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Elevated host lipid metabolism revealed by iTRAQ-based quantitative proteomic analysis of cerebrospinal fluid of tuberculous meningitis patients

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ABSTRACT

Purpose: Tuberculous meningitis (TBM) remains to be one of the most deadly infectious diseases. The pathogen interacts with the host immune system, the process of which is largely unknown. Various cellular processes of Mycobacterium tuberculosis (MTB) centers around lipid metabolism. To determine the lipid metabolism related proteins, a quantitative proteomic study was performed here to identify differential proteins in the cerebrospinal fluid (CSF) obtained from TBM patients (n = 12) and healthy controls (n = 12).

Methods: CSF samples were desalted, concentrated, labelled with isobaric tags for relative and absolute quantitation (iTRAQ™), and analyzed by multi-dimensional liquid chromatography-tandem mass spectrometry (LC-MS/MS). Gene ontology and proteomic phenotyping analysis of the differential proteins were conducted using Database for Annotation, Visualization, and Integrated Discovery (DAVID) Bioinformatics Resources. ApoE and ApoB were selected for validation by ELISA.

Results: Proteomic phenotyping of the 4 differential proteins was invloved in the lipid metabolism. ELISA showed significantly increased ApoB levels in TBM subjects compared to healthy controls. Area under the receiver operating characteristic curve analysis demonstrated ApoB levels could distinguish TBM subjects from healthy controls and viral meningitis subjects with 89.3% sensitivity and 92% specificity.

Conclusions: CSF lipid metabolism disregulation, especially elevated expression of ApoB, gives insights into the pathogenesis of TBM. Further evaluation of these findings in larger studies including anti-tuberculosis medicated and unmedicated patient cohorts with other center nervous system infectious diseases is required for successful clinical translation.

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1. Introduction

Tuberculosis(TB), caused by Mycobacterium tuberculosis (MTB) infection, remains one of the most deadly infectious diseases worldwide [1,2]. China, among the highest TB burden countries, accounts for 12% of the world's TB cases and a serious epidemic of drug-resistant tuberculosis [3]. Tuberculous meningitis (TBM), the most common and severe form of the TB affecting the central nervous system (CNS), may result in fatal death and permanent sequelae [4].

It is well known that MTB seeded and formed at the meninges or brain parenchyma during primary infection when they settled with peace within infected macrophages, and then reactivates and ruptures into the subarachnoid space causing TBM. However, it is largely unknown how MTB interacts with the host immune system during survival, latent infection, reactivation and progression. Clarifying the pathogenesis of TBM, faciliates quick diagnosis and

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urgent treatment [5–7]. The major aspect of MTB pathogensis is the interactions between macrophage membrane and the mycobacterial cell wall, which are mediated mainly by bacterial lipids[8]. Lipids constitute almost 60% of the MTB cell wall, provides source of energy and regulate various cellular processes. It functions as a barrier to evade eradication by the host immune system [8] and prevent entry of chemotherapeutic agents [9]. Two cell wall glycolipids even help MTB survive within macrophages until arriving the initial infection sites [10]. MTB is exotoxin and endotoxin free, nor has it invasive enzymes. Lipids serve as the virulence. The invasiveness is stronger with higher content of lipid. Most studies focusing on lipid metabolisms of MTB were based on pulmonary TB. During latency, MTB used host triacylglycerol to survive in lipid-loaded macrophages [11]. It is found when M. avium infected bone marrow-derived mouse macrophages exposed to very-low-density lipoprotein (VLDL) as a lipid source, mycobacteria formed intracytoplasmic lipid inclusions which resulted in a reversible block of division. When VLDL removed, mycobacterial division restored. This showed that VLDL might be key during latency and reactivation of tuberculosis [12]. Lipid rafts (LR) were known as the dynamic, cholesterol-dense regions on the plasma membrane. It was reported that there were dose- and time-dependent increases in LR aggregation after MTB infection in alveolar epithelial cells, which were utilized by MTB for internalization and survival in host cells [13]. MTB also metabolized cholesterol throughout the course of infection, as a result, degradation of cholesterol was crucial for bacterial persistence [14]. However, there is no research revealing the lipid profile in TBM patients.

Mass spectrometry-based quantitative proteomics has become an essential approach in identifying disease-specific lipid profiles. It yields information about differences of all proteins between samples in a single experiment. Among them, iTRAQ-based proteomics is a newly emerged valuable tool for the detection of relatively scarce proteins, like those in CSF [15,16].

In this study, an iTRAQ-based quantitative proteomic approach was applied to identify differentially expressed proteins from CSF of TBM patients as compared to patients with viral meningitis and healthy controls. As far as we know, this is the first research this proteomic approach be used in CSF of TBM patients. Several proteins were identified with differential expressions in TBM. These novel and lipid related proteins were further validated.

2. Subjects

The protocol of this study and the procedures employed for sample collection were approved by the Ethical Committee of Chongqing Medical University and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Written informed consents were obtained from all participants. Thirty seven TBM subjects were diagnosed following the standard criteria [17] and enrolled in the Department of Neurology at the First Affiliated Hospital of Chongqing Medical University and forty healthy control (HC) subjects were enrolled after acquiring written informed consents in the medical examination center at the First Affiliated Hospital of Chongqing Medical University. The HC subjects were required to have neither active TB nor any neurological complaints.

The recruited TBM and HC subjects were divided into two cohorts. 12 TBM and 12 demographically matched HC subjects were used to identify candidate biomarkers using iTRAQ™-based quantitative proteomic approach. Another 25 TBM and 28 HC subjects were used for validation by ELISA.

2.1. Protein digestion and iTRAQ™ labeling

Pooled CSF samples were generated by combining equal volumes of the 12 individual plasma samples from each group. Each sample (5 ml) was desalted and concentrated to 80 ml in a Vivaspin spin column with a molecular weight cutoff of 5 kDa (Vivascience AG, Hannover, Germany). Then, 120 μg of protein from each sample was dissolved in 30 μl of STD buffer (4% SDS, 100 mM DTT, 150 mM TrisHCl pH 8.0), boiled in water for 5 min, cooled to room temperature, diluted with 200 μl of UA buffer (8 M Urea, 150 mM TrisHCl pH 8.0), and transferred to 30 kd ultrafiltration. Samples were centrifuged at 14000 g for 15 min and 200 μl of UA buffer was added. The samples were centrifuged again for 15 min at the same conditions. Then, 100 μl of 0.05 M iodoacetamide in UA buffer was added, and the samples were incubated for 20 min in darkness. After 10 min of centrifugation at the above conditions, the filters were washed three times with 100 μl of UA buffer. Then, 100 μl of DS buffer (50 mM triethylammoniumbicarbonate, pH 8.5) was added to the filters, and the samples were centrifuged for 10 min at the above conditions. This step was repeated twice. Finally, 2 μg of trypsin (Promega) in 40 μl of DS buffer was added to each filter, and the samples were incubated overnight at 37 °C. The resulting peptides were collected by centrifugation. The filters were rinsed with 40 μl of 10 × DS buffer again. iTRAQ™ labeling was performed according to the manufacturer's instructions (Applied Biosystems). As each sample consisted of a pool of CSF from either 12 TBM subjects or 12 HC subjects, iTRAQ™ reagents 114 and 116 were applied to the HC sample and regents 115 and 117 were applied to the TBM sample. The analytic processes, including plasma depletion, protein digestion, iTRAQ™ labeling, SCX fractionation, and LC-MS/MS analysis, were repeated twice.

2.2. Peptide fractionation with strong cation exchange chromatography

Prior to liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis, peptides were purified from excess labeling reagent by strong cation exchange (SCX) chromatography: peptides were dried in a vacuum concentrator, dissolved in strong cation exchange buffer A (10 mM KH_2PO_4 in 25% of ACN, pH 3.0), and loaded onto a Polysulfoethyl 4.6 × 100 mm column (5 μm, 200 Å, PolyLC Inc., Maryland, U.S.A.) at a flow rate of 1 ml/min. A suitable gradient elution was applied to separate peptides at a flow rate of 1 ml/min with elution buffer (10 mM KH_2PO_4, 500 mM KCl in 25% acetonitrile, pH 3.0). Eluted peptides were collected and desalted by an offline fraction collector. The resulting fractions were combined to ten pools and desalted on C18 Cartridges (Empore™ SPE Cartridges C18 (standard density), bed I.D. 7 mm, volume 3 ml, Sigma, St. Louis, MO, USA). Each final fraction was concentrated by a vacuum concentrator and resuspended in 40 μl of 0.1% (v/v) trifluoroacetic acid. All samples were stored at −80 °C until later LC-MS/MS analysis.

2.3. LC-MS/MS analysis

For each fraction, 10 μl of solution was injected for nano LC-MS/MS analysis using an AB SCIEX TripleTOF 5600 MS (Toronto, Concord, Canada) equipped with a splitless Eksigent nanoUltra 2D Plus nanoLC system and a cHiPLC Nanoflex microchip system (Dublin, CA, USA). The Nanoflex system uses replaceable microfluidic traps and columns packed with ChromXP C18 (3 μm, 120 Å) for online trapping, desalting, and analytical separations. The sample was loaded, and trapping and desalting were performed at 2 μl/min for 10 min with 100% mobile phase A (2% acetonitrile/0.2% formic acid/98% water). For peptide elution, the gradient started at

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