Original Article
Analysis of differential proteins of pleural effusion cells from Mycobacterium tuberculosis infected patients

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Abstract: Our aim was to identify differential proteins in pleural effusion cells of M. tuberculosis infection patients and analyze their biological functions. Proteins of pleural effusion cells were screened by one-dimensional protein gel electrophoresis, then differentially expressed proteins were identified by LTQ MS mass spectrometry. LTQ MS mass spectrometry analysis identified nine differential proteins successfully, including six human proteins and three M. tuberculosis bacterial proteins. Human proteins were involved in skeleton structure, metabolism, and transport, etc., and pleural effusion cells contained M. tuberculosis antigens to transfer cellular immunity information to other immune cells.

Keywords: M. tuberculosis, pleural effusion cells, differential proteins

Introduction

Tuberculosis (TB) caused by intracellular pathogen M. tuberculosis (Mtb) remains one of the most prevalent and deadly infectious diseases. China is one of the worst infectious countries regarding the tuberculosis epidemic [1]. Tuberculosis pleurisy (TBP) is a common clinical manifestation of active TB disease and may account for up to 50% of all pleural effusions in areas with a high incidence of TB [2]. Tuberculosis pleural effusion (TPE) results from Mycobacterium tuberculosis infection of the pleura and is characterized by an intense chronic accumulation of fluid and inflammatory cells in pleural space. The pleura space is lined by a metabolically active monolayer of pleural mesothelial cells (PMCs) which may serve as the first-line defense against invading microorganisms [3]. In response to pleural infection, PMCs not only provide physical barriers but also produce cytokines, chemokines, ECM, and proteases that participate in the induction and resolution of inflammation and tissue repair [4]. Activated macrophages in the alveolar space are one of the major effector cells to resist the invasive M. tuberculosis [5], through the phagocytosis function to kill M. tuberculosis directly and through the secretion of cytokines such as IFN-γ, TNF-α, IL-12, IL-10, etc. to enhance the

function of immune cells [6, 7]. So far, no formal guidelines are available for diagnosis and treatment of tuberculosis pleurisy. At present, standard diagnostic assays for TBP including microbiological examination, adenosine deaminase (ADA) levels, cell infiltrate profile, and certain other biochemical tests do not provide satisfactory sensitivity and specificity [8].

In this study, pleural effusion cells of M. tuberculosis infection patients were analyzed by proteomic, bioinformatics, and Western blotting to investigate the protein composition of pleural effusion cells and to discuss the functions of these proteins.

Materials and methods

Sample collection

Pleural effusion of 50 tuberculosis patients and healthy controls were collected from the Department of Clinical Laboratory, Kunshan Hospital of Traditional Chinese Medicine and the Department of Clinical Laboratory, Kunshan First People's Hospital from 2015 to 2016.

Electrophoresis of isolated vesicle

Freshly isolated proteins of pleural effusion cells of non-tuberculosis patients or tuberculo-
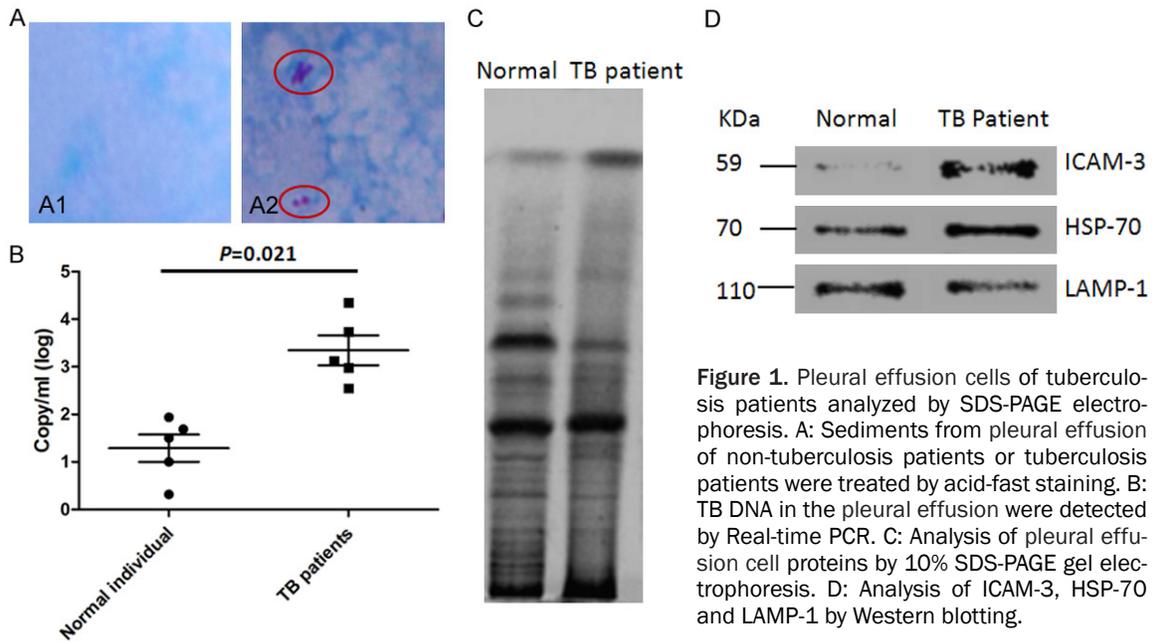
Figure 1. Pleural effusion cells of tuberculosis patients analyzed by SDS-PAGE electrophoresis. A: Sediments from pleural effusion of non-tuberculosis patients or tuberculosis patients were treated by acid-fast staining. B: TB DNA in the pleural effusion were detected by Real-time PCR. C: Analysis of pleural effusion cell proteins by 10% SDS-PAGE gel electrophoresis. D: Analysis of ICAM-3, HSP-70 and LAMP-1 by Western blotting.

sis patient samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for 200 V electrophoresis of about 1 hour. SDS-PAGE gels were then stained with Coomassie Blue at room temperature for 1 hour. They were washed with bleaching solution 3 times for 10 min/time. Finally, the differential proteins of vesicles in SDS-PAGE were compared for analysis. Then, characteristic proteins of vesicles such as ICAM-3, HSP-70, and LAMP-1 were examined by Western blotting.

MALDI TOF/TOF analysis and database searching

SDS-PAGE gels were analyzed with image scanning software to obtain differential protein bands. Protein bands of interest were then excised from SDS-PAGE gels, first washed twice in ddH$_2$O and then soaked in wash solution (50% ACN, 25 mmol/L NH$_4$HCO$_3$) twice for 30 minutes at 37°C and then dehydrated in 100% ACN. Each gel plug was then dried completely. Freshly prepared trypsin solution (4 μL, 20 μg/μL trypsin in 25 mmol/L NH$_4$HCO$_3$) was added to each plug for in-gel protein digestion at 37°C for 16 hours. Peptides were extracted first being incubated for 30 minutes at 37°C and then sonicated for 15 minutes twice in 20 μL 67% ACN, 0.1% TFA. For each sample, 0.5 μL of peptide solution was spotted on a MTP 384 massive target plate. Then, 0.5 μL matrix CCA solution was added to each spot.

Molecular weight information of peptides was obtained by using LTQ MS mass spectrometry and operated in reflector-delay extraction mode for LTQ MS peptide mass fingerprint. The peak list was generated by Flex analysis. Each spectrum was produced by accumulating data from 100 consecutive laser shots and spectra were interpreted with the aid of the Mascot software (Matrix Science Ltd). The peaks with S/N ≥ 5, resolution ≥ 2500 were selected and used for MS/MS from the same target.

Western blotting analysis

The protein concentration of vesicles were measured by Bradford method and we prepared 10% SDS-PGAE gels for electrophoresis. Vesicles (50 μg) were loaded on 10% SDS-PAGE gels, electrophoresed, and transferred onto PVDF membrane (Milipore, Bedford, MA) electrophoretically. The membranes were probed for CDK1, SLA, NOXO1, (BD, 1/800 dilution), COF1, LAMP-1, CD81, and HSPB1 (BD, 1:500 dilution). Immune-detected protein bands were quantified with ImageJ and statistically analyzed by ANOVA.

Diagnosis of tuberculosis by Atg85B, PRv1269c, and ESAT-6

Vesicles from pleural effusion samples of 50 tuberculosis patients were acquired by Exo-Quick Exosome Extraction Kit, then Atg85B, PRv1269c, and ESAT-6 in vesicles were ana-

Table 1. Results of differential proteins from pleural effusion cells of non-tuberculosis patients or tuberculosis patients identified and analyzed by LTQ mass spectrometry

Spot No	Swiss-prot AC	Protein name	MW/PI	Coverage	Match peptides	Score	Location
1	P11440	Cyclin-dependent kinase 1	34,107/8.40	41%	22	99	Nucleus, cytosol
2	Q60898	Src-like-adapter	31,681/8.14	56%	10	58	Cytosol, endosome
3	Q8VCM2	NADPH oxidase organizer 1	26,844/8.44	30%	23	123	Membrane
4	P18760	Cofilin-1	18,560/8.22	33%	24	68	Cytosol, nucleus
5	P35762	CD81 antigen	25,815/5.53	32%	11	89	Membrane
6	P14602	Heat shock protein beta-1	23,014/6.12	25%	7	94	Cytosol, nucleus

Table 2. Proteins of tuberculosis in pleural effusion cells of tuberculosis patients identified by LTQ mass spectrometry

Spot No	Swiss-prot AC	Protein name	MW/PI	Coverage	Match peptides	Score	Location
7	I6YBV4	Antigen 85-B	34,581/5.62	23%	11	67	Extracellular
8	P9WNK5	ESAT-6-like protein EsxB	10,000/7.79	22%	15	87	Extracellular
9	P0A5E1	Protein Rv1269c/MT1307	12,550/8.76	20%	13	82	Extracellular

lyzed by Western blotting. Detection rate of tuberculosis patients by three antigens were analyzed by statistical software.

Statistical analysis

Triplicates were performed for each condition/group. Data were analyzed by SPSS 16.0 and presented as mean ± SE at least 3 independent experiments. Differences within each group were subjected to t-test or q-test. Statistically significant differences (*$P \leq 0.05$) are indicated by asterisk between the groups being compared.

Results

Protein analysis of electrophoresis

Sediments from pleural effusion acquired by centrifugation were analyzed after being acid-fast stained and observed under the microscope (**Figure 1A**, **1B**). Meanwhile, DNA of Mycobacterium tuberculosis in pleural effusion was detected by real-time PCR. Proteins of pleural effusion cells were then analyzed by 10% SDS-PAGE electrophoresis (**Figure 1C**). Characteristic proteins of pleural effusion cells such as ICAM-3, HSP-70, and LAMP-1 were analyzed by Western blotting (**Figure 1D**).

LTQ MS analysis

Differential protein bands from pleural effusion cells of non-tuberculosis patients or tuberculo-

sis patients were bleached and enzymatically digested by trypsin hydrolysis, then were identified by LTQ mass spectrometry. Six proteins associated with human proteins (**Table 1**) and three antigen proteins of M. tuberculosis were obtained successfully (**Table 2**).
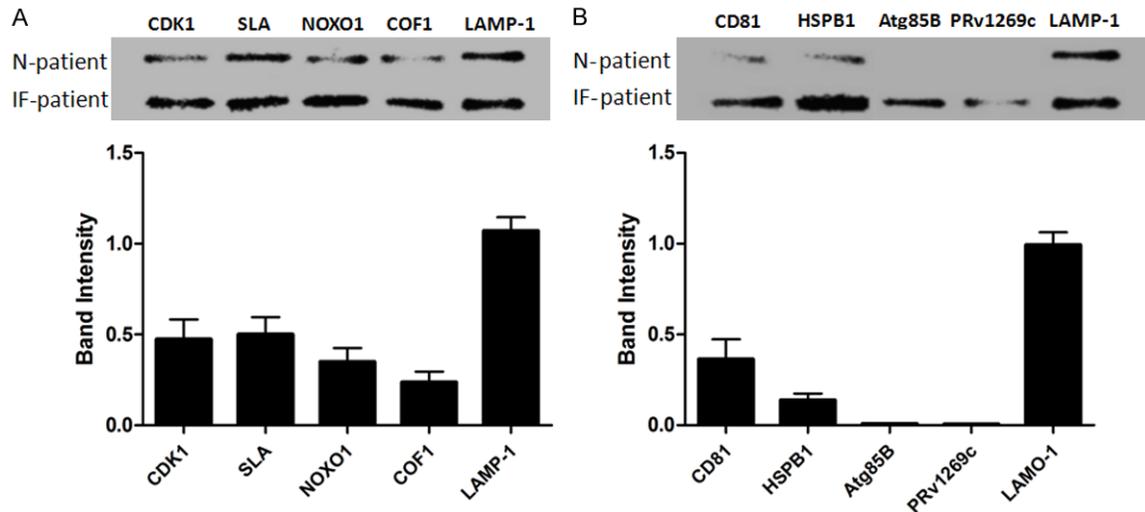
Western blotting

Nine proteins in vesicles from the serum of tuberculosis patients were analyzed by Western blotting (**Figure 2A**, **2B**). The gray values of proteins bands were scanned by Image J software, with the expression of LAMP-1 as a standard correction. Experimental results manifested that differential proteins were all upregulated in pleural effusion cells of tuberculosis patients and, moreover, proteins from pleural effusion cells of tuberculosis patients contained some antigen proteins of tuberculosis such as At85 and PRv1269c.
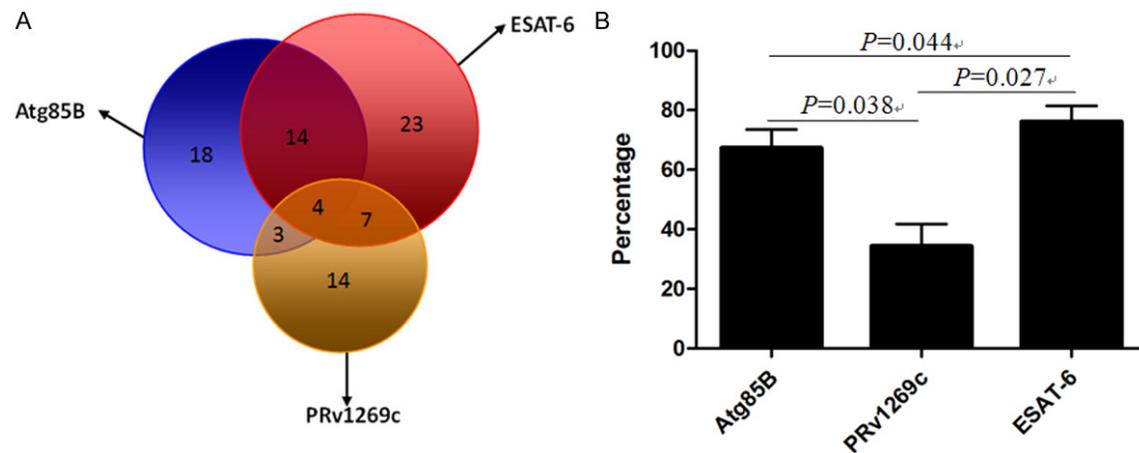
Analysis of Atg85B, PRv1269c, and ESAT-6 in vesicles from the serum of tuberculosis patients

According to analysis results of Atg85B, PRv-1269c, and ESAT-6 in pleural effusion cells from tuberculosis patients (50 cases), Atg85B protein was found in 39 cases of tuberculosis patients, PRv1269c detected in 28 cases, and ESAT-6 was not analyzed in 2 cases (**Figure 3**). Experimental results show that a good way to diagnose tuberculosis is by detecting the anti-

Figure 2. Analysis of six kinds of cellular proteins and two kinds of bacterial proteins by Western blotting. Quantitative analysis of differential proteins expression by Image J software and the lysosomal associated membrane protein-1 (LAMP-1) as a control (n = 3, compared with the control group).

Figure 3. Three proteins of tuberculosis Atg85B, PRv1269c, and ESAT-6 were detected by Western blotting in pleural effusion cells of fifty tuberculosis patients. A: Percentage map of Atg85B, PRv1269c, and ESAT-6 detected in pleural effusion cells of fifty tuberculosis patients by Western blotting; B: Detection rate of tuberculosis patients by three antigens Atg85B, PRv1269c, and ESAT-6 in pleural effusion cells were analyzed by statistical software (n = 3, compared with the control group).

gen proteins of M. tuberculosis in pleural effusion cells.

Discussion

Immune cells in the lung such as macrophages, DC cells, and lymphocytes are the most important cells in the original alveolar space are too weak to inhibit the growth of bacteria, so tuberculosis can spread to the surrounding cells and present antigens to sensitize peripheral T lymphocytes [9]. Macrophages phagocy-

tize M. tuberculosis, then the cells decompose and bacteria released through hydrolytic enzymes in lysosomes are able to kill or inhibit M. tuberculosis while attracting or recruiting other immune cells to the inflammatory site and present antigens to T-cells to acquire the specific cellular immune [10]. Antigen-presenting cells containing mycobacterial components released from infected macrophages have been reported to promote cellular recruitment during granuloma formation and regulate host cells during M. tuberculosis infection [11].

In cell supernatant of *M. avium*-infected macrophages, six differential proteins such as two actin isoforms, guanine nucleotide-binding protein β-1, cofilin-1, and so on were found by 2-D-MALDI-TOF/TOF mass spectrometry but proteins of bacteria were not found [12]. Meanwhile, exosomes in the cell supernatant from macrophages treated with M. tuberculosis culture filtrate proteins (CFP) contained a lot of mycobacterial proteins such as Antigen 85-C, LpdC, PstS1, HspX, Mpt51, and Alanine analyzed by proteomic analysis [13].

In this study, differential expression protein bands of pleural effusion cells of tuberculosis patients were analyzed by mass spectrometry. Nine proteins were identified successfully, of which six proteins came from human cells and three bacterial protein components. Among differential proteins were involved in cell membrane channels, cytoskeletal, regulation proteins and cell immunity. Importantly, we were interested in twelve proteins of them such as CDK1, SLA, NOXO1, COF1, LAMP-1, CD81, HSPB1, LAMP-1, Atg85B, and PRv1269c. Then, they were validated by Western blotting.

Differential proteins in vesicles are involved in multiple life functions of cells, for instance, CDK1 as a key player in cell cycle regulation [14], CD81 and SLAP1 participate in the activation or inhibition of lymphoma cell [15, 16], SLA takes part in the immune response and negatively regulates T-cell receptor signaling [17], NADPH oxidase organizer 1 up-regulates superoxide-producing activity by nine fold [18], and COF-1 is required for cytokinesis, endocytosis, and other cellular processes [19, 20]. It can be speculated that the above proteins from pleural effusion cells are influenced by the invasion of M. tuberculosis to protect immune ability, structural stability, and energy metabolism of host cells. Fortunately, Atg85B and PRv1269 in pleural effusion cells of tuberculosis patients are all important antigens of M. tuberculosis, which could be used as protein vaccine but their specific biological functions need to be studied further.

In summary, a number of proteins involved in various life activities of host cells could be interfered with by the infection of M. tuberculosis and pleural effusion cells might provide a novel diagnosis way for M. tuberculosis infection. However, the biological functions of differ-ential proteins and specific immune mechanisms of pleural effusion cells still require in depth research.

Disclosure of conflict of interest

None.

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