Tuberculosis xxx (2015) 1–8

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: http://intl.elsevierhealth.com/journals/tube

MOLECULAR ASPECTS

Discovery and verification of serum differential expression proteins for pulmonary tuberculosis

Cuiping Li [a, 1], Xiao He [b, 1], Hongtao Li [c], Yi Zhou [c], Ning Zang [c], Shuixiu Hu [d], Yanyan Zheng [a], Min He [a, *]

[a] School of Public Health, Guangxi Medical University, Nanning 530021, PR China
[b] Nanning Center for Disease Control and Prevention, Nanning 530023, PR China
[c] Medical Scientific Research Center, Guangxi Medical University, Nanning 530021, PR China
[d] Longtan Hospital of Guangxi Zhuang Autonomous Region, Liuzhou 545001, PR China

ARTICLE INFO

Article history:
Received 6 February 2015
Received in revised form
3 May 2015
Accepted 7 June 2015

Keywords:
Tuberculosis
Differentially expressed proteins
SHBG
iTRAQ
MALDI-TOF-MS

SUMMARY

Pulmonary tuberculosis (PTB) is a chronic disease and has remained a severe threat to public health. Valuable biomarkers for improving the detection rate are crucial for controlling this disease. The purpose of this study was to discover potential biomarkers in sera from PTB patients compared with pneumonia patients and normal healthy controls. A total of 336 human serum specimens were enrolled in this study. Differentially expressed proteins were identified using iTRAQ method combining with MALDI-TOF-MS. Data was analyzed using relative bioinformatics methods. Potential biomarkers were further validated by IHC, ELISA and Western blot. As a result, 489 non-redundant proteins were identified in the sera, and 159 of which could be quantified by calculating their iTRAQ ratios. Compared to the controls, 26 differentially expressed proteins were recognized among PTB patients, including 16 overexpressed proteins and 10 downregulated proteins. Analysis of their functional interactions revealed that 12 proteins appeared in the center of the functional network. One of these key proteins, sex hormone binding globulin (SHBG), was found to be significantly elevated among PTB patients as compared with the controls examined by IHC, ELISA and Western blot. This result was consistent with the iTRAQ result. An independent blinded testing set to examine serum SHBG by ELISA achieved an accuracy of 78.74%, sensitivity of 75.6% and specificity of 91.5% in diagnosing PTB. In summary, iTRAQ in combination with MALDI-TOF-MS technology can efficiently screen differentially expressed proteins in sera from the PTB patients. SHBG is suggested to be a possible and novel serum biomarker for PTB.

1. Introduction

Tuberculosis (TB) is a severe infectious disease around the world. In 2011, there were 12 million prevalent cases and 8.7 million incident cases of TB in the world was estimated by the World Health Organization (WHO), and 1.4 million people died from TB. Also in 2011, China estimated a total of 1.2–1.6 million prevalent TB cases [1]. Active TB infection has became a major disease reservoir and potential threat to the health of population. A sensitive and reliable diagnosis method is therefore needed to efficiently detect TB from the population.

Three conventional methods are used for detecting PTB in clinics, i.e., chest radiography, the sputum smear acid-fast staining test, and mycobacterial culture [2–4]. Generally, chest radiography is not sensitive enough to distinguish PTB [2,5]. In addition, sputum smear microscopy examination is infeasible for some patients with the number of sputum bacteria less than 10^4, and for those patients including young children who do not expectorate sputum, because patients with smear-negative pulmonary tuberculosis (SNP-TB) testing results are still considered important sources of PTB transmission. Furthermore, although mycobacterial culture is decisive, it requires quite amount of bacteria's sample size and a longer detecting period. The low efficiency and poor accuracy of these conventional screening methods hinder the early and efficient detection of PTB to a great degree.

* Corresponding author. School of Public Health, Guangxi Medical University, No. 22 Shuangyong Road, Nanning, PR China. Tel.: +86 771 5358146; fax: +86 771 5350084.
E-mail address: hemin@gxmu.edu.cn (M. He).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.tube.2015.06.001
1472-9792/

Detection of serum biomarkers is recognized as an effective approach for diagnosis, monitoring treatment efficacy and prognostic evaluation of PTB [5]. Some reports suggested that several markers in blood might be as important as TB markers, such as C-reactive protein (CRP) [6], IP-10 [7], human epididymis protein 4 [8], activator receptor, procalcitonin [9], krebs von den Lungen-6 [10], serum amyloid A and transthyretin [11]. These TB-associated proteins, however, have not been well established in clinical application because of their inadequate sensitivity and specificity [7]. In particular, no biomarker so far was reported to be able to distinguish the differences among TB patients, normal controls and pneumonia.

ITRAQ Labeling combining with MALDI-TOF-MS is able to quantify hundreds of proteins simultaneously, either relatively or absolutely. Thus, it has become a powerful analytical technique for discovering disease biomarkers and drug targets. This strategy has recently been employed successfully to identify new markers for certain diseases, such as adult obstructive sleep apnea [12], autoimmune diseases [13,14], head-and-neck cancer [15], lung Cancer [16], and hepatocellular carcinoma [17].

Our aim was to detect multiple differentially expressed proteins of TB in sera collected from smear-positive pulmonary tuberculosis (SPP-TB) patients, SNP-TB patients, and drug resistant tuberculosis (DR-TB) patients respectively in a single experimental setting by using iTRAQ Labeling and MALDI-TOF-MS technology. Differentially expressed proteins were further analyzed using bioinformatics methods, and SHBG protein was selectively validated by ELISA, Western blot and immunohistochemical staining (IHC). The predictive value of serum SHBG for distinguishing among PTB cases, pneumonia patients, as well as the normal controls was evaluated.

2. Materials and methods

2.1. Serum samples

This study was approved by the Ethics Committee of Guangxi Medical University. Written informed consent from all participants was collected. In the PTB group, sera were collected from PTB inpatients in the Fourth People's Hospital of Nanning City in September 2008. All PTB patients were diagnosed according to the combined clinical criteria from the WHO [18] including clinical, radiological, and bacteriological examinations. Sera from pneumonia patients were collected from the First Affiliated Hospital of Guangxi Medical University. Sera collection was underwent a full diagnostic assessment. The diagnosis of pneumonia was based on the presence of acute signs and symptoms suggesting lower respiratory tract infection on admission and radiographic evidence of a pulmonary infiltrate that had no other known cause. Sera from a control healthy group were also collected from the Guangxi Medical University First Affiliated Hospital and also underwent a full diagnostic assessment to exclude tuberculosis and pneumonia.

Screening study and Western blot validation study: A total of 87 participants (61 male and 26 female patients) were verified, including 30 healthy controls (mean age, 45.3 ± 3.6 years), 7 DR-TB (mean age, 42.7 ± 4.5 years), 10 SPP-TB (mean age, 43.2 ± 4.3 years), 30 SNP-TB (mean age, 42.8 ± 5.7 years), and 10 pneumonia cases (mean age, 42.8 ± 5.7 years). ELISA validation study: 207 participants (133 male and 74 female patients) were verified, including 32 healthy controls (mean age, 42.3 ± 3.6 years), 35 DR-TB (mean age, 42.7 ± 4.5 years), 55 SPP-TB (mean age, 42.4 ± 3.5 years), 70 SNP-TB (mean age, 40.1 ± 5.7 years), and 15 pneumonia cases (mean age, 43.5 ± 5.7 years). These five groups were matched for age and sex. There were 50 patients who did not accept PTB drug treatment including 33 male and 17 female patients (mean age, 44.7 ± 4.5 years) and 110 patients who accepted rifampicin treatment including 74 male and 36 female patients (mean age, 43.6 ± 5.2 years). All of the subjects were diagnosed as HIV-negative. All patients and controls were from the same geographic region (Guangxi, China) and ethnic origin (Han ethnicity). The general characteristics of the SNP-TB, SPP-TB, DR-TB and pneumonia cases for the control group are shown in Table 1. IHC validation study: 50 participants (16 male and 34 female patients) were verified, including 10 controls (mean age, 43.6 ± 15.6 years), and 40 PTB patients (mean age, 53.4 ± 10.4 years). Controls included 4 cancer adjacent normal pneumonic tissues and 6 normal pneumonic tissues.

2.2. ITRAQ and LC-MS/MS

Fourteen highest abundance proteins were extracted from the sera at room temperature with the Agilent 1200 HPLC system (Agilent Technologies, Waldron, Germany) and installed on the MARS Human 14 column (4.6 mm id × 100 mm, Agilent Technologies, Inc.). Briefly, prior to immunodepletion, the pooled serum samples from each group were diluted with Buffer A at a ratio of 1:3 (Agilent Technologies, Inc.), transferred to a 0.22 μm spin filter, and then centrifuged at 16,000 g for 1 min to remove particles. After collection of the less abundant protein fractions, the MARS columns were washed and the bound proteins were eluted with 100% buffer B. The procedures were conducted according to the protocol provided by the manufacturer. The collected fractions were further concentrated and desalted using 3000 MWCO Hydrosart Vivaspin 2 spin concentrators (Sartorius Stedim Biotech, Gottingen, Germany) at 8000 × g for 99 min for three times. On each occasion, the sample solution was buffer exchanged with 50 mM triethylammonium bicarbonate (TEAB, pH 8.5 buffer, Sigma–Aldrich Corporation, Saint Louis, MO, USA). The concentrated samples were determined using the BCA Protein Assay kit (Pierce, Ill, USA), and each 100 μg of protein was packed into 1.5 mL Eppendorf (EP) tubes, and dried and stored at −20 °C. Trypsin digestion and iTRAQ labeling were performed according to the manufacturer's protocol (Applied Biosystems) using 8-plex iTRAQ reagent and buffer kits (ABI, Framingham, MA). The samples were labeled with iTRAQ reagents as follows: controls, 113; DR-TB, 117; SPP-TB, 118; SNP-TB, 119; and pneumonia, 121. The peptide mixture was fractionated by SCX chromatography at a basic pH (pH 3) to reduce the complexity of the mixture using a polysulfoethyl column (2.1 × 200 mm, PolyLC, Columbia, MD). Data acquisition was accomplished in a positive ion mode using a 5800 analyzer equipped with time-of-flight (TOF)/TOF ion optics (Applied Biosystems). ProteinPilot software v.3.0 (AB Sciex) was used for protein identification and quantitation. The search parameters were set by MMTS as follows: homo sapiens, trypsin cleavage, and cysteine alkylation. Each mass spectrometry (MS/MS) spectrum was searched against the UniProt database. Proteins with at least one peptide and above the 95% confidence level (unused ProtScore > 1.3) were recorded. The normalization tools and statistical package from the ProteinPilot software were utilized. A $p < 0.05$ and an average iTRAQ ratio >9 or <0.1 were considered to be high significance. The protein was considered stably expressed if the differences in expression values in the triplicate experiments were less than 1. MS/MS data has been deposited in the PRIDE database with accession number PXD002298.

2.3. Informatics analysis

The cellular component, molecular function, and biological process were annotated by the GO database (http://www.geneontology.org/). The protein–protein interaction network was

Please cite this article in press as: Li C, et al., Discovery and verification of serum differential expression proteins for pulmonary tuberculosis, Tuberculosis (2015), http://dx.doi.org/10.1016/j.tube.2015.06.001