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Original Article

Auxiliary diagnostic value of monocyte chemoattractant protein-1 of whole blood in active tuberculosis

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Abstract: The aim of this study was to study the expression level of interferon-γ (IFN-γ) and monocyte chemoattractant protein-1 (MCP-1) in peripheral blood and its auxiliary diagnostic value in active tuberculosis. A chemiluminescence enzyme immunoassay method was used to detect the levels of IFN-γ and MCP-1 in peripheral blood. Then the receiver operating characteristic curve were drawn to determine the threshold of IFN-γ and MCP-1 for diagnosis of active tuberculosis and to evaluate their diagnostic performance. The specific IFN-γ and MCP-1 levels in the active tuberculosis group were significantly higher than those in the non-tuberculous pulmonary disease group ($P < 0.01$) and those in the healthy control group ($P < 0.01$). The IFN-γ levels in the healthy control group and the non-tuberculous respiratory disease group showed no statistically significant difference ($P > 0.05$), but the MCP-1 levels in the non-tuberculous respiratory disease group were significantly higher than those of the healthy control group ($P < 0.05$). The specific IFN-γ and MCP-1 level cut off values were 256 pg/ml and 389 pg/ml as an active tuberculosis diagnostic standard. The sensitivities of IFN-γ and MCP-1 were 57.3% and 92.8%, respectively; specificities were 80% and 80.7%, respectively; the positive predictive values were 76.9% and 84.9%, respectively; negative predictive values were 61.7% and 78.7%, respectively; and accuracy rates were 76.9% and 84.9%, respectively. Compared with the detection of IFN-γ, we observed a better diagnostic performance of MCP-1 in peripheral blood in active tuberculosis. MCP-1 may become one of the active tuberculosis auxiliary diagnostic targets.

Keywords: Tuberculosis, IFN-γ, monocyte chemoattractant protein-1, diagnosis

Introduction

Based on a recent global report, tuberculosis (TB) is a major cause of illness and death worldwide, with the heaviest burden in developing countries (World Health Organization, 2012). Early diagnosis and treatment of active TB are considered cornerstones of TB control. Current microbiologic methods (acid-fast stain smear and mycobacterial culture) are the gold standards for the clinical diagnosis of active TB. Unfortunately, acid-fast stain smears are relatively insensitive in TB diagnosis. Therefore, we urgently need a rapid and accurate method of laboratory diagnosis.

The early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) are both encoded in the region of difference 1 (RD1), which is present in *Mycobacterium tuberculosis* (MTB) and *M. bovis* but is absent in *M. bovis* BCG and most environmental Mycobacteria [1, 2]. RD1, therefore, can be used to distinguish *M. tuberculosis* infection from the BCG vaccination. The interferon gamma release assay (IGRA), is a quantitative detection method that measures interferon-γ (IFN-γ) release by sensitized T cells after stimulation with peptides of *M. tuberculosis*-specific antigens ESAT-6 and CFP-10. Quantiferon TB Gold (Cellestis Ltd., Carnegie, Australia), and IGRA, has proven both sensitive (75-97%) and specific (> 90%) as an immunodiagnostic test for TB infection, and appears to be more accurate than the tuberculin skin test (TST) [3, 4]. But the Quantiferon TB Gold method cannot differentiate active TB cases from those with latent TB infection. Ruhwald et al. and Frahm et al. screened large panels of potential biomarker candidates [5-8], in which monocyte-derived chemokine mono-

Table 1. The levels of MCP-1 and IFN-γ in the culture supernatant of whole blood (M)

Groups	Active TB disease group	Non-TB respiratory diseases group	Healthy control group
Example number	157	95	40
MCP-1 (pg/ml)			
Negative control well	114 (-38-940)	80.6 (3.9-520.4)[c]	46 (0-187)[d]
Non-specific antigen stimulation	826 (9-984)	872 (99-984)	973 (826-1035)
Tuberculosis -specific antigen stimulation	813 (-280-995)	212 (-59-954)[b,c]	87 (2-704)[c]
IFN-γ (pg/ml)			
Negative control wells	22.8 (0.5-345)[a]	22.2 (10.1-72.8)[a]	13 (6-99)[a]
Non-specific antigen stimulation	578 (58-1015)	510 (40-1057)	999 (124-1180)
TB-specific antigen stimulation	303 (11-1089)	99 (19-805)[c]	114 (7-365)[c]

Note: The data of MCP-1 and IFN-γ was in a skewed portion, therefore it was indicated by the median (M), the number in brackets was the minimum and maximum levels of the two cytokine. a: Comparison with the other groups $P > 0.05$. b: Comparison with the healthy control group $P < 0.05$. c: Comparison with active TB group $P < 0.05$. d: Comparison with non-tuberculous respiratory disease group $P < 0.05$.

cyte chemoattractant protein-1 (MCP-1) has shown the most promise in distinguishing between active and latent cases. MCP-1 is the most potent activator of monocytes and is essential for recruitment and migration of T lymphocytes to the lung. Responsiveness to MCP-1 is dependent on its receptor CCR2, and MCP-1 is a potent activator of not only monocytes, but all cells that express CCR2, including also macrophages, CD_4+ T cells, and immature dendritic cells [9]. MCP-1 has been shown essential for granuloma formation [10] and in playing a critical role in protection against tuberculosis in the murine model [11].

The primary objective of this study was to validate whether IFN-γ and MCP-1 responses to the fusion protein of ESAT-6 and CFP-10 in whole blood could identify active tuberculosis, and to compare their diagnostic values. To date, there have been no studies conducted in highly endemic settings that compare the sensitivities and specificities of IFN-γ and MCP-1 assays for active TB diagnosis.

Materials and methods

Study population

A total of 292 patients were recruited in the 309th Hospital of Chinese PLA, Beijing 100091, the Second Hospital of Jilin University, Changchun 130041, and the Tumor Hospital of Jilin Province, Changchun 130000, China, from October 2010 through November 2011. The patients were untreated or had received less than 2 weeks of therapy at the time of venipuncture for the test. The clinical data were surveyed retrospectively. The patients were separated into 3 groups based on their final diagnoses: 1) active TB group: 157 cases, mean age of 43 ± 21, 95 males and 62 females; 2) non-tuberculous pulmonary disease (non-TB) group: 95 cases, mean age of 53 ± 12, 62 males and 33 females; 3) healthy control group: 40 cases, mean age of 39 ± 9, 28 males and 12 females. The diagnosis of active TB was established according to China's 1992 revised guidelines, "Diagnostic Criteria and Classification of Tuberculosis." Details of patients and controls included in the study are given in **Table 1**. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of the Second Hospital of Jilin University. Written informed consent was obtained from all participants.

Purified protein derivative (PPD) skin test

The PPD skin test was performed according to the standard Mantoux technique by a trained nurse from the Institute for Tuberculosis Research, the 309th Hospital of Chinese PLA, Beijing, China. Five tuberculin units of PPD (0.1 ml) from *M. bovis* BCG (50 IU/ml, Chengdu Institute of Biological Products, China) was injected intradermally in the volar surface of the left forearm. The diameters of both axes of skin induration were measured and recorded by a certified doctor at 72 h after injection. A positive response to PPD was defined as an induration of greater than 5 mm in diameter.

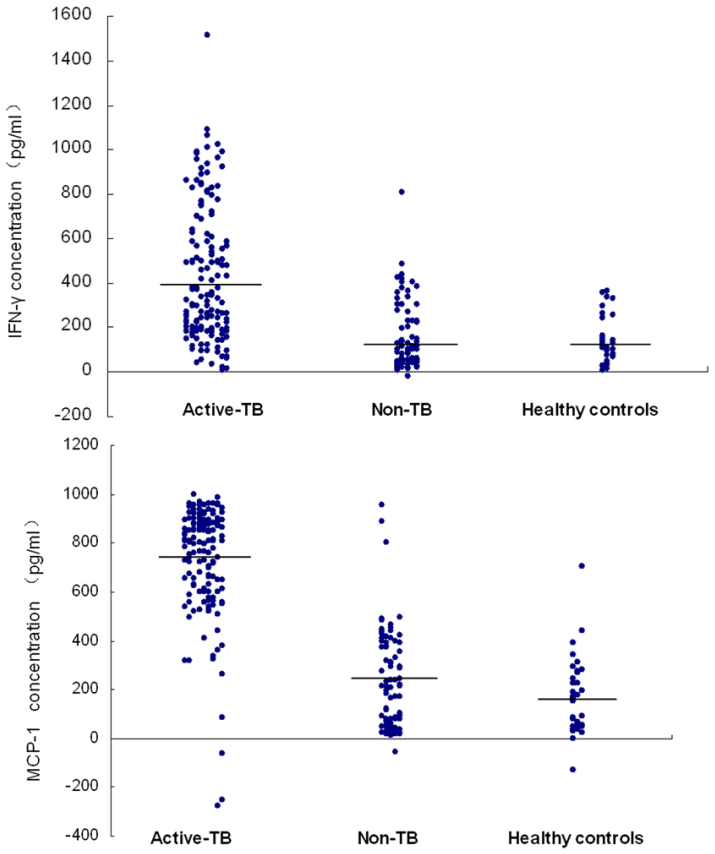

Figure 1. TB-specific IFN-γ and MCP-1 production. The TB-specific cytokine levels were obtained by subtracting the values of the negative control wells from the values in the rCFP-10/ESAT-6 antigen stimulated wells and expressed as pg/ml. Straight lines represent median values.

Whole blood stimulation and culture

M. tuberculosis CFP-10/ESAT-6 cloning, expression, and purification were performed as described previously [12], and the recombinant CFP-10/ESAT-6 (rCFP-10/ESAT-6) fusion protein was prepared by Institute of Tuberculosis Research, at the 309th Hospital of PLA, Beijing, China. For each person, 2 ml of venous blood was collected in a sodium citrate glass tube and processed within 6 h. Then, 400 μl aliquots of the whole blood was diluted 1:1 with serum-free medium (Gibco, Invitrogen, USA) and incubated in 24-well tissue culture plates (Shengyou, Hangzhou, Zhejiang Province, China) in a humidified box with 5% CO_2 at 37°C for 18 to 22 h in three separate conditions: 1) the rCFP-10/ESAT-6 fusion protein antigen at a concentration of 20 μl/ml; 2) a phytohemagglutinin (PHA, 20 μl/ml; Sigma, Missouri, USA) as a positive control; and 3) a mock stimulation as a negative control. Following the stimulations, 400 μl of supernatant was harvested from each well, and frozen at -80°C until analysis within 16 weeks.

IFN-γ and MCP-1 chemiluminescence enzyme immunoassay

The supernatants from each specimen were threw, for the simultaneous detection of IFN-γ and MCP-1. Capture antibodies against IFN-γ (BD OptEIATM Human IFN-γ ELISA Set, Becton Dickinson, New Jersey, USA) and MCP-1 (BD OptEIATM Human MCP-1 ELISA Set, Becton Dickinson) were coated in separate 96-well microtiter plates (Jinchanhua, Shenzhen Province, China) overnight at 4°C. After washing three times with phosphate-buffered saline (PBS-T), the plates were blocked with PBS containing 10% fetal bovine serum (FBS) for 1 h at room temperature. Subsequently, individual supernatant plasma samples (without dilution for the IFN-γ and 1:10 dilution for the MCP-1) were added in duplicate to the wells, and incubated for 2 h at room temperature. The plates were incubated at 37°C for 2 h. Following 5 recommended washes with wash buffer, biotinylated anti-human antibodies against each biomarker (1:250 biotinylated IFN-γ, BD OptEIATM Human IFN-γ ELISA Set, USA; 1:1000 biotinylated MCP-1, BD OptEIATM Human MCP-1 ELISA Set, USA), and streptavidin-horseradish peroxidase conjugate (1:250, BD Biosciences, 1:250, BD Biosciences, USA) were added to the wells and incubated at 37°C for 1 h. After the plate was washed 7 times with wash buffer, the chemiluminescent substrate solution A and B reagent (Chemiluminescence Substrate Reagent, Beijing Keyuezhongkai Co. Ltd., China) at a 1:1 mix, each hole after joining 100 μl to avoid light placed 5 minutes. The light values were measured with a chemiluminescence detector (type KPS-KM) (Beijing Kemeidongya Co. Ltd., Beijing, China) and converted

Table 2. The MCP-1 and IFN-γ levels of culture supernatant of whole blood of different groups of active TB

Groups	Number	TB -specific MCP-1	TB -specific IFN-γ
TB	108	807 (-280-996)	331 (15-1064)
Extrapulmonary TB	49	833 (-252-964)	279 (11-1089)
Acid-fast bacilli smear-positive	34	836 (83-961)	314 (11-1065)
Acid-fast bacilli smear-negative	123	809 (-280-996)	300 (15-1115)
PPD skin test positive	83	824 (83-996)	313 (19-1089)
PPD skin test negative	33	849 (-280-987)	241 (11-1015)

to pg/ml using a standard curve run on each plate. For each subject, the TB-specific cytokine levels were determined by subtracting the values of the negative control wells from the values in the stimulated wells and expressed in pg/ml.

Data management and statistical analysis

All data were entered into a Microsoft Office Excel file. Data conforming to the non-normal distribution using median (M) and the range indicates. The median and range of the cytokine levels in the test well minus the negative control well in individual groups were calculated. The differences between the groups were analyzed by nonparametric tests. The data was analyzed using SPSS 17.0. MCP-1 levels and IFN-γ levels of the three unstimulated groups were analyzed by Kruskal-Wallis H test. The difference between the two groups was analyzed by Mann-Whitney U test. All of the significance tests were two sided, and a $P < 0.05$ was considered to be statistically significant.

The sensitivities and specificities of tests with each antigen were calculated. The sensitivity is defined as the number of correctly detected cases in the active TB group divided by the total number in the active TB group, which shows the ability of the assay to correctly diagnose active TB. The specificity is defined as the number of correctly detected non-active cases divided by the total number in the control group, which shows the ability of the assay to correctly diagnose non-TB disease. Both non-TB patients and healthy controls were used as controls, while active-TB patients were the test group. Using SPSS 17.0 software (Chinese version), we drew tuberculosis-specific IFN-γ and MCP-1 receiver operating characteristics (ROC) curves. ROC curves were constructed and analyzed to determine the most accurate cut-off values for the diagnosis of active-TB.

Results

IFN-γ and MCP-1 levels in whole blood

One hundred and fifty-seven cases of active TB, 95 cases of non-TB, and 40 cases of healthy controls were studied. The two biomarkers' scatterplots are shown in **Figure 1**. The median and the range of the levels of the two biomarkers in whole blood in each group are presented in **Table 2**. The difference in MCP-1 levels was statistically significant between the 3 unstimulated groups ($P < 0.05$), while the difference in IFN-γ levels was not statistically significant between the 3 unstimulated groups ($P > 0.05$). The tuberculosis-specific IFN-γ levels in the active TB group were significantly higher than those of the non-tuberculosis respiratory disease group ($P < 0.001$) and the healthy controls ($P < 0.001$), while the difference in tuberculosis-specific IFN-γ levels between the non-tuberculous respiratory disease and healthy control groups were not statistically significant ($P > 0.05$).

IFN-γ and MCP-1 test performance

Both non-TB patients and healthy participants were used as control groups, and active-TB patients were used as the test group. Tuberculosis-specific IFN-γ and MCP-1 ROC curves are shown in **Figure 2**. The areas under the curves for IFN-γ and MCP-1 were 0.823 (95% CI: 0.775-0.870) and 0.948 (95% CI: 0.918-0.978), respectively. According to ROC curves, the cut off values of IFN-γ and MCP-1 were 256 pg/mL and 389 pg/ml, respectively. With IFN-γ detection, there were 90 positive cases in the active-TB group, and 108 negative cases in the control group (including both the non-TB patients and healthy controls), and the sensitivity, specificity, and diagnostic efficiency was 57.3%, 80.0%, and 67.8%, respectively. With MCP-1 detection, there were 146 positive cases in the active-TB group, and 109 negative cases in the control group, and the sensitivity, specificity, and diagnostic efficiency for MCP-1 was 92.9%, 80.7%, and 87.3%, respectively. Although both tests showed higher sensitivities, specificities, and diagnostic efficiencies that expected in the differentiation between active TB and non-TB, the sensitivity and diag-

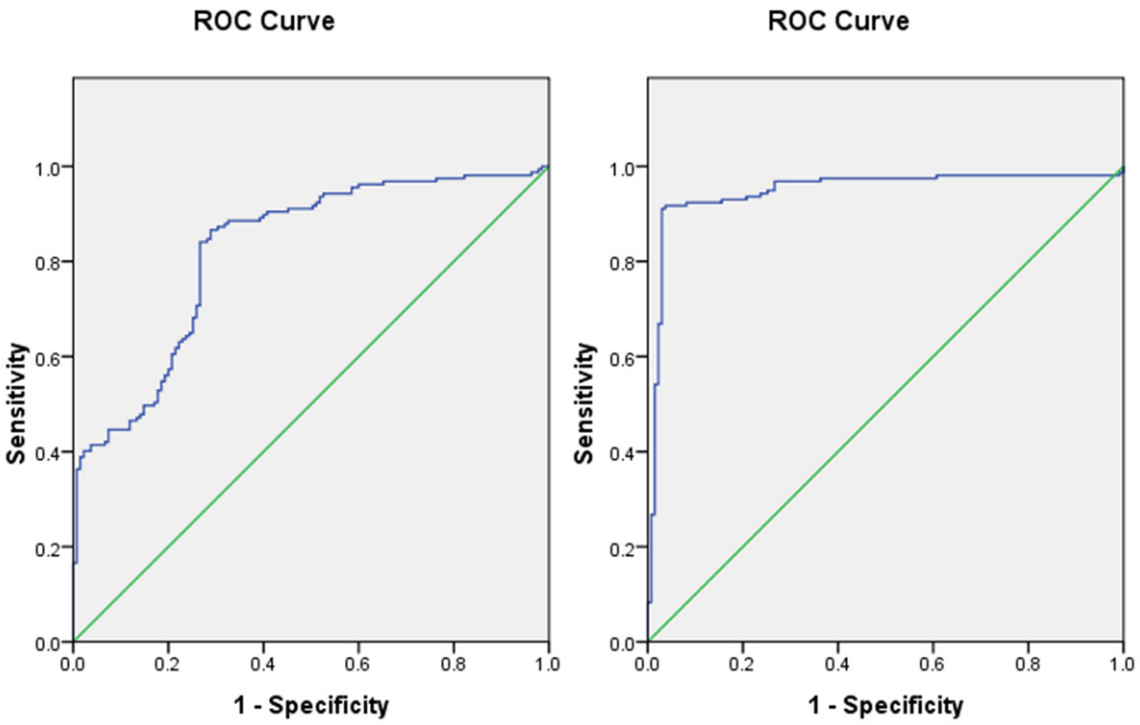

Figure 2. Tuberculosis-specific IFN-γ and MCP-1 ROC curve.

Table 3. Comparations of clinical diagnostic value in active TB between three kinds of cytokines

	MCP-1	IFN-γ
Sensitivity	92.80%	57.30%
Specificity	80.70%	80%
The positive predictive value	84.90%	76.90%
The negative predictive value	90.80%	61.70%
Accuracy	84.9%	76.9%

nostic efficiency of IFN-γ was lower than MCP-1.

The 157 cases of active TB were divided into a pulmonary TB group and an extra-pulmonary TB group according to the site of tuberculosis, and into a smear-positive group and a smear-negative group according to the acid-fast stain smear results. The results are shown in **Table 3**. The IFN-γ levels in the pulmonary TB group was lower than those in the extra-pulmonary TB group, and the levels of MCP-1 in the extra-pulmonary TB group were higher than those in the pulmonary TB group, but there was no significant difference between them ($P > 0.05$). The levels of IFN-γ in smear-positive TB patients were higher than those in smear-negative cases, and the MCP-1 level in the smear-positive group was lower than those in the smear-negative group, but there were no significant difference between them ($P > 0.05$).

Discussion

This study suggests that biomarkers, other than those currently in use, with high sensitivity and specificity may be useful for the diagnosis of active TB, as well as for differentiating active-TB from non-TB. We obtained two important results. First, this study confirmed the findings from Ruhwald et al. and Frahm et al. [5-8], showing that high levels of IFN-γ and MCP-1 are produced in stimulated whole blood from TB patients, but not from non-TB and healthy controls. Second, MCP-1 used to differentiate active TB from non-TB respiratory diseases has a higher diagnostic value than IFN-γ. Because IFN-γ and MCP-1 are released by CD_4+ T lymphocytes, CD_4+ T lymphocyte number in the blood may affect the levels of biomarkers Arias et al [13]. This study did not examine CD_4+ T lymphocyte numbers to compare results with the previous study, but roughly analyzed the influence of peripheral blood lymphocyte

counts on the levels of whole blood IFN-γ and MCP-1.

Results from testing of the negative controls of the IFN-γ groups were not significantly different from the experimental group. The results of this study are consistent with the findings presented by Alessandri et al. [14]. Results from testing of the three groups of the MCP-1 group were significantly different, with the active TB group and the non-TB group both showing higher levels of MCP-1 than the healthy control group. The active TB group showed an even more significance difference in MCP-1 levels from controls. MCP-1 is one of the chemotactic factor subfamily members, and it plays an important role in the inflammatory response in the human body. MCP-1 can promote monocyte and lymphocyte migration to sites of inflammation, which is the initiating factors that trigger the inflammatory response. The healthy control group had less of an inflammatory response, so MCP-1 was lower than in the active TB group and the non-TB group. This has been observed in other studies: Saukkonen et al. [15] showed a higher MCP-1 concentration in BALF of pulmonary TB compared with non-TB, and Arias et al [13] showed that PBMC secrete more MCP-1 in TB patients compared with non-TB patients. This indicates that MCP-1 is closely related to the tuberculosis pathological process.

The levels of the two cytokines were not significantly different between the smear-positive and smear-negative cases with active TB. This is consistent with previous studies in other countries [16-19], and indicates that TB specific IFN-γ and MCP-1 secretion may depend more on the MTB induced T-cell immune response. Acid-fast bacilli smear-negative TB is often false-negative because results are limited by the quality of the specimen, the impact of the detection means and the level of detection. These cases were diagnosed as active TB cases, two groups of peripheral blood of patients with specific antigen to effector T cell number and response level is close, which resulted in two groups of patients with IFN-γ and MCP-1 secretion is close to. The levels of these two cytokines showed no significant difference between pulmonary TB and extra-pulmonary TB cases with active TB. This indicates that TB specific IFN-γ and MCP-1 levels may be not associated with the location of TB infection, which is consistent with the Nishimura et al. study [20]. This experiment demonstrated an effective diagnostic method for extra-pulmonary TB. **Table 2** shows that 33 PPD-negative cases were clinically diagnosed with active TB. These PPD tests may have been negative due to the body's immune dysfunction, malnutrition, new TB infection, or severe tuberculosis. The IFN-γ levels in the PPD skin test-positive group was significantly higher than those of the PPD skin test-negative group, but there was no difference in MCP-1 levels between the two groups, indicating that secretion of IFN-γ may be lower with a more impaired immune status, as has been confirmed by other researchers [9]. In contrast, the secretion of MCP-1 was not affected by a poor immune status, showing that MCP-1 was more sensitive for diagnosis of active TB than IFN-γ.

The sensitivity (92.9%), specificity (80.7%), and diagnostic efficiency (87.3%) for MCP-1 was higher than for IFN-γ (57.3%, 80.0%, and 67.8%). The diagnostic efficiency of MCP-1 appeared greater than that of IFN-γ, a result that is consistent with Ruhwald et al. and collaborator's reports [6, 18, 19]. Our study, however, had two distinctions: first of all, the experimental groups were different, since this study do not include LTBI. Second, we used IFN-γ for the diagnosis of active TB rather than the diagnosis of LTBI. The immunological mechanisms underlying these differences in IFN-γ are not fully understood, but they can likely be attributed to the fact that IFN-γ is a cytokine produced by specific T cells when stimulated by the interaction with an antigen presenting cell. Beyond IFN-γ, we detected increased MCP-1 production as an indicator of active TB. In vitro studies [13] have shown that a large number of activated CD_4+ Thl cells can secrete IL-2 and IFN-γ after MTB invasion. IL-2 and IFN-γ both stimulate monocytes and T cells expressing MCP-1, and thus MCP-1 activates macrophages and lymphocytes to migrate to infection sites so that MTB will be killed by phagocytosis. Although MCP-1 plays an important role in combatting a TB infection, its protective effect is still controversial. Multiple studies have indicated that high MCP-1 production may be detrimental to the host immune response [21-23]. A single nucleotide polymorphism in the MCP-1 promoter was found to correlate with increased MCP-1 expression and increased susceptibility to active TB disease [24]. While this finding held true in Mexican, Korean, and Peruvian patient

populations, the SNP had no effect in a Russian population, and even correlated with protection in patients presented in Ganachari et al. [25, 26]. Additional genetic and/or environmental factors are probably important for determining the role of MCP-1 in fighting TB disease. Owing to these contradictory findings, it is also important that the present study be extended to diverse MTB strains around the world. In this study, the diagnostic specificity of the two cytokines was 80%-87%, the difference is significant compared to 92%-100% of foreign literature about IGRA. This study take "non TB group and the healthy control group" may exist in a LTBI. In low TB endemic countries, TST specificity was better because most people without a history of BCG vaccination, research tended to the TST-negative patients included in the non-TB group to assess the specificity of IGRA. China, however, is a country with a high TB incidence and high TB infection rate, and has a policy of BCG vaccination. In the choice of disease control groups, the TST negative as control group made a better specificity of diagnosis. However, in clinical work, the TST false-positive rate and false negative rate was high. Therefore, this experiment tries to select from Tuberculosis symptoms and signs as control group. Thus, the final specific indicator was lower than other countries. This will be the main obstacle of application of IGRA principle to diagnose active TB in China.

Conclusion

Our findings showed that MCP-1 have a higher sensitivity and specificity as a novel biomarker for active TB than IFN-γ [27]. Further studies are needed to explore the potential of MCP-1 as a replacement for or an addition to IFN-γ for the diagnosis of active and latent TB infection in children and immunocompromised patients.

Disclosure of conflict of interest

None.

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