

Proteomic analysis of cerebrospinal fluid in amyotrophic lateral sclerosis

YAN CHEN¹, XIAO-HUI LIU², JIAN-JUN WU¹, HUI-MING REN¹,
JIAN WANG¹, ZHENG-TONG DING¹ and YU-PING JIANG¹

¹Department of Neurology, Huashan Hospital, Fudan University, Shanghai 200040;

²Institute of Biomedical Science, Fudan University, Shanghai 200032, P.R. China

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Abstract. The present study used comparative proteomic analysis of cerebrospinal fluid (CSF) in amyotrophic lateral sclerosis (ALS) patients in order to identify proteins that may act as diagnostic biomarkers and indicators of the pathogenesis of ALS. This analysis was performed using isobaric tags for relative and absolute quantitation (iTRAQ) technology, coupled with 2-dimensional liquid chromatography/mass spectrometry. Database for Annotation, Visualization and Integrated Discovery software was utilized for bioinformatic analysis of the data. Following this, western blotting was performed in order to examine the expression of 3 candidate proteins in ALS patients compared with healthy individuals [as a normal control (NC) group] or patients with other neurological disease (OND); these proteins were insulin-like growth factor II (IGF-2), glutamate receptor 4 (GRIA4) and leucine-rich α -2-glycoprotein 1 (LRG1). Clinical data, including gender, age, disease duration and ALS functional rating scale (ALSFRS-R) score, were also collected in the ALS patients. Multiple linear regression analysis was performed between the clinical data and the results of western blot analysis. A total of 248 distinct proteins were identified in the ALS and NC groups, amongst which a significant difference could be identified in 35 proteins; of these, 21 proteins were downregulated and 14 were upregulated. These differentially-expressed proteins were thus revealed to be associated with ALS. The western blot analysis confirmed a proportion of the data attained in the iTRAQ analysis, revealing the differential protein expression of IGF-2 and GRIA4 between the ALS and NC groups. IGF-2 was significantly downregulated in ALS patients ($P=0.017$) and GRIA4 was significantly upregulated ($P=0.016$). These results were subsequently validated in the 35-patient ALS and OND groups ($P=0.002$), but no significant difference was

identified in LRG1 expression between these groups. GRIA4 protein expression was higher in male than female patients and was positively correlated with the ALSFRS-R score, meaning that GRIA4 expression was negatively correlated with the severity of ALS, while IGF-2 and LRG1 expression did not correlate with any clinical data. The present study thus demonstrated that GRIA4 expression levels, as a marker of severity, may be used as a reference for the timing of treatment, and that IGF-2 may serve as an effective biomarker of ALS progression.

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease specifically affecting the upper and lower motor neurons. Due to frequent early misdiagnosis, patients do not benefit from early drug intervention and clinical drug studies have been largely unsuccessful; a correct, early diagnosis of ALS is therefore crucial.

Such a clinical diagnosis, and study of the pathogenesis of ALS, could occur through analysis of changes to the cerebrospinal fluid (CSF) proteins. Insulin-like growth factor-1, vascular endothelial growth factor, transactive response DNA-binding protein 43, monocyte chemotactic protein 1 and other proteins have been reported as possible diagnostic indicators of ALS (1-4), but a definitive diagnostic indicator has yet to be established.

CSF quantitative proteomics, including differential in gel electrophoresis (DIGE) and isotope-coded affinity tags, have been reported in studies on Alzheimer's disease and Parkinson's disease (5,6), but have not been widely used to investigate ALS. In 2005, a study by Ranganathan *et al* (7) was the first to investigate the CSF in ALS patients using surface-enhanced laser desorption/ionization (SELDI) technology and proteomics; three proteins, cystatin C, transthyretin and a carboxy-terminal fragment of the neuroendocrine protein 7B2, were screened and validated for their sensitivity and specificity as biomarkers. Other previous studies examined the CSF of ALS with two-dimensional gel electrophoresis, DIGE and SELDI (8,9), but use of isobaric tags for relative and absolute quantitation (iTRAQ) technology in this context has not been reported, to the best of our knowledge.

The present study compared the CSF protein expression of ALS patients and healthy [normal control [NC] group]

Correspondence to: Dr Yu-Ping Jiang, Department of Neurology, Huashan Hospital, Fudan University, 12 Wulumuqi Zhong Road, Shanghai 200040, P.R. China
E-mail: yupingjiang@163.com

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patients using iTRAQ labeling and 2-dimensional liquid chromatography/tandem mass spectrometry (2D LC-MS/MS) technology, screened the resulting proteins and verified their differential expression by western blotting, in order to determine the most effective biomarkers for ALS diagnosis.

Patients and methods

Patients

ALS-A group. A total of 35 patients with ALS who presented to Huashan Hospital between March 2008 and October 2010 were selected for the study. Informed consent was obtained from all patients, or their families. Tension headache sufferers were selected as the normal control (NC) group. The other neurological disease (OND) group consisted of patients who, during clinical diagnosis, were subjected to a lumbar puncture; these patients suffered from conditions such as chronic non-inflammatory peripheral neuropathy, Parkinson's disease, spastic paraplegia and hydrocephalus. Patient ages ranged between 30 and 75 years old.

ALS-B group. A total of 10 cases of ALS were randomly selected from the ALS-A group and used to screen additional proteins.

CSF sample collection. Under fasting conditions, each patient was treated with the 2 ml local anesthetic lidocaine hydrochloride injection (2%; Shanghai Harvest Pharmaceutical Co., Ltd., Shanghai, China) and subjected to a lumbar puncture, from which 8–10 ml of CSF was collected. A volume of 4–5 ml of CSF was immediately centrifuged at 2,000 × g for 10 min; the resulting supernatant was collected and placed in 1.5 ml Eppendorf tubes (Eppendorf AG, Hamburg, Germany) at -80°C. The remaining CSF was used for biochemical and immunological detection, as subsequently described.

Determination of protein concentration using iTRAQ and 2D LC-MS/MS. Following the removal of 22 high-abundance proteins, including albumin and IgG, using ProteoMiner low abundance protein enrichment kits (Bio-Rad Laboratories, Inc., Hercules, CA, USA), protein quantification was conducted using a Protein Assay reagent kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) based on Bradford methods, according to manufacturer's protocol. iTRAQ labeling was performed according to the manufacturer's protocol (Applied Biosystems Life Technologies, Foster City, CA, USA). Briefly, 100 µg CSF proteins from the ALS and NC groups were precipitated with cold acetone (ratio of acetone:sample, 5:1) for 1 h at -20°C and resuspended in 20 µl dissolution buffer, respectively. Following centrifugation at 2,000 × g for 15 min and disposal of the supernatant, the precipitant was dissolved into 20 µl iTRAQ solution and 1 µl 1% sodium dodecyl sulfate (SDS). Subsequently, 1 µl cysteine sealing reagent was added for 10 min at room temperature. Proteins were trypsinized (Sigma-Aldrich, St. Louis, MO, USA) at 37°C overnight (ratio of enzyme:protein, 1:20). Peptides were labeled with iTRAQ reagents for 1 h at room temperature. iTRAQ reagents 113 and 118 were used to label the peptides from the NC and ALS groups, respectively. Following this, samples were mixed, desalted with Sep-Pak Vac C18 cartridges (Waters Corporation, Milford, MA, USA) and dried in a vacuum concentrator.

2D LC-MS/MS analysis. High-performance liquid chromatography and time-of-flight mass spectrometry (API QSTAR XL Hybrid LC-MS/MS; Applied Biosystems Life Technologies) were used for protein separation and analysis. For 2D LC-MS/MS analysis, the iTRAQ-labeled mixed peptides were fractionated using strong cation exchange (SCX) chromatography on a 20AD HPLC system (Shimadzu Corporation, Kyoto, Japan) with a polysulfoethyl column (2.1×100 mm; 5 µm; 200 Å; The Nest Group, Inc., Southborough, MA, USA). Peptide mixture was reconstituted in Buffer A (SCXA), which contained 10 mM KH₂PO₄ in 25% acetonitrile (pH 2.6; Thermo Fisher Scientific, Waltham, MA, USA), and loaded onto the column. Peptides were separated at a flow rate of 200 µl/min for 60 min with a gradient of 0–80% Buffer B (Buffer A supplemented with 350 mM KCl) in Buffer A. Absorbances of 214 nm and 280 nm were identified by tandem mass spectrometry. A total of 20 SCX fractions were collected.

Protein identification. All data from tandem mass spectrometry were obtained from the UniProtKB/Swiss-Prot database using ProteinPilot 3.0 software (AB Sciex, Framingham, MA, USA), and the identification and quantification results were recorded. Search parameters were as follows: At least 1 matching peptide, a confidence interval (CI) of the peptide of >95% (P<0.05) and results in accordance with the peak of the spectrum.

Protein annotation and classification. The Database for Annotation, Visualization and Integrated Discovery (DAVID) was used for functional annotation of proteins and gene ontology (GO) was used to classify these proteins, including their involvement in biological processes, as cellular components and their molecular function.

Differential expression of proteins. Western blotting was performed to analyze differential protein expression in the CSF between the ALS-B and NC groups, in order to verify the iTRAQ results. A total of 1 ml CSF sample was added into a 3 kD ultrafiltration centrifugal tube (EMD Millipore, Billerica, CA, USA) for desalination and concentration. Protein concentrations were subsequently measured via the Bradford method using Bio-Rad protein assay reagent (Bio-Rad Laboratories, Inc.). A total of 20 µg protein was separated by 12% SDS polyacrylamide gel electrophoresis followed by electro-blotting onto a polyvinylidene difluoride membrane. The membrane was subsequently incubated with 5% nonfat dry milk in Tris-buffered saline at room temperature for 2 h, in order to block non-specific binding. Following this, the membrane was incubated with the following primary antibodies: Rabbit anti-human insulin-like growth factor II (IGF-2; 1:1,250; ab9574); mouse anti-human leucine-rich α-2-glycoprotein 1 (LRG1; 1:800; ab57992); and rabbit anti-human glutamate receptor 4 (GRIA4; 1:500; ab61171; all Abcam, Cambridge, UK), diluted in blocking buffer overnight at 4°C. The membrane was subsequently incubated with horseradish peroxidase-conjugated AffinPure goat anti-rabbit (KC-RB-035) and anti-mouse (KC-MM-035) immunoglobulin G (H+L) secondary antibodies (both 1:5,000; Shanghai Kangcheng Biotechnology Co., Ltd., Shanghai, China) diluted with nonfat dry milk and Tris-buffered saline and Tween 20 (TBST). After rinsing three times with TBST, the western blot protein band

Table I. Proteins analyzed in the present study.

Unused ProtScore (CL, %)	Proteins detected, n	Proteins prior to grouping, n	Distinct peptides, n	Spectra identified, n	% of total spectra
>2.0 (99)	211	285	18106	37075	33.8
>1.3 (95)	248	347	19568	38823	35.4 ^a
>0.47 (66)	294	448	21271	40761	37.2

^aCutoff applied at an unused protein score of >1.3. CL, confidence level.

Table II. Proteins in ALS and NC groups by cerebrospinal fluid.

Protein name	iTRAQ ratio (ALS/NC)	Accession no.
Serum albumin	0.9262	splP02768I
Complement C4-A	1.0317	splP0C0L4I
Complement C3	1.0003	splP01024I
Transthyretin	1.0717	splP02766I
α -1-antitrypsin	0.7250	splP01009I
α -2-macroglobulin	0.9938	splP01023I
Serotransferrin	0.8150	splP02787I
Fibronectin	1.0084	splP02751I
Apolipoprotein A1	1.0930	splP02647I
Ig γ 1 chain C region	0.9304	splP01857I
Apolipoprotein E	1.1323	splP02649I
Gelsolin	1.0509	splP06396I
Apolipoprotein A-IV	1.1446	splP06727I
Clusterin	1.0969	splP10909I
Cystatin C	1.0671	splP01034I
Vitamin D-binding protein	0.8710	splP02774I
Contactin-1	1.0430	splQ12860I
Complement factor	1.0036	splP08603I
Pigment epithelium-derived factor	0.9803	splP36955I
Secretogranin-1	1.0670	splP05060I
Ceruloplasmin	0.8720	splP00450I
Serum albumin	1.0588	splP51693I
Haptoglobin	0.6926	splP00738I
Secretogranin-3	1.1640	splQ8WXD2I
Antithrombin-III	0.8452	splP01008I
Chromogranin-A	1.0098	splP010645I
α -1-B glycoprotein	0.9835	splP04217I
β -Ala-His dipeptidase	1.1591	splQ96KN2I
Neuronal cell adhesion molecule	1.0097	splQ92823I
Ig γ 2 chain C region	1.0383	splP01859I
Monocyte differentiation antigen CD14	0.8775	splP08571I
Fibrinogen α chain	1.0375	splP02671I
α -1-antichymotrypsin	0.9855	splP01011I
Neurosecretory protein VGF	1.0510	spl015240I
α -2-HS-glycoprotein	1.0036	splP02765I
Angiotensinogen	1.0014	splP01019I
Ig α 1 chain C region	1.0096	splP01876I
Collagen α -1(I) chain	1.0412	splP02452I
Plasminogen	0.8738	splP00747I
Kininogen-1	0.8529	splP01042I
Fibulin-1	0.9324	splP23142I

Table II. Continued.

Protein name	iTRAQ ratio (ALS/NC)	Accession no.
Hemoglobin subunit β	1.4623	splP688711
Prostaglandin-H2 D-isomerase	0.9310	splP412221
<i>N</i> -acetyllactosaminide β -1,3- <i>N</i> -acetylglucosaminyltransferase	1.0294	splO435051
Neuronal pentraxin receptor	1.0815	splO955021
Hemopexin	0.8432	splP027901
Retinol-binding protein 4	0.9796	splP027531
Apolipoprotein D	0.9616	splP050901
Ectonucleotide pyrophosphatase/phosphodiesterase family member 2	0.9689	splQ138221
β -2-glycoprotein 1	0.9413	splP027491
Carboxypeptidase E	1.0193	splP168701
Collagen α -2(I) chain	1.0000	splP081231
Calsyntenin-1	1.1224	splO949851
Vitronectin	0.8401	splP040041
Nucleobindin-1	1.0513	splQ028181
Ig μ chain C region	0.8467	splP018711
Ig κ chain C region	1.0135	splP018341
Ig γ 3 chain C region	0.9289	splP018601
Extracellular superoxide dismutase (Cu-Zn)	1.0356	splP082941
Cathepsin D	0.9478	splP073391
Afamin	1.0176	splP436521
Complement component C7	0.9460	splP106431
Apolipoprotein A-II	1.2524	splP026521
Contactin-2	1.0433	splQ022461
Inter- α -trypsin inhibitor heavy chain	1.0549	splQ146241
Neural cell adhesion molecule 1	1.0091	splP135911
EGF-containing fibulin-like extracellular matrix protein	0.9392	splQ128051
Ig λ chain C regions	1.0045	splP018421
Complement component C9	0.7597	splP027481
Neural cell adhesion molecule L1-like protein	1.0405	splO005331
Procollagen C-endopeptidase enhancer 1	1.0410	splQ151131
Mimecan	0.9845	splP207741
Fibrinogen β chain	1.0713	splP026751
Hemoglobin subunit α	1.5451	splP699051
ProSAAS	1.0492	splQ9UHG21
Neuronal pentraxin-1	1.1167	splQ158181
β -2-microglobulin	1.0138	splP617691
Collagen α -1(VI) chain	1.0602	splP121091
Neural cell adhesion molecule 2	0.9561	splO153941
Leucine-rich α -2-glycoprotein	0.6430	splP027501
Insulin-like growth factor-binding protein 2	0.9574	splP180651
Insulin-like growth factor-binding protein 6	0.9883	splP245921
Protein kinase C-binding protein NELL2	0.9929	splQ994351
Keratin, type II cytoskeletal 1	0.9729	splP042641
Dickkopf-related protein 3	1.0396	splQ9UBP41
Ig κ chain V-III region	0.9945	splP016231
Complement C1r subcomponent	0.9240	splP007361
Prothrombin	0.9113	splP007341
Dystroglycan	1.0292	splQ141181
Tetranectin	0.9282	splP054521
α -2-antiplasmin	0.9126	splP086971
Complement factor B	0.8143	splP007511

Table II. Continued.

Protein name	iTRAQ ratio (ALS/NC)	Accession no.
Cartilage acidic protein 1	1.0590	splQ9NQ79I
Peptidylglycine α -amidating monooxygenase	0.8763	splP19021I
Major prion protein	1.0478	splP04156I
Zinc- α -2-glycoprotein	0.7912	splP25311I
Neuroendocrine protein 7B2	1.1447	splP05408I
Multiple epidermal growth factor-like domains 8	0.9706	splQ7Z7M0I
Insulin-like growth factor-binding protein 7	1.0327	splQ16270I
SPARC	0.8425	splP09486I
Trypsin-1	1.2077	splP07477I
Secretogranin-2	0.9307	splP13521I
Voltage-dependent calcium channel subunit $\alpha_2\delta$ -1	0.9343	splP54289I
Pyruvate kinase isozymes M1/M2	1.0611	splP14618I
Cadherin 13	1.0163	splP55290I
GM2 Ganglioside activator	1.0083	splP17900I
Fibrinogen γ chain	1.0925	splP02679I
Extracellular matrix protein 1	1.0849	splQ16610I
Collagen α -1(XVIII) chain	1.0000	splP39060I
Cadherin-2	1.0560	splP19022I
Semaphorin 7A	0.9433	splO75326I
Ig κ chain V-II region GM607	0.9526	splP06309I
Ig λ chain V-III region LOI	0.7060	splP01617I
Transmembrane protein 132A	1.1680	splQ24JP5I
Metalloproteinase inhibitor 2	0.9855	splP16035I
Osteopontin	1.0354	splP10451I
Kallikrein-6	0.9713	splQ92876I
Sex hormone-binding globulin	0.6051	splP04278I
Actin, cytoplasmic 1	0.8566	splP60709I
Ig γ -4 chain C region	1.1808	splP01861I
Protein FAM3C	0.9182	splQ92520I
Chorionic somatomammotropin hormone	0.5234	splP01243I
Keratin, type I cytoskeletal 9	0.9161	splP35527I
Limbic system-associated membrane protein	0.9398	splQ13449I
Phospholipid transfer protein	1.1687	splP55058I
Ig heavy chain V-III region BRO	0.9650	splP01766I
SPARC-like protein 1	0.9325	splQ14515I
Fructose-bisphosphate aldolase	0.9490	splP04075I
<i>N</i> -acetylmuramoyl-L-alanine amidase	0.9820	splQ96PD5I
Complement C1s subcomponent	0.9598	splP09871I
Ig κ chain V-IV region B17	0.8581	splP06314I
Lumican	1.0259	splP51884I
Opioid-binding protein/cell adhesion molecule	0.8758	splQ14982I
Ribonuclease pancreatic	0.7527	splP07998I
Ig κ chain V-III region CLL	0.8486	splP04207I
Immunoglobulin superfamily member 8	0.8751	splQ969P0I
78-kDa glucose-regulated protein	0.9751	splP11021I
Protein AMBP	0.7950	splP02760I
Coagulation factor V	1.0938	splP12259I
Histidine-rich glycoprotein	0.9048	splP04196I
Ig heavy chain V-III region KOL	0.9839	splP01772I
L-lactate dehydrogenase B chain	0.9649	splP07195I
Complement component C6	0.9164	splP13671I
Ephrin type-A receptor 4	0.9178	splP54764I

Table II. Continued.

Protein name	iTRAQ ratio (ALS/NC)	Accession no.
Cerebellin-3	1.0609	splQ6UW011
Proenkephalin A	1.0079	splP012101
Insulin like growth factor binding protein 4	0.8461	splP226921
Apolipoprotein C-III	1.1181	splP026561
Trypsin -3	1.1478	splP350301
Transforming growth factor- β -induced protein ig-h3	1.0709	splQ155821
IgG Fc-binding protein	1.0775	splQ9Y6R71
Plasma serine protease inhibitor	0.9604	splP051541
Coagulation factor XII	0.9422	splP007481
Biotinidase	1.2970	splP432511
Ig κ chain V-III region VG (Fragment)	1.09987	splP044331
Collagen α -3(VI) chain	0.9422	splP007481
Neuroserpin	1.0459	splQ995741
Keratin, type I cytoskeletal 10	0.8858	splP136451
Fibulin-5	0.9587	splQ9UBX51
Receptor-type tyrosine-protein phosphatase S	1.1670	splQ133321
Complement factor I	0.8627	splP051561
Ig heavy chain V-III region TRO	1.1189	splP017621
Basement membrane-specific heparan sulfate proteoglycan core protein	0.9080	splP981601
α -1 acid glycoprotein 1	0.7355	splP027631
Chitinase-3-like protein 1	0.9904	splP362221
Cell adhesion molecule 3	0.8572	splQ8N1261
Galectin-3-binding protein	0.9876	splQ083801
Ig heavy chain V-III region POM	1.0712	splP017741
Endonuclease domain-containing 1 protein	1.0166	splP017761
Ig λ chain V-I region HA	1.0838	splP017791
Complement C1q subcomponent subunit B	1.0301	splP027461
Leucine-rich repeat-containing protein 4B	1.0174	splQ9NT991
Peroxiredoxin-2	1.6278	splP321191
Glyceraldehyde-3-phosphate dehydrogenase	1.2506	splP044061
Serum paraoxonase/arylesterase 1	0.8635	splP271691
Calcium/calmodulin-dependent protein kinase type II α chain	1.1677	splQ9UQM71
Fibrillin-1	0.2204	splP355551
Complement C2	0.9405	splP066811
Cell growth regulator with EF hand domain protein 1	1.3740	splQ996741
Myopalladin	0.6801	splQ86TC91
Neuronal growth regulator 1	1.0667	splQ7Z3B11
Serum amyloid A-4 protein	1.0645	splP355421
Protocadherin Fat 2	1.1409	splQ9NYQ81
Cathepsin F	1.1142	splQ9UBX11
DNA repair protein RAD50	0.9463	splQ928781
α -enolase	1.1591	splP067331
Insulin-like growth factor II	0.4053	splP013441
Ig λ chain V-III region SH	1.0399	splP01714
Reelin	1.1149	splP785091
Pregnancy-specific β -1-glycoprotein 1	0.7522	splP114641
Retinoic acid receptor responder protein 2	1.0850	splQ999691
Lymphocyte antigen 6H	1.0322	splO947721
Receptor-type tyrosine-protein phosphatase N2	1.0020	splQ929321
Multimerin-2	1.0029	splQ9H8L61

Table II. Continued.

Protein name	iTRAQ ratio (ALS/NC)	Accession no.
Apolipoprotein L1	0.9537	splO147911
Ig κ chain V-I region Roy	a	splP016081
Neurofascin	1.0305	splO948561
V-type proton ATPase	0.8780	splQ159041
Heparin cofactor 2	1.0087	splP055461
Plasma glutamate carboxypeptidase	1.0663	splQ9Y6461
Hypoxia upregulated protein 1	1.0213	splQ9Y4L11
Ig κ chain V-I region Ka	0.9834	splP016031
Protein DJ-1	1.2886	splQ994971
Laminin subunit γ -1	0.8128	splP110471
Cell surface glycoprotein MUC18	0.7681	splP431211
Neuroendocrine convertase 2	1.2290	splP165191
Inter- α -trypsin inhibitor heavy chain H5	0.9165	splQ86UX21
Exostosin-like 2	0.9342	splQ9UBQ61
Metalloproteinase inhibitor 1	1.0673	splP010331
Immunoglobulin J chain	1.0429	splP015911
Ig κ chain V-I region BAN	a	splP044301
Ig κ chain V-I region DEE	1.0241	splP015971
Ig κ chain V-I region Wes	0.8814	splP016111
Serum amyloid A-1 protein	0.6516	splP027351
Glutamate receptor 4	1.3098	splP480581
Amyloid β A4	1.0164	splP050671
Zinc finger protein	0.9751	splB1APH41
Nidogen-2	1.0441	splQ141121
72-kDa type IV collagenase	0.8378	splP082531
WAP, kazal, immunoglobulin, Kunitz and NTR domain-containing protein 2	1.0204	splQ8TEU81
Kallistatin	0.8933	splP296221
45-kDa calcium-binding protein	1.0575	splQ9BRK51
Tissue α -L-fucosidase	1.1211	splP040661
protein Cut A	1.0521	splO608881
Ig heavy chain V-I region	0.9126	splP063261
Ig heavy chain V-I region	0.9126	splP063261
γ -glutamyl hydrolase	1.2209	splQ928201
Complement component C8 γ chain	0.9202	splP073601
Phosphatidylethanolamine-binding protein 1	1.1293	splP300861
Thy-1 membrane glycoprotein	0.7535	splP042161
Cell adhesion molecule 4	0.9868	splQ8NFZ81
Sjogren syndrome/scleroderma autoantigen 1	0.9615	splO602321
Uncharacterized protein C6orf170	1.1061	splQ96NH31
<i>N</i> -acetylglucosamine-1-phosphotransferase subunit γ	1.0938	splQ9UJJ91
Testican-2	1.2140	splQ925631
Fructose-bisphosphate aldolase C	a	splP099721
Lysozyme C	0.8222	splP616261
V-type proton ATPase subunit D	1.2915	splQ9Y5K81
Coagulation factor XI	a	splP039511
Complement C1q subcomponent subunit C	0.8441	splP027471
Dermcidin	0.7257	splP816051
Ig κ chain V-II region RPMI 6410	0.7960	splP063101
Hemoglobin subunit δ	a	splP063101
Titin	0.9960	splQ8WZ421
Tumor protein 63	0.7445	splQ9H3D41

Table II. Continued.

Protein name	iTRAQ ratio (ALS/NC)	Accession no.
Cysteine-rich with EGF-like domain protein 1	1.0219	splQ96HD11
Putative α -1-antitrypsin-related protein	0.8877	splP208481
Scrapie-responsive protein 1	1.0576	splO757111

^aNot identified. ALS, amyotrophic lateral sclerosis; NC, normal control; iTRAQ, isobaric tags for relative and absolute quantitation.

Table III. Proteins decreased in ALS group.

Protein	Ratio of ALS vs. control	Accession no.
α -1-antitrypsin α 1	0.7250	splP010091
Haptoglobin	0.6926	splP007381
Complement component 9	0.7597	splP027481
Leucine-rich α -2-glycoprotein	0.6430	splP027501
Zinc- α -2-glycoprotein	0.7912	splP253111
Sex hormone-binding globulin	0.6051	splP042781
Chorionic somatomammotropin hormone 1	0.5234	splP012431
Ribonuclease pancreatic	0.7527	splP079981
Protein AMBP	0.7950	splP027601
α -1-acid glycoprotein 1	0.7355	splP027631
Fibrillin-1	0.2204	splP355551
Myopalladin	0.6801	splQ86TC91
Insulin-like growth factor II	0.4053	splP013441
Pregnancy-specific β -1-glycoprotein 1	0.7522	splP432511
Cell surface glycoprotein MUC18	0.7681	splP431211
Serum amyloid A protein	0.6516	splP027351
Thy-1 membrane glycoprotein	0.7535	splP042161
Dermeidin	0.7257	splP816051
Ig λ chain V-III region LOI	0.7060	splP016171
Ig κ chain V-II region RPMI 6410	0.7960	splP063101
Tumor protein 63	0.7444	splQ9H3D41

ALS, amyotrophic lateral sclerosis.

was detected using chemiluminescence, and the gray scales of the bands were quantified using software Image Lab 3.0 (Bio-Rad Laboratories, Inc.).

Statistical analysis. SPSS17.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analyses, GraphPad Prism 4 (GraphPad Software, Inc., La Jolla, CA, USA) was used to draw graphs and ProteinPilot 3.0 was used to detect the protein threshold [where Unused ProtScore >1.3 (95% CI)]. An error (ProtScore) of 2.0 indicated a credible identified protein; an error of >1.2 or <0.8 indicated an identifiable significant difference ($P < 0.05$).

All data were normally distributed when examined with a one-sample Kolmogorov-Smirnov test. A t-test was used to compare two groups and data are expressed as the mean \pm standard deviation; $P < 0.05$ was considered to indicate a statistically significant difference.

Correlation analysis used multiple linear regression analysis and the disaggregated data was assigned a conversion score, as follows: i) Gender: male, 1; and female, 2; ii) diagnostic level: diagnosed, 1; suspected, 2; suspected and clinically supported, 3; iii) involvement: medullary, 1; cervical, 2; and lumbar, 3.

Results

Clinical data. The average ages of the ALS-B and NC groups were 52.7 ± 12.13 and 51.1 ± 10.62 years old, respectively, and there were 6 men and 4 women in each group. No significant difference in age or gender balance between these groups was identified ($P > 0.05$).

The average ages of the ALS-A and OND groups were 52.80 ± 11.98 and 51.17 ± 12.44 years old, respectively, and there were 22 men and 13 women in the ALS-A group,

Table IV. Increased proteins in ALS group.

Protein	Ratio of ALS vs. control	Accession no.
Peroxiredoxin-2	1.6278	splP321191
Glutamate receptor 4	1.3097	splP027351
Apolipoprotein A-II	1.2523	splP480581
Hemoglobin subunit α	1.5451	splP699051
Trypsin-1	1.2076	splP699051
Biotinidase	1.2970	splP432511
Hemoglobin subunit β	1.4623	splP688711
Glyceraldehyde-3-phosphate dehydrogenase	1.2505	splP044061
Cell growth regulator with EF hand domain protein 1	1.3748	splQ996741
Protein DJ-1	1.2886	splQ994971
Neuroendocrine convertase 2	1.2294	splP165191
γ -glutamyl hydrolase	1.2209	splQ928201
Testican-2	1.2140	splQ925631
V-type proton ATPase subunit D	1.2915	splQ9Y5K81

ALS, amyotrophic lateral sclerosis.

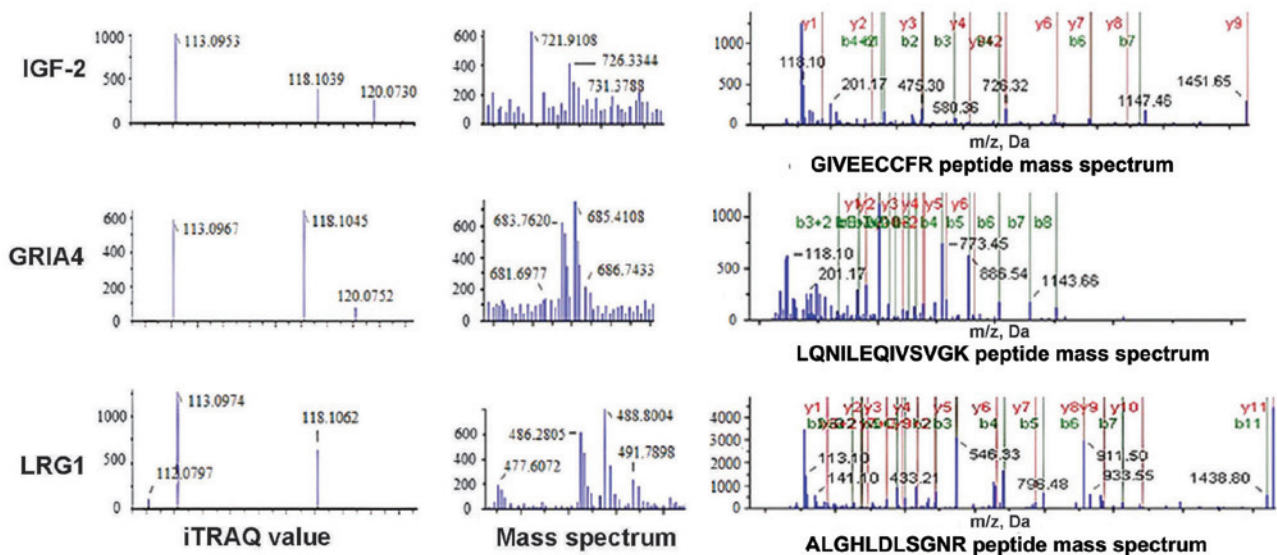


Figure 1. Sample data of 3 differentially-expressed proteins. GIVEECCFR, ALGHLDLSGNGR and LQNILEQIVSVGK are enzyme-specific peptides. IGF-2, insulin-like growth factor II; GRIA4, glutamate receptor 4; LRG1, leucine-rich α -2-glycoprotein 1; iTRAQ, isobaric tags for relative and absolute quantitation.

and 11 men and 7 women in the OND group. No significant difference was identified in age or gender balance between these groups ($P>0.05$). The protein concentration of CSF was 350.46 ± 110.09 mg/l in the ALS-A group and 377.56 ± 85.85 mg/l in the control group, with no significant difference revealed between the two ($P>0.05$).

CSF protein identification. iTRAQ and 2D-LC-MS/MS analyses were performed and used to analyze the protein content of the CSF in the ALS and NC groups. A total of 248 proteins were identified, and their names, the iTRAQ ratio (where available) and the UniProtKB/Swiss-Prot database accession number of 243 of these proteins are provided (95% CI; Tables I and II).

Analyses of differential protein expression. A total of 35 differentially-expressed proteins were compared between the ALS and NC groups; of these, 14 were upregulated and 21 were downregulated (Tables III and IV). These proteins had a ProtScore between the values of >1.2 and <0.8 , corresponding to $P<0.05$.

Sample data of specific differentially-expressed proteins. IGF-2 and LRG1 protein expression was decreased in the experimental groups, whereas GRIA4 expression was increased (Fig. 1).

DAVID results and the classification of proteins by biological role. The function of all identified proteins was analyzed using

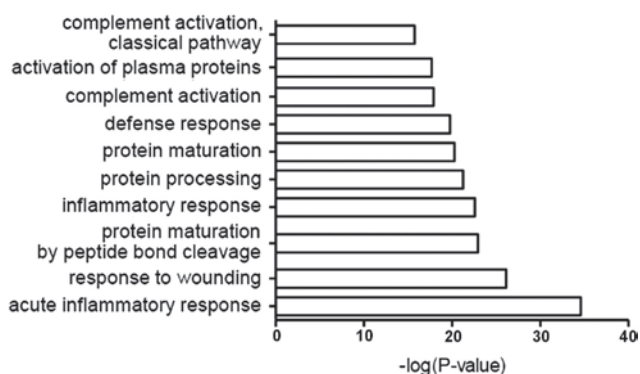


Figure 2. Identified cerebrospinal fluid proteins, classified by the biological processes that they are involved in. Activation of plasma proteins refers to this process in the acute inflammatory response.

GO in conjunction with DAVID software. The most common biological roles of CSF proteins were in acute inflammation, damage response, protein maturation, inflammation, defense response, complement activation and other associated immune pathways (Fig. 2).

Classification by cellular localization. The most common localization of CSF proteins relative to cells included the extracellular domain, extracellular space, extracellular matrix and protein-lipid complexes (Fig. 3).

Classification by molecular function. The most common molecular functions of CSF proteins were endopeptidase, peptidase, enzyme and serine-type endopeptidase inhibitors, and antigen-, calcium- and heparin-binding proteins (Fig. 4).

Western blotting. A total of 3 candidate proteins were randomly selected to be examined by western blot analysis in the ALS and the NC groups (Fig. 5); of these, IGF-2 was revealed to be significantly downregulated and GRIA4 was significantly upregulated in the ALS group when compared with the normal control group ($P < 0.05$; Table V), but LRG1 expression was not significantly altered ($P = 0.224$; Table V). These proteins were also examined by western blot analysis in the ALS-A and OND groups, again demonstrating a significant downregulation of IGF-2 and a significant upregulation of GRIA4 in the ALS group compared with the OND group ($P < 0.01$; Table VI), but no significant difference in LRG1 expression between these groups ($P = 0.196$; Table VI).

Correlation between GRIA4 and gender. GRIA4 expression in the ALS-A group was significantly higher in male patients than in female patients ($765,483 \pm 583,227$ and $319,766 \pm 224,242$, respectively; $r = -0.574$; $P = 0.003$; Fig. 6).

GRIA4 expression in the ALS-A group was also positively correlated with ALS clinical scores ($r = 0.487$; $P = 0.017$), indicating a negative correlation with clinical severity (Fig. 7).

Discussion

In the present study, 248 different low-abundance proteins were identified in human CSF and the details of these proteins were established in ALS patients. All proteins were subjected

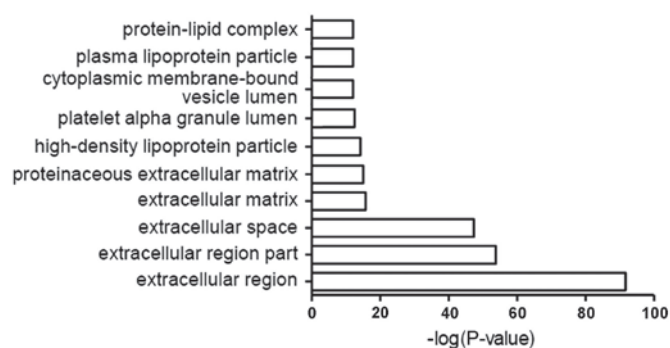


Figure 3. Identified cerebrospinal fluid proteins, classified by their cellular localization. Extracellular region refers to the space external to the outermost structure of the cell, indicating gene products that are not attached to the cell surface. Extracellular region part refers to any constituent part of the extracellular region, and is not used to specifically indicate gene products.

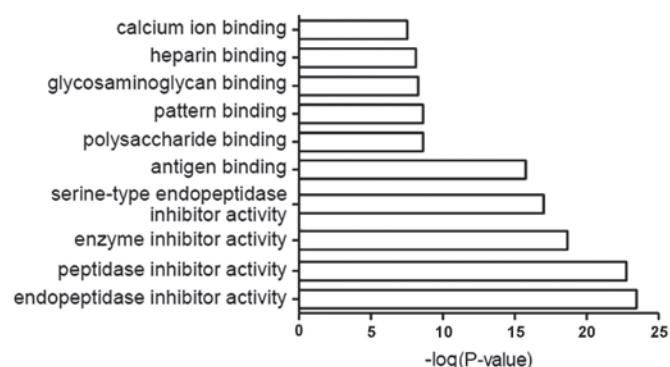


Figure 4. Identified cerebrospinal fluid proteins, classified by their molecular function.

to GO analysis with DAVID software and were classified according to their involvement in biological processes, their cellular localization and their molecular function. Data indicated that the primary roles of these proteins were in the acute inflammatory response and injury response, that the proteins were predominantly localized to extracellular regions and that the majority of these proteins were endopeptidase and peptidase inhibitors. These data aid the understanding of CSF protein profiles in patients with ALS, and provide possible biomarkers of the disease. A screening of 35 of these proteins revealed significant differences in protein expression between the ALS and NC groups, primarily in inflammation-associated proteins, neurotrophic factors and signal transduction proteins.

IGF-2, GRIA4 and LRG1 were randomly selected to verify their differential expression in ALS patients using western blot analysis. Consistent with the results of the proteomic analysis, IGF-2 and GRIA4 expression was altered in the CSF of ALS patients, but there was no significant difference in LRG1 expression between the ALS and NC groups; this led to the conclusion that additional verification of the altered protein expression reported in the present study is necessary to confirm these proteomic results.

To confirm the expression specificity of IGF-2, GRIA4 and LRG1, expression levels of these proteins were compared in patients with ALS and patients with OND; IGF-2 expression

Table V. Western blotting results of ALS-B and NC groups.

Protein	Molecular weight, KDa	ALS group (n=10)	NC group (n=10)	P-value
IGF-2	7.5	225700±126090	436857±212550	0.017 ^a
GRIA4	102	715730±432220	305796±130600	0.016 ^a
LRG1	38	1278000±702040	1807000±1115500	0.224

Data are presented as the mean ± standard deviation. ^aP<0.05 vs. NC group. ALS, amyotrophic lateral sclerosis; NC, normal control; IGF-2, insulin-like growth factor II; GRIA4, glutamate receptor 4; LRG1, leucine-rich α -2-glycoprotein 1.

Table VI. Western blotting results of ALS-A and OND groups.

Protein	ALS group (n=35)	OND group (n=18)	P-value
IGF-2	222200±123648	452500±255620	0.002 ^a
GRIA4	608502±519012	200100±150810	0.002 ^a
LRG1	1097255±961025	746070±703690	0.196

Data are presented as the mean ± standard deviation. ^aP<0.01 vs. OND group. ALS, amyotrophic lateral sclerosis; OND, other neurological disease; IGF-2, insulin-like growth factor II; GRIA4, glutamate receptor 4; LRG1, leucine-rich α -2-glycoprotein 1.

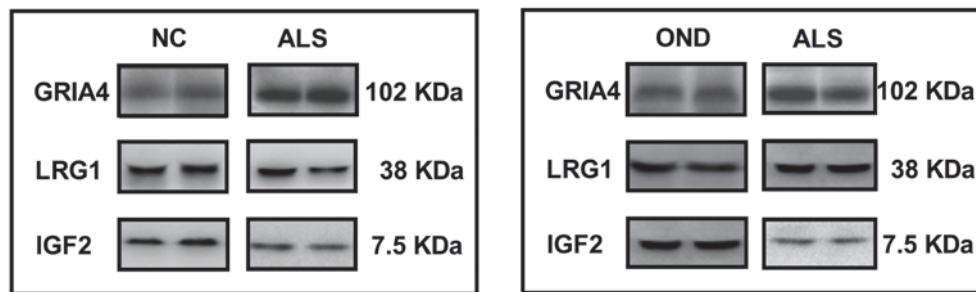


Figure 5. Western blot analysis of the three candidate proteins, glutamate receptor 4 (GRIA4), leucine-rich α -2-glycoprotein 1 (LRG1) and insulin-like growth factor II (IGF-2). NC, normal control; ALS, amyotrophic lateral sclerosis; OND, other neurological disease.

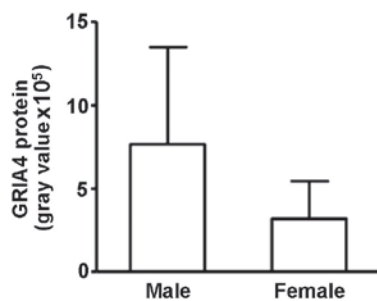


Figure 6. Correlation between GRIA4 and clinical features. GRIA4, glutamate receptor 4.

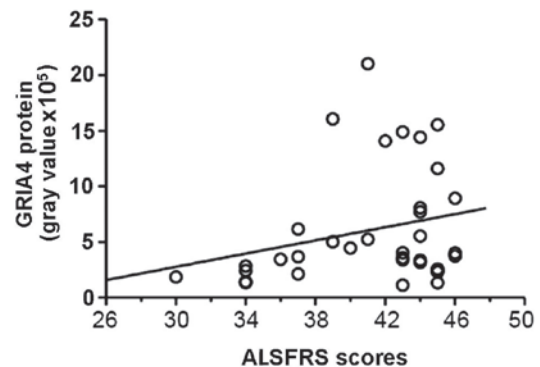


Figure 7. Correlation of ALS value with GRIA4. ALS, amyotrophic lateral sclerosis; GRIA4, glutamate receptor 4; ALSFRS, ALS functional rating scale.

was significantly decreased, but GRIA4 expression was significantly increased.

Alterations to protein expression are complex with regard to disease progression, age, gender and duration of illness; it was thus important to examine the correlation between alterations to protein expression and clinical features. Clinical data of 35 ALS patients was collected and were subjected to multiple linear regression analysis to reveal any confounding factors.

The clinical data in the present study revealed a higher male incidence of ALS (male to female ratio, 1.7:1), which was in support of a previous study; the 2009 European epidemiological study revealed a similar ratio of 1.4:1 (10). The present results demonstrated a correlation of GRIA4 expression with

gender; male GRIA4 levels were 2.5-fold those of female levels ($P < 0.01$).

To the best of our knowledge, the association between glutamate receptor levels and clinical characteristics has not been studied; however, glutamate excitotoxicity damage is widely recognized in the pathogenesis of ALS. Fiszman *et al* (11) reported no significant correlation between glutamate ligand concentration in the CSF of patients with different severities of ALS, suggesting that glutamate is involved in the occurrence of ALS and not in the severity of the disease. Excitotoxicity of glutamate also requires the presence of a glutamate receptor, meaning that high expression of glutamate receptors may be responsible for the neuronal toxicity injury induced by glutamate. As the concentration of glutamate is increased in the CSF of ALS patients (11), and GRIA4 expression was increased in ALS in the current study, the high incidence of ALS may be associated with the expression of GRIA4.

In the present study, the ALS score was estimated using the ALSFRS-R scale; a lower score on this scale corresponded to more severe disease. A multivariate analysis indicated that GRIA4 expression was positively correlated with the ALS score, revealing a negative correlation with the severity of the disease. However, ALS patients with mild symptoms were selected, defined in accordance with a previous scoring system attributing a score >25 to less severe ALS and scores of <25 to moderate and severe phases of ALS (12). As the glutamate concentration is significantly increased in the CSF of ALS patients (7), glutamate is likely to be involved in the pathogenesis of the disease. From the present results, it was concluded that GRIA4 expression is likely to be involved in the pathogenesis of ALS, resulting in a negative feedback regulatory mechanism to subsequently reduce its expression. The glutamate receptor antagonist, riluzole, is effective in the early treatment of ALS (13). In conjunction with the present report suggesting the early-stage overexpression of GRIA4, these data indicate that early treatment with anti-glutamate-associated drugs may prove a useful therapeutic measure.

The multivariate analysis examining IGF-2 and LRG1 expression and the clinical data revealed no significant correlations. This may be attributable to the sample size of the present study being too small or too few clinical factors being included. Based on the standard deviation values, the expression levels of IGF-2 and LRG1 were relatively balanced, as compared with the standard deviation of the GRIA4 expression levels, which suggested that IGF-2 may be a valuable biomarker of ALS with higher credibility due to fewer interference factors.

In summary, GRIA4 expression varied based on gender and may be reflective of ALS severity, providing a meaningful reference value for the timing of treatment. Furthermore, IGF-2 may prove an effective diagnostic marker of ALS.

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