An iTRAQ approach to quantitative proteome analysis of cerebrospinal fluid from patients with tuberculous meningitis

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Summary   **To study the cerebrospinal fluid (CSF) protein profiles of tuberculous meningitis (TBM) and discover potential biomarkers for TBM, differential expression of proteins in the CSF of patients with TBM, patients with cryptococcal meningitis, and a control group were compared using isobaric tags for relative and absolute quantitation labelling (iTRAQ) coupled with 2-dimensional liquid chromatography-tandem mass spectrometry (LC-MS). As a result, a total of 208 unique proteins with a molecular weight ranging from 10 KD to 135 KD were identified and quantified in CSF samples from patients with TBM. Of the proteins, 9 were expressed at levels differing 2.0 fold, 6 were up-regulated, and 3 were down-regulated. These proteins appear to be involved in calcium ion binding, lipoprotein metabolism, immune response, and signal conduction. Two differentially expressed proteins were identified using ELISA. The present study represents the successful use of iTRAQ to examine CSF from patients with TBM. The differentially expressed proteins identified may be potential diagnostic biomarkers and provide valuable insight into the underlying mechanisms of TBM. This study also demonstrated that the differential protein profiles of diseases can be quickly determined using iTRAQ-LC-MS, a potential method for quantitative comparative proteomics.**

Keywords: Tuberculous meningitis (TBM), iTRAQ, LC-MS, biomarker

1. Introduction

Tuberculous meningitis (TBM) is an infection of the meninges caused by *Mycobacterium tuberculosis* (MTB) and is associated with high mortality rates and severe neurological sequelae. Its prognosis largely depends on early diagnosis as well as timely treatment (*1,2*). Currently, however, the diagnosis of TBM remains a major challenge due to inadequate diagnostic methods and poor sensitivity and/or specificity of existing markers. Thus, the search for more potential biomarkers to facilitate diagnosis and predict prognosis is of great importance.

Central nervous system (CNS) diseases can cause changes in protein expression in cerebrospinal fluid (CSF) (*3*). At present, differential proteomics provides a powerful approach to screen for variations in protein levels and posttranslational modifications associated with disease, culminating in the identification of many potential therapeutic targets and disease-related biomarkers. Numerous researchers have discovered potential biomarkers of disease using proteomics (*4-6*). Kumar *et al.* performed quantitative protein expression profiling of the brains of patients with TBM and normal individuals using isobaric tags for relative and absolute quantitation labelling (iTRAQ) labelling and LC-MS/MS, and they found several potential diagnostic biomarkers for tuberculous (*7*). However, the global proteomic profiling of CSF from individuals with TBM has proceeded very slowly, and changes in the CSF proteomes of patients with TBM are rarely reported.

Although 2-dimensional gel electrophoresis is commonly used in conventional proteomics, its drawbacks have been noted; these include low sensitivity,

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taking an excessive amount of time, and failure to detect low-abundance proteins (*8*). A new labelling method that uses isobaric tags for relative and absolute quantitation labelling (iTRAQ) avoids these drawbacks and offers several advantages (*9*). iTRAQ reagents have been developed for the relative and/or absolute quantification of complex protein mixtures by labelling tryptic fragments of protein mixtures with reporter tags at the N-terminus and lysine residues (*10*). Quantification is achieved by comparing reporter ion abundance (*i.e.*, m/z 114, 115, 116, and 117) in the MS/MS spectra. Isotope-coded affinity tag (ICAT) reagents react with cysteine residues, but iTRAQ labels the unblocked N-termini of tryptic peptides and lysines, thus improving protein coverage.

In the present study, iTRAQ and liquid chromatography-tandem mass spectrometry (LC-MS) were used to identify differential proteins in patients with TBM. Nine proteins that were differentially expressed in CSF from patients with TBM were identified. These proteins may be potential diagnostic protein markers for tuberculous. Two of these potential biomarkers were identified using ELISA.

2. Material and Methods

2.1. *Subjects*

Subjects were patients with TBM and patients with cryptococcal meningitis seen by the Department of Infectious Diseases of Shanghai Public Health Clinical Center from January 2009 to December 2010 and healthy volunteers. *i*) Patients with TBM: twenty patients definitively diagnosed with TBM (having the disease no longer than 2 weeks) consisting of 13 males and 7 females. Their ages ranged from 18 to 52 years, with an average age of 32.6 years. All patients had typical clinical manifestations of TBM and CSF that tested positive for *M. tuberculosis* DNA according to acid-fast bacillus smears, cultures, or PCR results. The patients were yet to receive treatment. *ii*) Patients with cryptococcal meningitis: twenty patients consisting of 12 males and 8 females. Their ages ranged from 18 to 65 years, with an average age of 35.8 years. These patients had typical clinical manifestations, positive smear or culture results, and were yet to receive treatment. *iii*) Normal control group: twenty healthy volunteers consisting of 13 males and 7 females. Their ages ranged from 16 to 55 years, with an average age of 38.4 years. Individuals in this group were free of CNS infections and had normal CSF biochemical and routine test results.

There were no statistically significant differences between the patients and healthy individuals in terms of age and gender ($p > 0.05$). The present study was approved by the Ethics Committee of Shanghai Public Health Clinical Center. All subjects provided written informed consent for the collection of samples and subsequent analysis.

2.2. *Preparation of CSF samples*

CSF was extracted *via* a lumbar puncture. Pooled TBM, cryptococcal meningitis, and control samples were created by combining 800 μL of each individual CSF sample. Proteins were precipitated by mixing 10 volumes of cold acetone containing 10% trichloroacetic acid (TCA) with the CSF samples. The mixtures were then incubated overnight at –20°C, followed by centrifugation at 8,000 rpm/min for 15 min at 4°C. The concentrations of pooled samples were measured using the Bradford method (*11*).

2.3. *iTRAQ labelling and strong cation exchange (SCX) chromatography*

About 100 μg of each of three pooled samples was labelled. iTRAQ labelling was performed in accordance with a kit protocol (Applied Biosystem Inc, Foster City, CA, USA) and a previous report (*12*). After trypsin digestion, iTRAQ reagents (114, 115, and 117) in 70 μL ethanol were added separately to each tube and incubated at room temperature for 1 h (114 for patients with TBM, 115 for patients with cryptococcal meningitis, and 117 for the control group). All labelled peptide solutions were then pooled in a new vial and dried using a rotary vacuum concentrator (Christ RVC 2-25, Christ, Germany). Pooled iTRAQ-labelled peptide samples were desalted prior to SCX chromatographic fractionation and LC-MS/MS analysis.

iTRAQ-labelled mixed peptides were fractionated using SCX chromatography in a 20 AD HPLC system (Shimadzu, Kyoto, Japan) using a polysulfoethyl column (2.1 mm × 100 mm, 5 μm, 200 Å, The Nest Group, Southborough, MA, USA). The peptide mixture was reconstituted in Buffer A [10 mM KH_2PO_4 in 25% ACN (Fisher Scientific, Fair Lawn, New Jersey), pH 2.6] and loaded onto the column. The peptides were separated at a flow rate of 200 μL/min for 60 min with a gradient of 0% to 80% Buffer B (Buffer A containing 350 mM KCl) in Buffer A. Fractions were collected in 3-min increments and vacuum-dried.

2.4. *Protein identification by MS*

Fractions were vacuum-dried and resuspended in 50 μL HPLC Buffer A (5% ACN, 0.1% formic acid (TEDIA, Fairfield, USA)), loaded onto the RPLC column (ZORBAX 300 SB-C18 column, 5 μm, 300 Å, 0.1 mm × 15 mm, Microm, Auburn, CA, USA) and analyzed on a QSTAR XL System (Applied Biosystem, Inc.) and a 20AD HPLC system (Shimadzu). MS data were acquired automatically using Analyst QS 1.1 Service Pack 8 software (ABI/MDS SCIEX, Concord, Canada).

Survey scans were acquired from m/z 400 to 1,800, with up to 6 precursors selected for MS/MS from m/z 100 to m/z 2,000.

Ratios of 114.1, 115.1, and 117.1 amu signature mass tags were generated from MS/MS fragmentation of iTRAQ-labelled tryptic peptides and calculated using Protein Pilot (version 3.0, Applied Biosystem, Inc.). MS and MS/MS tolerances were set to 0.2 Da. The International Protein Index (IPI) (version 3.45, HUMAN) database was used to search for peptides. All proteins identified must have ≥ 95% confidence, and the protein confidence threshold cut-off was set at 1.3 (unused) with at least more than two peptides above the 95% confidence level. Fold-changes > 2.0 or < 0.5 were set as cut-off values to designate significant protein expression changes. The subcellular location and function of identified proteins were determined according to the UniProt knowledgebase (Swiss-Prot/UniProtKB) and Gene Ontology (GO) database.

2.5. *ELISA analysis of individual samples*

Differential proteins were measured in an independent sample set of subjects (25 patients with TBM, 25 patients with cryptococcal meningitis and 25 healthy volunteers) by commercially available ELISA in accordance with the manufacturer's instructions. Data were expressed as mean ± S.D. and statistical analysis was performed with SPSS 13.0 software (SPSS, Chicago, IL). $p < 0.05$ was considered statistically significant.

3. Results

3.1. *CSF proteomes in patients with TBM*

After LC-MS/MS analysis, 208 proteins of CSF in TBM were identified and quantified. According to the GO database, most of the proteins identified were located outside cells or in the membrane, cytoplasm, or cytoskeleton, and most function through binding, catalytic activity, enzyme regulator activity, and transporter activity. Most of the proteins identified may be involved in biological regulation, cellular processes, response to stimulus, metabolic process, or the like (Figure 1).

3.2. *Identification of differentially expressed proteins*

Nine differentially expressed proteins were identified: 6 proteins were up-regulated and 3 proteins were down-regulated. Representative MS/MS spectra for one peptide, identified from the APOB antigen, are shown in Figure 2.

Up-regulated and down-regulated proteins are showed in Tables 1 and 2. These proteins were related to cholesterol metabolism, response to stimulus, calcium ion binding, and prostaglandin-D synthase activity.
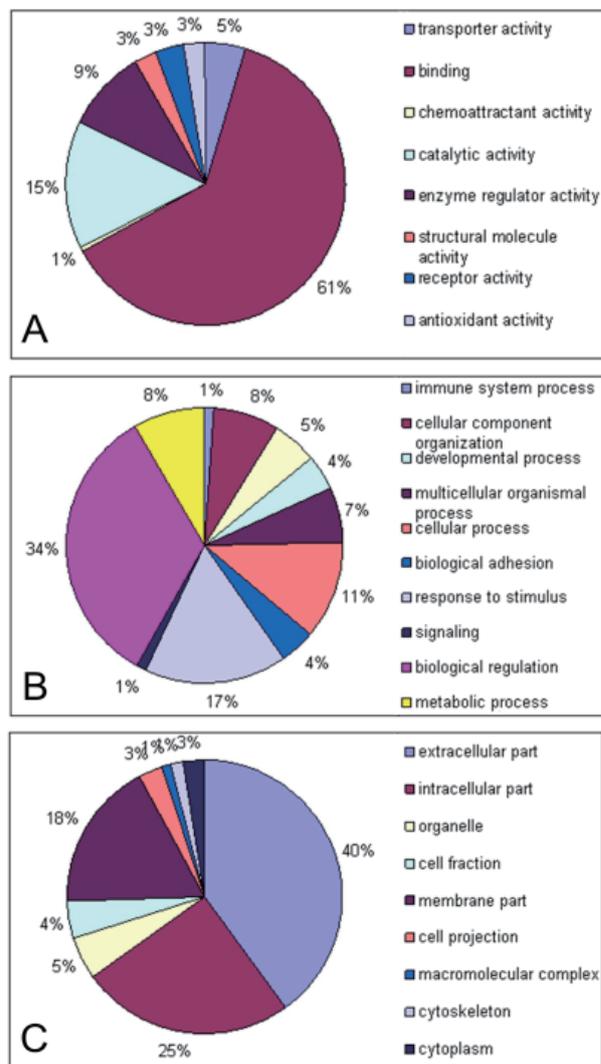

Figure 1. Gene ontology (GO) analysis of 208 proteins in CSF from patients with TBM. (A) molecular function; **(B)** biological process; **(C)** cellular component.

3.3. *Differential expression of S100A8 and APOB according to ELISA*

The levels of S100A8 and APOB expression in patients with TBM, patients with cryptococcal meningitis, and the control group were examined with ELISA to further ascertain differences in protein expression observed using iTRAQ. Differences between patients with TBM and patients with cryptococcal meningitis and the control group ($p < 0.05$) were noted. This result validates the current findings regarding the proteomic probing of differential CSF proteins (Table 3).

4. Discussion

The iTRAQ approach was first described in 2004. The method uses new isobaric reagents that consist of a charged reporter group, a peptide-reactive group, and a neutral balance portion, and the method can identify more proteins than other proteomic methods. It has
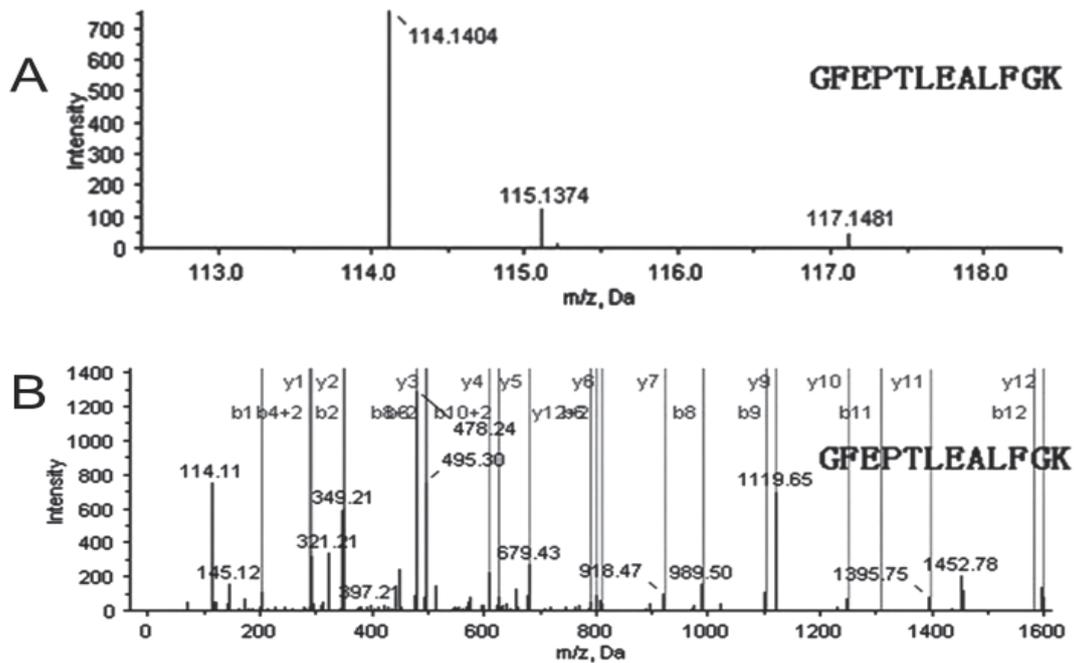
Figure 2. APOB Precursor MS region (GFEPTLEALFGK). The ion assignments were as follows: 114 for patients with TBM, 115 for patients with cryptococcal meningitis, and 117 for the control group. The intensity of reporter ions from precursor peptides indicates protein expression levels **(A)**. The MS/MS spectra revealed peptide sequences with GFEPTLEALFGK, leading to the identification of APOB **(B)**.

Table 1. Up-regulated proteins in CSF from individuals with TBM

IPI	Protein name	MW (D)	Peptide (95%)	Ratio 114:117	Ratio 114:115	Subcellular Location	Molecular function
IPI00847179.1	Apolipoprotein A-I (APOA4)	45372	8	4.4	2.2	secreted	cholesterol transporter activity, antioxidant activity, and copper ion binding
IPI00884926.1	Orosomucoid 1 (ORM1)	23540	20	9.8	2.1	secreted	regulation of immune system processes and protein binding
IPI00292532.6	Cathelicidin antimicrobial peptide (CAMP)	19591	2	2.9	7.4	secreted	antibacterial activity and antibiotic activity
IPI00007047.1	S100 Calcium-binding Protein A8 (S100A8)	10835	4	3.9	9.9	cytoplasm; cell membrane	calcium ion binding and protein binding
IPI00022229.1	Apolipoprotein B-100 (APOB)	515563	7	13.4	3.9	secreted	cholesterol transporter activity, enzyme binding, and low-density lipoprotein receptor binding
IPI00847635.1	a1-antichymotrypsin (SERPINA3)	47652	17	3.1	2.3	secreted	serine-type endopeptidase inhibitor activity and protein binding

CSF: cerebrospinal fluid; TBM: tuberculous meningitis; IPI: international protein index; MW: molecular weight; D: daltons.

Table 2. Down-regulated proteins in CSF from individuals with TBM

IPI	Protein name	MW (D)	Peptide (95%)	Ratio 114:117	Ratio 114:115	Subcellular location	Molecular function
IPI00514285.2	Prostaglandin D2 synthase (PTGDS)	21080	86	0.35	0.42	nucleus membrane	fatty acid binding, transporter activity, and prostaglandin-D synthase activity
IPI00021842.1	Apolipoprotein E (APOE)	36154	31	0.44	0.36	secreted	antioxidant activity, low-density lipoprotein particle receptor binding, and beta-amyloid binding
IPI00607600.1	Calsyntenin-1 (CLSTN1)	108643	6	0.11	0.48	postsynaptic cell membrane	calcium ion binding

CSF: cerebrospinal fluid; TBM: tuberculous meningitis; IPI: international protein index; MW: molecular weight; D: daltons.

Table 3. Differential expression of S100A8 and APOB in CSF

Protein	Patients with TBM (ng/mL)	Patients with cryptococcal meningitis (ng/mL)	Control group (ng/mL)
S100A8	$48.9 \pm 9.1^{*,\#}$	16.9 ± 5.2	13.9 ± 6.7
APOB	$480.1 \pm 215.2^{*,\#}$	166.5 ± 241.9	125.6 ± 131.2

Compared to patients with cryptococcal meningitis, $^{\#}p < 0.05$; Compared to the control group, $^{*}p < 0.05$.

been extensively used to identify biomarkers in various disease contexts ([13,14]).

The present study successfully used iTRAQ-LC-MS/MS to analyze the expression of proteins in CSF from individuals with TBM. A total of 208 proteins with a molecular weight ranging from 10 KD to 135 KD were identified through LC-MS/MS and a database search. GO analysis indicated that these proteins are involved in transport, biological regulation, cellular processes, immune response, metabolic process, and the like. Nine of the 208 proteins were found to be differentially expressed in patients with TBM compared to patients with cryptococcal meningitis and the control group. Six of the 9 proteins were up-regulated and three were down-regulated. Two differential proteins were identified using ELISA. The 9 differentially expressed proteins may be involved in the host response to TBM at the molecular level and may be potential diagnostic biomarkers for TBM.

S100A8 belongs to the S100 protein family and originates from neutrophil granulocytes and macrophages. This protein favors cell growth and restrains proliferation, has the ability to mediate apoptosis and enzymatic activity, promotes a dynamic cytoskeleton, maintains calcium ion stability, and stimulates the development of cancer ([15,16]). In recent years, S100A8 has been found to be able to regulate inflammatory activity and S100A8 is overexpressed in many infectious diseases ([17]). In the present study, patients with TBM had higher levels of S100A8 expression in CSF than did patients with cryptococcal meningitis and the control group, which implies that S100A8 may be related to the pathological and biological progression of TBM. CLSTN1 is allocated in the postsynaptic membrane of CNS synapses and is a proteolytically processed protein of the postsynaptic membrane with a calcium-binding cytoplasmic domain ([18]). It is a dynamic modulator of postsynaptic calcium *via* extracellular proteolysis and may modulate local Ca transport either beneath the postsynaptic membrane or around intracellular Ca stores. In the present study, CLSTN1 levels in the CSF of patients with TBM were lower than those in patients with cryptococcal meningitis and the control group. Symptoms such as loss of memory and impaired cognition are presumed to be related to the abnormal expression of CLSTN1. However, further research is needed to determine the exact mechanism of this process.

APOB, a component of low-density lipoprotein, can participate in endogenous cholesterol and triacylglycerol transport ([19]). Upregulation of APOB can cause arterial atherosclerosis, which is responsible for coronary disease and arteriosclerosis ([20]). APOA4 plays a key role in human cholesterol homeostasis, secretion of chylomicron particles, and antioxidant activity ([21]). The present study found that levels of APOB and APOA4 expression were higher in patients with TBM than in patients with cryptococcal meningitis and the control group, which suggests that the onset and development of TBM is related to abnormal cholesterol metabolism. Further studies are needed to prove this hypothesis. APOE in human brains is mainly synthesized and secreted by astrocytes, oligodendrocytes, neurons, and activated neuroglia cells ([22]). It modulates cholesterol and phospholipid homeostasis and adjusts the mobilization and redistribution of cholesterol and phospholipids during remolding of the neurilemma to mediate the maintenance of synapse flexibility and repair of impaired nerve cells ([23]). The *APOE* gene can affect memory and facilitate neuron maintenance and repair. Research has shown that APOE-knockout mice have learning and memory deficits compared to normal controls ([24]). In the present study, APOE levels in the CSF of patients with TBM were lower than those in patients with cryptococcal meningitis and the control group, presumably because astrocyte cells or neurons that have been attacked exhibit impeded composition and secretion of APOE. The lack of APOE degrades the repair function of nerve cells. An insufficient supply of acetylcholine leads to nervous system sequelae, such as memory loss and dementia. *APOE* gene polymorphism has been associated with Alzheimer's disease, ischemic stroke, hemorrhagic stroke, multiple sclerosis, Parkinson's disease, schizophrenia, and other diseases ([25-27]). Further studies are needed to determine the effects of *APOE* gene polymorphism on TBM.

ORM1, SERPINA3, and CAMP are proteins known to be related to acute inflammatory reactions. When MTB attacks meninges, it causes a severe inflammatory reaction. Large amounts of cell factors and mediators of inflammation are released and cause an acute reaction, resulting in an increase in ORM1 and SERPINA3 secretion. Several studies have found that SERPINA3 is overexpressed in CSF from patients with Alzheimer's disease and that it plays an important role in the pathology and pathogenesis of the disease ([28,29]). Presumably there are common factors involved in the pathogenesis of both TBM and Alzheimer's disease. CAMP plays an important part in natural immune systems. It functions as a antimicrobial, regulating the immune system and the release of inflammation mediators and accelerating revascularization ([30,31]). The present study found that MTB can cause high levels of CAMP expression, indicating that CAMP serves as an immediate and effective frontline defense.

Further studies are needed to clarify the effect of high levels of ORM1, SERPINA3, and CAMP expression on the onset of TBM and assessment of prognosis.

PTGDS is a member of the prostaglandin synthetases and a transporter of retinoids and thyroxine. PTGDS is expressed in tissues of the blood-brain barrier and secreted into the CSF. Changes in PTGDS expression may influence signal transmission and the activities of vitamin A and thyroxine (*32*). PTGDS may be involved in blood-brain development and maintenance. It may play an important role in the maturation and maintenance of both the CNS and the male reproductive system. A retrospective analysis of published data that quantified relative amounts of PTGDS in patients with multiple sclerosis, schizophrenia, and Parkinson's disease compared to controls revealed significant dysregulation (*33*). In the present study, levels of PTGDS in CSF from patients with TBM were lower than those in CSF from patients with cryptococcal meningitis and the control group. This implies that the pathogenesis of TBM may be related to the abnormal expression of PTGDS, although the exact mechanism of this process remains unclear.

In conclusion, 9 differentially expressed proteins associated with TBM were identified using an iTRAQ-based quantitative proteomic approach. Some proteins such as PTGDS, CLSTN1, and S100A8 were found to be expressed at high levels in TBM for the very first time. These promising potential markers warrant further study and evaluation in patients with TBM to determine their clinical utility. The present study also demonstrated that the differential protein profiles of diseases can be quickly determined by iTRAQ-LC-MS/MS, a commercially available method of quantitate comparative proteomics.

Acknowledgements

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