lens (cat. no. BX43F; Olympus Corporation). In each experiment, one positive control and one negative control were included to ensure the accuracy of the experimental results. Positive signals were required to meet three criteria: Irregular patchy dark brown granules; more than five clusters; and, located in the necrotic area but not in the non-necrotic granuloma. Two professional pathologists were invited to observe the results and, according to the manufacturer’s protocol of each marker, they gave a score (0 points, negative; 1 point, mildly positive; 2 points, moderately positive; 3 points, strongly positive). Additionally, ImageJ software (ImageJ Software) was used to analyze the IHC results. The final result was the positive area multiplied by the score.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Peripheral blood (2 ml) was taken from each participant and red blood cell lysis was performed. Total RNA was extracted from the leukocytes using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA concentration (>80 ng/μl) and purity (OD260/OD280=1.8-2.0) were determined using a nucleic acid quantifier. cDNA was synthesized by reverse transcription according to
manufacturer’s protocols of the PrimeScript™ RT reagent kit (Takara Bio, Inc.). The RT-qPCR system was as follows: 2 µl cDNA, 1 µl forward primers and 1 µl reverse primers, 12.5 µl TB Green Premix Ex Taq (Takara Bio, Inc.) and 8.5 µl DEPC water. The thermocycling was as follow: Pre-denaturation (95˚C, 30 sec, 1 cycle) and PCR reaction (95˚C, 5 sec, 60˚C, 30 sec, 40 cycles in total) followed by a dissolution curve. Primer sequences are presented in Table I. GAPDH was used as the internal control. The relative expression was calculated by the comparative cycle threshold method \((2^{-\Delta\Delta C_t})\) (13).

**ELISA.** Serum levels of IFN-γ, CXCR3, CXCL9 and CXCL10 were detected according to the manufacturer’s protocols of the ELISA kit (cat. no. KHC4021; eBioscience; Thermo Fisher Scientific, Inc.). Standard wells, blank control wells and sample wells were set on 96-well plates. The diluted standard was added to the standard well, the sample dilution was added to the blank control well, and the test serum was added to the sample well. Next, they were placed in a 37˚C incubator for 60 min. Following washing, color development and termination, the absorbance (A) was detected at a wavelength of 450 nm. The concentration was determined according to the standard curve.

**Statistical analysis.** All data in the present study were statistically analyzed using SPSS 22.0 (IBM Corp.) and GraphPad Prism 8.0 software (GraphPad Software, Inc.). Quantitative data with normal distribution are expressed as (mean ± standard deviation). t-tests were used for comparisons between groups, including paired t-test (close vs. distant) and unpaired t-test (ST vs. control). For quantitative data that did not conform to the normal distribution, the median (interquartile range) was used, and the difference between groups was compared using the Wilcoxon rank sum test. The receiver operating characteristic (ROC) curve was established to evaluate the diagnostic value of the markers. The area under the curve (AUC) was calculated. The Youden index was used to determine the optimal cut-off value. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Clinical data analysis.** The clinical data of the subjects are presented in Table II. The mean age of the 36 enrolled patients with ST was ~40 years old. In the early stage of the disease, they were admitted to the hospital due to chest and back pain and paralysis of both lower extremities. Laboratory data are presented in Table III. Compared with healthy controls, the levels of C-reactive protein (CRP; P<0.001) and platelets (PLT; P<0.001), and the percentage of monocytes (P<0.01) were increased in patients with ST, while the levels of hemoglobin (Hb; P<0.01) and hematocrit (Hct; P=0.012) were decreased. In addition, T-SPOT.TB was positive in 16 subjects.
AFB was identified in the TB tissues of patients with ST. In ZN staining, a large amount of AFB ~3 µm in length, was observed inside the non-necrotic TB granuloma. The AFB was slender, red-stained, non-refractive, and slightly curved (Fig. 1A). A total of 36 patients with ST were included in the present study. Among them, AFB was identified in the sections of 10 patients, accounting for 27.7% of the total subjects. The AFB\(^+\) and T‑SPOT.TB\(^+\) patients did not entirely overlap.

In the inflammatory lesion tissue of the patients with ST, Ag85B was stained tan and distributed in caseous necrotic tissues, Langerhans cells and epithelioid cells (Fig. 1B). However, Ag85B was not expressed in the distant tissue (Fig. 1C). The difference in Ag85B expression between TB tissues and distant tissues was significant (Fig. 1D; P<0.001).

Pathological manifestations of TB lesions in patients with ST. H&E staining demonstrated that there was a typical tuberculous granuloma in TB tissue. In the center of the granuloma, caseous necrosis was observed, which was surrounded by radially arranged epithelioid cells, as well as a large number of macrophages and Langerhans multinucleated giant cells (typical multinucleated giant cells in tuberculous granuloma). Furthermore, there was lymphocyte infiltration in the outermost layer. Fibrotic tissues were occasionally observed. The morphology of Langerhans multinucleated giant cells was mostly horseshoe-shaped, large in size, rich in cytoplasm and pink in pulp (Fig. 2A). There was occasional inflammatory infiltration or hemorrhage in the distant tissues (Fig. 2B). A total of 36 patients with spinal TB were included in the present study. Among them, TB granuloma was identified in sections from 28 patients, accounting for 77.8% of the total subjects.

High expression of IFN-\(\gamma\), and CXCR3 and its ligands in patients with ST. IHC staining revealed that IFN-\(\gamma\) and CXCL10 exhibited a secretory positive result in the nucleus and cytoplasm of caseous necrosis (Fig. 3A and B) compared with the distant tissue (Fig. 3C and D), and the area with a positive expression area of IFN-\(\gamma\) and CXCL10 in TB tissues was significantly increased (Fig. 4A; P<0.01; P<0.001). A positive expression of CXCR3 and CXCL9 presented as brown granules and could be observed in the cytoplasm of caseous necrosis and Langerhans multinucleated giant cells of TB tissue (Fig. 3E and F); however, there was a weak...
expression in the distant tissues (Fig. 3G and H). The area for positive expression of CXCR3 and CXCL9 in TB tissue was significantly increased compared with that in distant tissues (Fig. 4A; P<0.0001; P<0.0001).

To investigate the mRNA expression of CXCR3 and its ligands (CXCL9 and CXCL10), RT-qPCR was performed. The results demonstrated that IFN-γ (P<0.05), and CXCR3 (P>0.05) and its ligands, CXCL9 (P<0.05) and CXCL10 (P<0.001), were increased in patients with ST. The differences in IFN-γ, CXCL9 and CXCL10 expression were statistically significant, compared with the control group (Fig. 4B).

ELISA was conducted to detect serum chemokine levels. The expression levels of serum IFN-γ (P<0.0001), CXCR3 (P<0.0001), CXCL9 (P<0.001) and CXCL10 (P<0.001) in the patients with ST were significantly higher than those in the healthy controls (Fig. 4C).

Discussion

A total of 56 subjects were included in the present study, including 36 patients with ST and 20 healthy controls. In order to improve understanding regarding whether there is any difference between the ST patients or healthy controls, their clinical symptoms were observed and biochemical analysis was performed. The mean age of the patients with ST was ~40 years old, and they tended to be middle-aged. In the early stages of the disease, patients often have clinical symptoms, including chest, waist and back pain, and paralysis of both lower limbs (14). At this point, the patient’s quality of life is poorer than it was previously, and timely medical treatment is required to relieve symptoms. In previous studies, the cause of ST was that M.tb invaded the host, reproduced and migrated to the lesion. The incidence of ST appears to be unrelated to the occupation of the patient in the present study (Table II).

CRP as an acute phase response protein usually rises rapidly during the acute phase of the host's inflammatory response. Elevated CRP may be associated with the regulation of phagocytosis of M.tb by immune cells in patients. An increase in monocyte count further confirms that, when M.tb infects a host, a large number of inflammatory cells are recruited in the host to serve a pro-inflammatory role.

ST is the most common type of bone and joint TB. M.tb in the primary focus of ST as it can spread directly to the edge of the vertebral body through blood and lymphatic vessels (15,16), causing destruction of the vertebral body or intervertebral disc, spinal deformities, dysfunction, paraplegia and death, particularly in certain severe cases (4,17). The immune response of M.tb infection is mainly cell-mediated immune response, during which specific Th1 cells are sensitized with the M.tb antigen to produce IFN-γ. IFN-γ is an important factor for cells to resist M.tb infection, and may induce chemokines to function, causing macrophages to be activated to serve an immune role against M.tb (18). The pathological characteristics of the lesion tissue were identified microscopically in the present study. The presence of a
typical tuberculous granulomatous structure indicates that the course of disease in patients with ST is continuing. It is well known that *M. tb* is not abundant in tissue; however, in the present study acid-fast bacilli was found in ST tissue in several cases. Ag85B is the most abundant protein expressed by *M. tb*. It is a myocoly transferase in the myc pathway and catalyses, in the same way as Ag85A and Ag85C, the transfer of the fatty acid mycolate from one trehalose monomycolate to another, resulting in trehalose dimycolate and free trehalose and helping to build the cell wall. Positive expression of *M. tb* was identified in the ZN staining and Ag85B IHC staining. These two experimental methods further verified that there may be a large amount of *M. tb* replication in patients with ST.

Chemokine receptors are mainly expressed in various leukocytes from bone marrow, as well as in epithelial cells and vascular endothelial cells. Chemokines and their receptors may regulate leukocyte transport and homing of cells in tissues, and they serve a physiological and pathological role in the development of immune cells and immune organs, immune response, inflammatory response and pathogen infection (19). CXCR3 is an important chemokine receptor in activated T cells, which is rapidly upregulated and highly expressed on T cells when stimulated. In the present study, the positive expression area of IFN-γ and CXCL9 in the areas affected by ST was higher than that in the distant control tissue. This result demonstrated that when the ST patients were infected with *M. tb*, T cells could recognize them and fight against them, as well as producing IFN-γ. IFN-γ may further induce the production of CXCR3 ligands, promote the binding of CXCR3 and CXCR3 ligands on Th1 cells and recruit more Th1 cells into the inflammatory site to serve an inflammatory role (20).

CXCL9 is originally obtained from the mouse IFN-γ-stimulated macrophage cell line, RAW264.7 (21). The chemotaxis of CXCL9 is mainly mediated by CXCR3. After the combination of CXCL9 and CXCR3, CXCR3 is activated. This further stimulates Src phosphorylation and Src kinase activity and increases the activity of phosphatidylinositol 3 kinase (P13K) and Akt to participate in the pathological process of the inflammatory reaction (22). Increased expression of CXCR3 and CXCL9 in the ST group may strengthen the combination of CXCR3 and CXCL9, and activate the downstream pathway and promote inflammatory reaction during *M. tb* infection.

CXCL10 was first identified from activated U937 cells in 1985 (23). CXCL10 is one of the chemokines in the CXC non-ELR subfamily. The cells stimulated by IFN-γ can secrete CXCL10, directly, and CXCL10 may then selectively activate and enhance the expression of IFN-γ gene in antigen-activated T cells (24), therefore positive feedback may recruit more T cells, participating in cell growth, differentiation, apoptosis and inflammation. In the present study, the expression of IFN-γ and CXCL10 in patients with ST was increased, which was consistent with the expression trend of CXCL9. These results indicated that the expression of CXCR3 and its ligands may have clinical significance for ST.

Chemokines also have a chemotactic role due to inflammation in other diseases. However, a previous study reported that CXCR3 and its ligands are of profound significance in TB (11).
Table IV. Diagnostic performance evaluation of markers for ST patients and healthy controls.

| Marker          | AUC    | 95% CI      | Sensitivity (%) | Specificity (%) |
|-----------------|--------|-------------|-----------------|-----------------|
| IFN-γ           | 0.9861 | 0.9641-1.008| 97.22           | 90              |
| CXCR3           | 0.9778 | 0.9458-1.010| 94.44           | 95              |
| CXCL9           | 0.7861 | 0.6589-0.9133| 75              | 75              |
| CXCL10          | 0.9181 | 0.8232-1.013| 91.67           | 95              |

AUC, area under the curve; CI, confidence interval; IFN-γ, interferon-gamma; CXCR3, CXC chemokine receptor 3; CXCL9, CXC chemokine receptor ligand 9; CXCL10, CXC chemokine receptor ligand 10.

Therefore, the present study aimed to investigate whether CXCR3 and its ligands may have a role in ST. Other causes of spinal damage may be neoplastic lesions and metabolic disorders. However, the inclusion criteria used in the present study were strict, as the enrolled subjects were patients with typical symptoms of TB infection, spinal cord compression, positive M.tb antibody and MRI suggesting ST.

In the present study, the protein expression of IFN-γ, CXCR3, CXCL9 and CXCL10 in spinal tissue and surrounding connective tissue of patients with ST were detected by IHC. It was revealed that their expression levels were increased, and they were primarily expressed around and inside the granuloma of caseous necrosis, which may be associated with the enhancement of the chemotactic effect of CXCL9 and CXCL10 mediated by CXCR3 during M.tb infection. Additionally, the mRNA expression of IFN-γ, CXCR3, CXCL9 and CXCL10 in peripheral blood leukocytes was also increased. The secretion of four markers in the serum was the same as that in IHC and RT-qPCR. The results demonstrated that the ligands induced by CXCR3 and IFN-γ were highly expressed during M.tb infection, and the high expression of CXCL9 and CXCL10 may enhance the pro-inflammatory effect of CXCR3 in ST. In addition, the present study evaluated the diagnostic performance of the four markers using ROC curve analysis (Table IV), which revealed that the AUC values of IFN-γ, CXCR3 and CXCL10 were >0.9, and their sensitivity and specificity were >90%. It has been demonstrated that IFN-γ, CXCR3 and CXCL10 were helpful for the diagnosis of ST. In conclusion, the ROC results of IFN-γ, CXCR3 and CXCL10 may be optional indicators for the diagnosis of ST.

The mechanism of bone destruction caused by tuberculous granuloma is very complicated. ST, a common form of TB, seriously affects people's quality of life in the early stages of infection and has attracted much attention. The present study focused on the expression of CXCR3 and its IFN-γ-induced ligands, CXCL9 and CXCL10, in tissues, peripheral blood leukocytes and serum of patients with ST in order to evaluate the role of these four markers. The results of the present study indicated that IFN-γ, CXCR3, CXCL9 and CXCL10 were highly expressed in lesion tissue and peripheral blood in patients with ST. However, further ROC curve analysis was more likely to indicate whether IFN-γ, CXCR3 and CXCL10 were useful in the diagnosis of ST. Therefore, more attention should be paid to elucidate the role of IFN-γ, CXCR3 and their ligands in ST. Future experimental studies should include more in-depth research into the diagnostic performance and mechanism of action of these biomarkers.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

XS and LW conceived and designed the study; HW and SN acquired the data of experimental results; XS, YL, XL, JL, XZ analyzed and interpreted the data; XS, LW and XM drafted the article or revised it critically for important intellectual content; JW and XM agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or
integrity of any part of the work are appropriately investigated and resolved. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All participants provided written informed consent and the present study was approved by the Ethics Committee of Xinjiang Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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