iTRAQ-based quantitative proteomic analysis of cerebrospinal fluid reveals NELL2 as a potential diagnostic biomarker of tuberculous meningitis

YONGTAO YANG[1-3*], JUN MU[1-3*], GUANGHUI CHEN[2,3*], YUAN ZHAN[2,3*], JIAJU ZHONG[2-4], YOUDONG WEI[1-3], KE CHENG[1-3], BIN QIN[1-3], HONGMIN YOU[2,3] and PENG XIE[1-3]

[1]Department of Neurology, The First Affiliated Hospital of Chongqing Medical University; [2]Institute of Neuroscience, Chongqing Medical University; [3]Chongqing Key Laboratory of Neurobiology; [4]Department of Neurology, Yongchuan Hospital of Chongqing Medical University, Chongqing, P.R. China

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Abstract. Tuberculous meningitis (TBM) is a serious complication of tuberculosis that affects the central nervous system. As TBM may result in permanent sequelae and death, rapid, accurate diagnostic tests using novel biomarkers are required for the early diagnosis and treatment of TBM. A quantitative proteomic study was therefore performed to identify differential proteins in the cerebrospinal fluid (CSF) obtained from TBM patients (n=12) and healthy controls (n=12). CSF samples were labelled with iTRAQ™ and analyzed by LC-MS/MS. Gene Ontology and Pathway Analysis were conducted using DAVID bioinformatics resources. Neural epidermal growth factor-like like 2 (NELL2) with the largest fold-change value was selected for validation by western blotting. Proteomic phenotyping revealed over-representation in two inflammation-associated processes, complement and coagulation cascades as well as cell adhesion molecules. Western blotting showed a significant decrease in NELL2 levels in TBM subjects compared to healthy controls. The AUC analysis revealed NELL2 was able to distinguish TBM subjects from healthy controls with 83.3% sensitivity and 75% specificity. In conclusion, the results showed that CSF NELL2 is a potential diagnostic biomarker for TBM. Further evaluation of these findings in larger studies including anti-tuberculosis medicated and unmedicated patient cohorts with other intracranial infectious diseases is required for clinical translation.

Correspondence to: Professor Peng Xie, Department of Neurology, The First Affiliated Hospital of Chongqing Medical University, 1 Yixue Road, Yuzhong District, Chongqing 400016, P.R. China
E-mail: xiepeng@cqmu.edu.cn

*Contributed equally

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Introduction

Each year, almost 10 million individuals worldwide are newly diagnosed with tuberculosis (TB), an infectious disease caused by Mycobacterium tuberculosis (MTB) with 1.7 million mortalities owing to TB (1). Moreover, two billion individuals worldwide with latent MTB infection represent a potential risk of TB reactivation (2). The burden of disease of TB is highest in Asia, with China listed only second to India. In the latest Global Tuberculosis Report by the World Health Organization (WHO), China accounts for 12% of the TB cases worldwide. According to the National Survey of Drug-Resistant TB conducted by the Chinese Center for Disease Control, China also has a serious epidemic of drug-resistant TB (3), with 5.7% of newly diagnosed cases and 25.6% of previously treated cases being multidrug-resistant (MDR) TB.

TB affects the central nervous system (CNS); a condition termed CNS tuberculosis, and is the most severe complication of TB infection. Tuberculous meningitis (TBM) is the most common form of CNS tuberculosis. In TBM patients, MTB in Rich foci (previously seeded and formed in the meninges or brain parenchyma through primary infection) reactivate and rupture into the subarachnoid space, producing meningitis. As TBM may result in permanent sequelae and death, prompt diagnosis and treatment are required (4). Prognosis depends on the clinical stage at which TBM is diagnosed (5). Effective TBM control requires early, efficient, and accurate diagnostic biomarkers in addition to drug-resistance identification and treatment response evaluation (6-8). However, the currrent gold standard for TBM diagnosis, MTB isolation from cerebrospinal fluid (CSF) culture, usually takes weeks to reach a positive outcome and has a relatively low sensitivity of 25-70% (9).

Since these conventional methods do not satisfy the requirements for diagnosis of TBM, it is crucial to target complementary disease-specific biomarkers that can facilitate earlier diagnosis and treatment in TBM patients. However, most studies on TB biomarkers have focused on pulmonary TB. In previous studies it was suggested that diagnostic biomarkers for TBM, were adenosine deaminase isoenzyme-2

(ADA2) (10), adenosine deaminase (ADA) (11,12), 65-kDa heat shock protein antigen (13), CSF lactate, CSF lactate dehydrogenase (14) and arachidonate 5-lipoxygenase (15). However, none of these biomarkers have been sufficiently validated (16).

Therefore, rapid, accurate diagnostic tests using novel biomarkers for TBM remain to be developed. To that end, mass spectrometry (MS)-based quantitative proteomics has become a useful tool in identifying disease-specific biomarkers (17) by yielding information about differences across a spectrum of proteins within samples in a single experiment. Kumar *et al* (18) have applied an MS-based quantitative proteomic approach to compare postmortem brain tissues from confirmed cases of TBM with uninfected brain tissues and found upregulated levels of signal-regulatory protein α (SIRPA), protein disulfide isomerase family A, member 6 (PDIA6), amphiphysin (AMPH) and neurofascin (NFASC) in addition to downregulated levels of ferritin light chain (FTL). Although all the postmortem brain tissue samples were collected within 8-16 h of death with the patients' bodies maintained at 4˚C, proteins are rapidly degraded after death. Thus, there is a reasonable risk that TBM-specific proteins may have been missing or severely altered in the study of Kumar *et al*. As opposed to brain tissue, CSF can more easily be collected from live patients by lumbar puncture. As the CSF is in constant exchange with the brain's interstitial fluid and thus alterations in protein expression within the brain may be closely reflected in the CSF (17,19), the CSF appears to be a more promising source of biomarkers for TBM. Among MS-based approaches, the isobaric tag for relative and absolute quantitation (iTRAQ™)-based proteomics has emerged as particularly valuable in detecting relatively scarce proteins, such as those in the CSF (18,20).

In the present study, an iTRAQ™-based quantitative proteomic approach was used to identify differential proteins in the CSF obtained from live TBM patients and healthy controls. Proteomic phenotyping of the 81 differential proteins revealed over-representation in two inflammation-associated processes, complement and coagulation cascades as well as cell adhesion molecules. Western blotting of the neuron-specific differential protein with the largest absolute fold-change value, neural epidermal growth factor-like like 2 (NELL2), showed significantly decreased NELL2 levels in TBM subjects compared to healthy controls. Area under the receiver operating characteristic curve analysis demonstrated NELL2 was able to distinguish TBM subjects from healthy controls with 83.3% sensitivity and 75% specificity, indicating that CSF NELL2 shows promise as a diagnostic biomarker for TBM.

Materials and methods

Subjects. The protocol of the present study and the procedures employed for sample collection were approved by the Ethics 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 consent was obtained from all participants. Twelve TBM subjects were diagnosed following the standard criteria (21) and enrolled in the Department of Neurology at the First Affiliated Hospital of Chongqing Medical University. Twelve healthy control (HC) subjects were enrolled after written informed consent was obtained 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.

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 cut-off 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 Tris-HCl 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 Tris-HCl pH 8.0), and transferred to 30 kDa ultrafiltration. The samples were centrifuged at 14,000 x g for 15 min and 200 μl of UA buffer was added. The samples were centrifuged again for 15 min under 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 the dark. 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. Subsequently, 2 μg of trypsin (Promega, Madison, WI, USA) 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 10X DS buffer again. iTRAQ™ labeling was performed according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). As each sample consisted of a pool of CSF from 12 TBM subjects or 12 HC subjects, iTRAQ™ reagents 114 and 116 were applied to the HC sample and reagents 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.

Peptide fractionation with strong cation exchange chromatography. Prior to liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis, the peptides were purified from excess labeling reagent by strong cation exchange (SCX) chromatography. Briefly, the 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 x 100 mm column (5 μm, 200 Å; PolyLC Inc., Columbia, MD, USA) 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 10 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 the samples were stored at -80˚C until subsequent LC-MS/MS analysis.

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, ON, Canada) equipped with a splitless Eksigent nanoUltra 2D Plus nanoLC system and a cHiPLC Nanoflex microchip system (Eksigent, 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 was initiated at 5% mobile phase B (98% acetonitrile/0.2% formic acid/2% water) and increased to 24% linearly in 70 min at a flow rate of 300 nl/min. The total gradient length was 120 min. MS data acquisition was performed in the information-dependent acquisition (IDA) mode. For TOF MS scans, the TripleTOF 5600 MS was operated with a resolving power of 30,000 FWHM. IDA survey scans were acquired in 250 msec with a mass range of m/z 350-1250. Specifically, 30x100 msec MS/MS were followed at a 120 cps (counts/sec) trigger with a precursor charge state of +2 to +5. Dynamic exclusion was set for 18 sec. Collision energies were calculated on-the-fly for all precursor ions using empirical equations based on mass and charge, and the Enhance iTRAQ™ function was turned on to improve the efficiency of the collision-induced dissociation.

Protein identification and quantification. All LC-MS/MS data were processed using ProteinPilot v4.2 and used to search the Uniprot_HUMAN.20140108_134997 database using the 'thorough search' option. The 'iTRAQ™ 8-plex peptide labeled' sample type and a 'biological modification ID focus' were selected for the analysis method. Trypsin was selected as the digestion enzyme, with cysteine alkylation by iodoacetamide as a modification. Data were normalized by ProteinPilot. The reported data were based on a false discovery rate (FDR) ≤1% confidence for protein identification. Differential proteins were analyzed by ProteinPilot for significant downregulation or upregulation. A significance threshold of 95% was selected as a criterion for each individual experiment. Peptides that matched multiple proteins were not included in the software quantification. The final ratios were calculated from the average of the ratios obtained in the two independent experiments in which the protein abundance was significant (P<0.05). In each experiment, bias correction for unequal mixing in the differently labeled samples was performed based on the assumption that most proteins do not change in expression. Thus, if the sample from each experimental condition was not combined in exactly equal amounts, this bias correction would fix the systematic error. The software identifies the median average protein ratio and corrects it to unity, and then applies this factor to all quantification results.

Gene Ontology and Pathway Analysis. Functional clustering was conducted using DAVID Bioinformatics Resources v6.7 (http://david.abcc.ncifcrf.gov/home.jsp) (22). A functional annotation analysis was performed with the Gene Ontology tool (GOTERM_CC_ALL) using the UniProt accession numbers for all the differential proteins. Only those GO terms in each gene list that yielded a P-value of <0.05 using Fisher's exact test were considered significantly enriched. A Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was subsequently performed using the same software, in which a corrected P-value of <0.05 was deemed to indicate a statistically significant difference.

Table I. Demographic and clinical characteristics of the participants.

Variables	HC	TBM	P-value (HC vs. TBM)
Sample size	12	12	
Gender (M/F)	7/5	5/7	0.44
Age (years)[a]	27.7±5.3	28.3±8.2	0.09
BMI[a]	46.1±10.0	36.9±14.6	0.85
TB culture	12/12	0/12	-
Fungal culture	0/12	0/12	-

HC, healthy controls;TBM, patients infected with tuberculous meningitis; M, male; F, female; BMI, body mass index; TB, tuberculosis. [a]Age and BMI are presented as the means ± SD.

Western blotting validation. Western blotting was performed on the 12 individual TBM samples and 12 individual HC samples. CSF samples were separated by 10% SDS-PAGE, blocked in 5% skim milk in TBST, and transferred onto PVDF membranes. The membranes were incubated overnight at 4˚C with an anti-NELL2 (diluted 1:1,000; Abcam, Cambridge, MA, USA) primary antibody. The membranes were washed and incubated with goat polyclonal to rabbit IgG horseradish peroxidase-coupled secondary antibody (diluted 1:10,000; Bio-Rad Laboratories, Hercules, CA, USA). After extensive washing, the protein bands detected by the antibodies were visualized by the enhanced chemiluminescence method and exposed to autoradiography film. After immunodetection, the membranes were stained with Ponceau S as an internal control for normalization. The western blot signals were densitometrically quantified in each sample with Quantity One (Bio-Rad Laboratories).

Statistical analysis. Statistical analysis was performed using the Statistical Package of Social Science (SPSS) for Windows v19.0. Data are expressed as the means ± SD. The Student's t-test was applied to identify proteins with significant abundance differences between the TBM and HC subjects. Areas under the receiver operating characteristic (ROC) curve (AUC) were calculated to evaluate classifier performance. All the tests were two-tailed. The significance threshold was set at P<0.05.

Results

Subject characteristics. Demographic and clinical data obtained from TBM and HC subjects are provided in Table I. The two groups of subjects were not differentiable by any key demographic characteristics, such as age, gender or body mass index (BMI).

Table II. Differential proteins identified by iTRAQ coupled with LC-MS/MS ordered by absolute fold-change.

Accession no.	Gene name	Protein name	Pep count	Cover %	Biological function	Fold-change (TBM/HC)
P01024	C3	Complement C3	147	52.5	Inflammation: acute-phase	2.25
P01023	A2M	α-2-macroglobulin	137	52.5	Inflammation: acute-phase	5.45
C0JYY2	APOB	Apolipoprotein B	48	18.8	Lipid metabolic, inflammation	13.55
P00738	HP	Haptoglobin	116	67.5	Inflammation: acute-phase	33.4195
A8K5T0	CFH	Complement factor H	62	38.7	Inflammation: acute-phase	3.37
A5PL27	CP	Ceruloplasmin	66	41.3	Inflammation: acute-phase; copper metabolism	5.25
B7Z9A0	GSN	Gelsolin	44	39.2	Motility, signaling, apoptosis, cancer	-14.60
G3V5I3	SERPINA3	α-1-antichymotrypsin	55	47.1	Inflammation: acute-phase	10.19
P10645	CHGA	Chromogranin-A	28	41.8	Regulation of blood pressure	-31.35
E9PGN7	SERPING1	Plasma protease C1 inhibitor	29	28.4	Regulation of complement activation blood coagulation, fibrinolysis and the generation of kinins	5.65
Q8WXD2	SCG3	Secretogranin-3	12	23.1	Platelet activation platelet degranulation	-7.94
P02671	FGA	Fibrinogen α chain	14	17.1	Coagulation, inflammation	13.80
Q12860	CNTN1	Contactin-1	6	14.4	Notch signaling	95.24
P06702	S100A9	Protein S100-A9	9	70.2	Inflammation: acute-phase	40.93
P02647	APOA1	Apolipoprotein A-I	61	86.5	Lipid metabolic, inflammation	4.45
B2RMS9	ITIH4	Inter-α (Globulin) inhibitor H4	22	25.7	Inflammation: acute-phase; development or regeneration	4.25
P36955	SERPINF1	Pigment epithelium-derived factor	16	29	Cell proliferation	-8.47
Q5VVQ8	C4BPA	Complement component 4 binding protein	9	24.8	Inflammation: acute-phase	3.56
Q92876	KLK6	Kallikrein-6	17	49.2	Regulation of cell differentiation	-30.77
P02649	APOE	Apolipoprotein E	56	48.6	Regulation of cell growth, lipid metabolic	-3.66
Q68CK4	LRG1	Leucine-rich α-2-glycoprotein	12	23.3	Inflammation: acute-phase	9.29
F8VVB6	NELL2	Protein kinase C-binding protein	11	19.9	Cell growth and differentiation	-20.88
P16870	CPE	Carboxypeptidase E	9	27.9	Protein modification process	-72.46
Q14CA1	NRCAM	NRCAM protein	12	17	Cell morphogenesis	-22.47
E7EUF1	ENPP2	Ectonucleotide pyrophosphatase/ phosphodiesterase family member 2	18	24.6	Immune system process, lipid metabolic process	-15.85
Q16270	IGFBP7	Insulin-like growth factor-binding protein 7	9	33.7	Regulation of cell growth	-43.67

Table II. Continued.

Accession no.	Gene name	Protein name	Pep count	Cover %	Biological function	Fold-change (TBM/HC)
B4E1Z4	CFB	Complement factor B	40	28.9	Inflammation: acute-phase	2.27
P02790	HPX	Hemopexin	96	55.6	Inflammation: acute-phase	4.57
P01031	C5	Complement C5	22	19	Inflammation: acute-phase	4.02
P05109	S100A8	Protein S100-A8	5	38.7	Defense response	29.1
P02763	ORM1	α-1-acid glycoprotein	69	55.2	Inflammation: acute-phase	9.38
Q8TCF0	LBP	Lipopolysaccharide-binding protein	3	10.5	Negative regulation of cytokine	12.13
P19021	PAM	Peptidyl-glycine α-amidating monooxygenase	6	9.5	Central nervous system development	-22.88
D9YZU5	HBB	Hemoglobin	8	81.6	Inflammation: acute-phase	-79.36
P01871	IGHM	Ig μ chain C region	26	33.2	Inflammation: acute-phase	13.06
F8W7I2	PTGDS	Prostaglandin-H2 D-isomerase	44	45.5	Lipid metabolic	-18.87
O15240	VGF	Neurosecretory protein	6	16.8	Ovarian follicle development	-14.99
D3DVW9	PTPNS1	Protein tyrosine phosphatase	4	18.7	Enhances cell proliferation, cell motility and invasive activity, and promotes cancer metastasis	-22.27
P02748	C9	Complement component C9	22	37.4	Inflammation: acute-phase	4.88
Q14520	HABP2	Hyaluronan-binding protein 2	7	22	Blood coagulation	3.69
P51693	APLP1	Amyloid-like protein 1	15	23.9	Blood coagulation	-11.06
Q9NQ79	CRTAC1	Cartilage acidic protein 1	5	11.7	Calcium ion binding	-2.70
E9PEV0	APP	γ-secretase C-terminal fragment 59	15	26.2	Apoptosis, cell adhesion, endocytosis, Notch signaling pathway	-18.87
A6XGL1	Ttr	Transthyretin	80	85.3	Transport thyroxine	-11.38
P00734	F2	Prothrombin	38	45.2	Blood coagulation	1.85
P01008	SERPINC1	Antithrombin-III	44	53	Blood coagulation, inflammation	3.13
P02741	CRP	C-reactive protein	2	12.1	Inflammation: acute-phase	22.28
O43866	CD5L	CD5 antigen-like	5	20.2	Inflammation: acute-phase	3.37
Q9BX83	HBA1	Hemoglobin α 1 globin chain	6	43	Inflammation: acute-phase	-11.59
B2R4C5	LYZ	Lysozyme	6	42.6	Bacteriolytic function	19.41
O95502	NPTXR	Neuronal pentraxin receptor	10	18.4	Synapse remodeling	-3.77
K9JA46	EL52	Epididymis luminal secretory protein 52	3	10.4	Neuron migration, cardiac muscle cell apoptotic process	2.22
Q92859	NEO1	Neogenin	2	5.5	Glycolysis and gluconeogenesis	-7.11
J3KPS3	ALDOA	Fructose-bisphosphate aldolase	7	29.1	Glycolysis and gluconeogenesis	4.20
P13521	SCG2	Secretogranin-2	3	6.3	MAPK cascade, angiogenesis, endothelial cell migration	-20.49

Table II. Continued.

Accession no.	Gene name	Protein name	Pep count	Cover %	Biological function	Fold-change (TBM/HC)
D3DNU8	KNG1	Kininogen 1	32	46.6	Blood coagulation, inflammation	4.49
D3DQX7	SAA1	Serum amyloid A protein	4	36.1	Inflammation: acute-phase	13.55
Q32Q65	FGB	Fibrinogen β chain	25	46.6	Coagulation, inflammation	5.445
Q53FR6	COMP	Cartilage oligomeric matrix protein variant	7	15.6	Coagulation, apoptosis	-13.55
Q9NT99	LRRC4B	Leucine-rich repeat-containing protein 4B	3	9.5	Positive regulation of synapse assembly	-12.47
P12109	COL6A1	Collagen α-1(VI) chain	4	9.6	Cell adhesion, blood coagulation	-11.48
D3DTX7	COL1A1	Collagen, type I, α 1, isoform CRA_a	9	22.2	Blood vessel development	-1.29
Q6FHM9	CD59	CD59 antigen, complement regulatory protein, isoform CRA_b	6	32.8	Inflammation	-17.54
Q06033	ITIH3	Inter-α-trypsin inhibitor heavy chain H3	5	10.3	Carbohydrate metabolic process	4.25
P19022	CDH2	Cadherin-2	3	12.1	Blood vessel development	-5.60
D6RD17	IGJ	Immunoglobulin J chain	5	20.3	Inflammation: acute-phase	14.99
P17900	GM2A	Ganglioside GM2 activator	4	24.9	Carbohydrate metabolic process	-6.98
Q86XR1	PRNP	Major prion protein (fragment)	4	13.5	Neuronal development	-16.45
A4D2D2	PCOLCE	Procollagen C-endopeptidase enhancer	8	21.2	Developmental process	-10.57
Q86U78	AGT	Angiotensinogen	15	34.9	Regulation of blood pressure, body fluid and electrolyte	-3.02
Q53HB8	MOG	Oligodendrocyte myelin glycoprotein variant	3	12.7	Inflammation	-8.63
C9JEU5	FGA	Fibrinogen γ chain	32	53.7	Coagulation, inflammation	12.13
P01034	CST3	Cystatin-C	63	56.9	Negative regulation of blood vessel remodeling	-17.70
Q7Z532	OGN	Osteoglycin	2	19.1	Induces bone formation	-8.02
P23471	PTPRZ1	Receptor-type tyrosine-protein phosphatase ζ	3	5.3	Cell morphogenesis	-5.44
P00558	PGK1	Phosphoglycerate kinase 1	5	32.1	Carbohydrate metabolic process	4.33
P25311	AZGP1	Zinc-α-2-glycoprotein	18	39.6	Inflammation: acute-phase	2.65
P24592	IGFBP6	Insulin-like growth factor-binding protein 6	7	23.3	Regulation of cell growth	-4.74
P23142	FBLN1	Fibulin-1	18	33	Cell adhesion and migration	-2.63
Q5NV91	IGLV3-27	V2-19 protein	7	48.4	Inflammation, complement activation	8.32
P13591	NCAM1	Neural cell adhesion molecule 1	10	21.5	Regulation of signal	-6.08

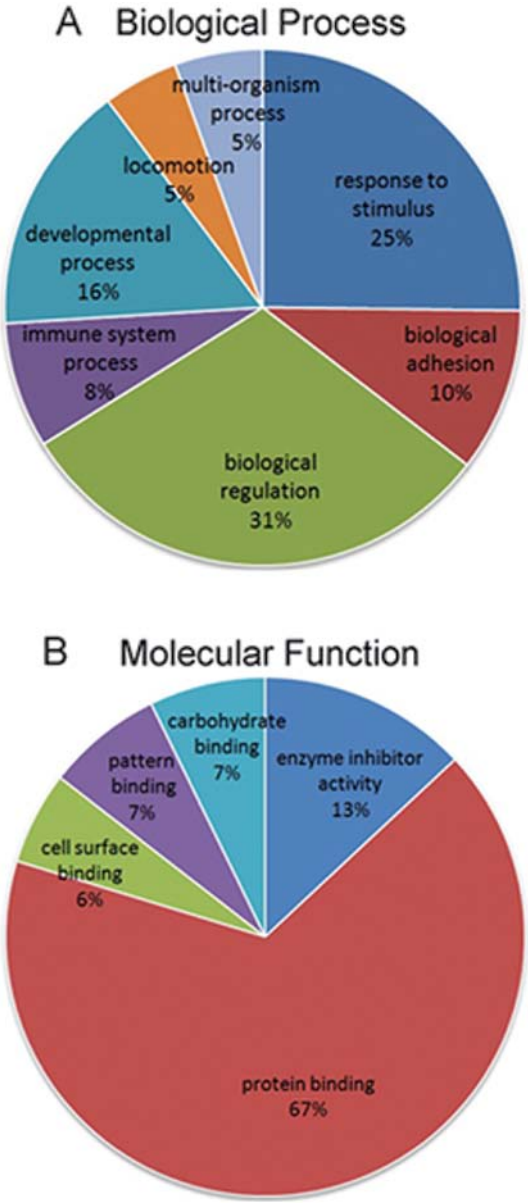

Figure 1. Gene Ontologies. The most significantly enriched (A) biological processes and (B) molecular functions according to DAVID Bioinformatics Resources based on the 111 differential proteins identified in TBM subjects.

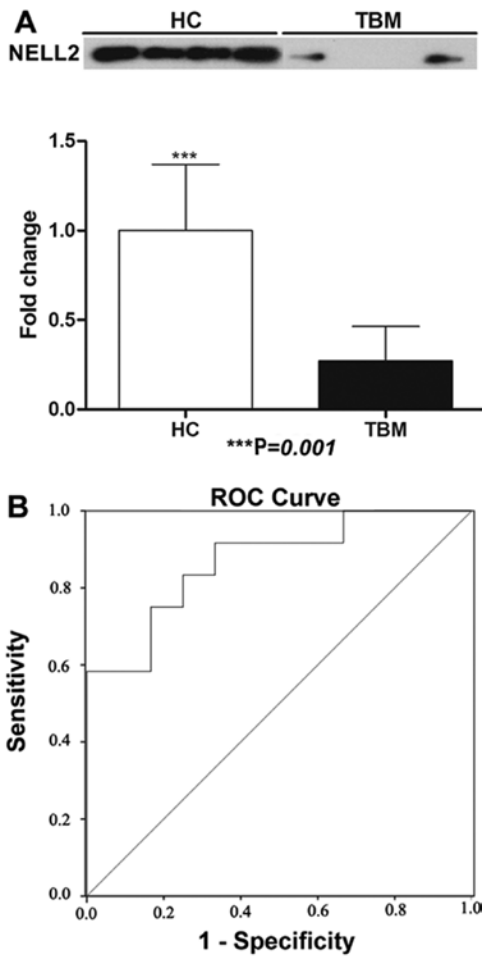

Figure 2. Western blot validation of NELL2. (A) NELL2 was significantly downregulated in tuberculous meningitis (TBM) subjects compared to healthy control (HC) subjects by western blotting (HC, n=12; TBM, n=12). (B) Receiver operating characteristic (ROC) analysis of NELL2 western blot data showing detection of TBM from HC with an AUC of 0.868, indicating that NELL2 in cerebrospinal fluid (CSF) shows promise as a diagnostic biomarker for TBM.

Qualitative results. Data from the two MS repetitions identified 572 unique proteins according to the previously described parameter set. Only the 81 differential proteins with a ±1.2-fold-change and P<0.05 were selected for further analysis (Table II). The threshold criteria were selected based on literature investigating the reproducibility of iTRAQ™ quantification (23).

Annotation of differential proteins. Some differential proteins exhibited multiple functions and subcellular locations, which resulted in individual proteins being counted more than once. Considering that a given protein can be attributed to several functions and/or processes, we categorized differential proteins by their biological processes (Fig. 1A) and molecular functions (Fig. 1B) by pie chart. 'Response to stimulus', 'biological adhesion' and 'biological regulation' were the most over-represented biological processes. 'Enzyme inhibitor activity' was the most over-represented molecular function, distantly followed by 'protein binding' and 'cell surface binding'.

Altered biological pathways by KEGG. The 81 differential proteins were analyzed for KEGG over-representation of pathways ('proteomic phenotyping') (24) to obtain insights into the functional differences between the CSF proteomes of TBM and HC subjects. The top three-ranking canonical KEGG pathways are listed in Table II. Inflammatory processes were the most statistically over-represented, including complement and coagulation cascades ranking first (P<0.001) and cell adhesion molecules (CAMs) ranking third (P<0.01).

Validation of NELL2 by western blotting. The neuron-specific differential protein with the largest absolute fold-change value, neural epidermal growth factor-like like 2 (NELL2), was selected for western blot validation (Table II). In accordance with iTRAQ™, NELL2 decreased significantly in TBM compared to HC subjects (Fig. 2A). ROC curves were constructed using the western blotting data, in which the AUC, sensitivity, and specificity were 0.868, 83.3 and 75%, respectively (Fig. 2B).

Table III. Top ten-ranking canonical KEGG pathways associated with differential proteins.

KEGG pathway	No. of molecules		P-value
	Mapping	%	
Complement and coagulation cascades	10	16.7	$2.40e^{-11}$
Prior diseases	4	6.7	$7.00e^{-4}$
Cell adhesion molecules (CAMs)	5	8.3	$10.20e^{-3}$

Discussion

In the present study, using an iTRAQ™-based quantitative proteomic approach, we compared 12 CSF samples from TBM subjects to 12 CSF samples from demographically-matched HC subjects. Among the 81 differential proteins identified, the neuron-specific protein with the largest absolute fold-change value, NELL2, was selected for western blotting validation and was found to be significantly decreased in TBM subjects compared to HC subjects. NELL2 had 83.3% sensitivity and 75% specificity and shows promise as a diagnostic biomarker for TBM.

Inflammation. KEGG analysis revealed over-representation in two inflammation-associated processes, namely complement and coagulation cascades and CAMs (Table III). As MTB infects and persists inside host macrophages, MTB infection is naturally accompanied by a host inflammatory response (25). In the initial stage, MTB activates several pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α) (26), interleukin 1 (IL-1) (27), and reactive oxygen/nitrogen intermediaries (ROI/RNI) that promote MTB termination (28). Simultaneously, MTB induces the release of anti-inflammatory molecules, such as transforming growth factor-β (TGF-β) (29) and IL-10 (30), to suppress the pro-inflammatory response and maintain MTB survival. In ~90% of MTB cases, the host inflammatory response results in granuloma formation, which is sufficient to contain the infection and prevent disease. However, in 5-10% of MTB cases, the pathogen evades or subverts the host's granulomatous inflammatory response, producing either a latent or active infection (31). Therefore, the outcome of MTB infection depends on a complex set of host-pathogen interactions, and further investigation into the differential proteins associated with the host inflammatory response can offer insight into the pathogenesis and treatment of TBM. For example, drugs that intervene in the host inflammatory response, such as TGF-β blockers, have been suggested as a method to prevent the spread of MTB infection (32).

Glycolysis/gluconeogenesis. Two significantly altered proteins including neogenin and fructose-bisphosphate aldolase related to glycolysis/gluconeogenesis were identified. MTB prefers to utilize host fatty acids as an energy source. Thus, glycolytic/gluconeogenic proteins that are involved in fatty acid utilization would be required for the growth and persistence of MTB *in vivo* (33).

NELL2. TBM is characterized by meningeal inflammation, CSF flow obstruction, fibrogelatinous exudates, and arterial vasculitis that traverse the exudates, resulting in cerebral infarction (34,35). TBM sometimes results in tuberculoma formation (36), which is most commonly observed in the cerebellum, cerebrum and pons (37). In the present study, by magnetic resonance imaging (MRI), the 12 TBM subjects excibited basal meningeal enhancement consistent with TBM, seven of whom excibited cerebral infarction shown by high DWI signal intensity. These findings suggest a strong possibility of brain neuronal cell damage via vasculitis and tuberculoma formation. Considering that CSF neuron-specific enolase (NSE) is the most commonly applied biomarker of nervous tissue damage and can act as an early diagnostic marker of TBM (38,39), we selected NELL2 for western blotting validation due to its neuronal specificity and high absolute fold-change value. NELL2, also known as neural epidermal growth factor-like like 2, NEL-like protein 2, Nel-related protein 2 (NRP2), and protein kinase C-binding protein, is a cytoplasmic enzyme that is expressed almost exclusively in the nervous system (40), predominantly in neurons with the highest density in the hippocampus and cerebral cortex (41). NELL2 promotes neuronal differentiation and survival during embryonic development (42) through mitogen-activated protein kinases (MAPK) (43), stimulates motor and sensory neuron differentiation in dorsal root ganglia (44), and promotes the differentiation of neural progenitor cells into neurons (45). NELL2 knock-out mice have demonstrated learning impairment in the Morris water maze task.

The present study revealed significantly decreased levels of NELL2 in TBM patients. As neuron-produced NELL2 supports neuronal survival and differentiation and neurons are compromised in TBM, it is reasonable to surmise that the reduced NELL2 levels observed in TBM patients result from brain neuronal cell damage or death. Additional study is required to determine whether there is a TBM disease stage in which NELL2 significantly increases to compensate for brain neuronal cell damage.

There are several limitations to the present study. First, eight of the 12 TBM patients also had pulmonary TB, and another TBM patient had vertebral TB. As 25% of miliary TB patients also have TBM, presumably by transmission across the blood-brain barrier (46), future studies should include TBM patient cohorts with and without pulmonary TB, which may provide additional novel biomarkers of TBM. Second, the World Health Organization (WHO) recommends that all newly diagnosed human immunodeficiency virus (HIV) and syphilis patients should be tested for TB, as individuals with HIV or syphilis and latent TB infection are at a significantly higher risk of developing active TB (47). However, since no subjects of the present study were co-infected with HIV or syphilis, future studies should include cohorts of HIV or syphilis co-infected patients to improve our understanding of MTB host interaction in these patient populations. Third, the influence of anti-TB agents on differential protein expression was not assessed and should be taken into consideration in future studies. Fourth, in order to improve specificity for

TBM, the current findings require further validation in larger sample sets that include other intracranial infectious diseases.

In conclusion, an iTRAQ™-based quantitative proteomic approach was utilized in the present study to identify differential proteins in the CSF of TBM patients and healthy controls. A total of 111 differential proteins were identified, and NELL2 was validated by western blotting as a promising diagnostic biomarker for TBM. Further evaluation of these findings in larger studies including medicated and unmedicated cohorts with other intracranial infectious diseases is required for clinical translation.

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