Proteomic analysis of cerebrospinal fluid in amyotrophic lateral sclerosis

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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]...
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 x 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 x g for 15 min and disposal of the supernatant, the precipitant was dissolved into 20 ul iTRAQ solution and 1 ul 1% sodium dodecyl sulfate (SDS). Subsequently, 1 ul 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 regents for 1 h at room temperature. iTRAQ regents 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 (2.1x100 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; l:1,250; ab9574); mouse anti-human leucine-rich α-2-glycoprotein 1 (LRG1; l: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 AffiniPure 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 spectra, 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              |
| >0.47 (66)              | 294                  | 448                         | 21271             | 40761                | 37.2              |

*Cutoff 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                | sp|P02768I| |
| Complement C4-A               | 1.0317                | sp|P0C0L4| |
| Complement C3                 | 1.0003                | sp|P01024I| |
| Transthyretin                  | 1.0717                | sp|P02766I| |
| α-1-antitrypsin               | 0.7250                | sp|P01009I| |
| α-2-macroglobulin             | 0.9938                | sp|P01023I| |
| Serotransferrin                | 0.8150                | sp|P02787I| |
| Fibronectin                   | 1.0084                | sp|P02751I| |
| Apolipoprotein A1             | 1.0930                | sp|P02647I| |
| Ig γ1 chain C region          | 0.9304                | sp|P01857I| |
| Apolipoprotein E              | 1.1323                | sp|P02649I| |
| Gelsolin                      | 1.0509                | sp|P06396I| |
| Apolipoprotein A-IV           | 1.1446                | sp|P06727I| |
| Clusterin                     | 1.0969                | sp|P10909I| |
| Cystatin C                    | 1.0671                | sp|P01034I| |
| Vitamin D-binding protein     | 0.8710                | sp|P02774I| |
| Contactin-1                   | 1.0430                | sp|P012860I| |
| Complement factor             | 1.0036                | sp|P08603I| |
| Pigment epithelium-derived factor | 0.9803            | sp|P36955I| |
| Secretogranin-1               | 1.0670                | sp|P05060I| |
| Ceruloplasmin                 | 0.8720                | sp|P00450I| |
| Serum albumin                 | 1.0588                | sp|P01042I| |
| Haptoglobin                   | 0.6926                | sp|P00738I| |
| Secretogranin-3               | 1.1640                | sp|P08603I| |
| Antithrombin-III              | 0.8452                | sp|P01008I| |
| Chromogranin-A                | 1.0098                | sp|P010645I| |
| α-1-B glycoprotein            | 0.9835                | sp|P04217I| |
| β-Ala-His dipeptidase         | 1.1591                | sp|P096KN2I| |
| Neuronal cell adhesion molecule | 1.0097           | sp|P02765I| |
| Ig γ2 chain C region          | 1.0383                | sp|P01859I| |
| Monocyte differentiation antigen CD14 | 0.8775          | sp|P08571I| |
| Fibrinogen α chain            | 1.0375                | sp|P02671I| |
| α-1-antichymotrypsin          | 0.9855                | sp|P01011I| |
| Neurosecretory protein VGF    | 1.0510                | sp|P015240I| |
| α-2-HS-glycoprotein           | 1.0036                | sp|P02765I| |
| Angiotensinogen               | 1.0014                | sp|P01019I| |
| Ig α1 chain C region          | 1.0096                | sp|P01876I| |
| Collagen α-1(I) chain         | 1.0412                | sp|P02452I| |
| Plasminogen                   | 0.8738                | sp|P00747I| |
| Kininogen-1                   | 0.8529                | sp|P01042I| |
| Fibulin-1                     | 0.9324                | sp|P23142I| |
| Protein name | iTRAQ ratio (ALS/NC) | Accession no. |
|--------------|----------------------|---------------|
| Hemoglobin subunit β | 1.4623 | sp|P68871|
| Prostaglandin-H2 D-isomerase | 0.9310 | sp|P41222|
| N-acetyllactosaminide β-1,3-N-acetylg glucosaminyltransferase | 1.0294 | sp|O43505i|
| Neuronal pentraxin receptor | 1.0815 | sp|O95502i|
| Hemopexin | 0.8432 | sp|P02790|
| Retinol-binding protein 4 | 0.9796 | sp|P02753i|
| Apolipoprotein D | 0.9616 | sp|P05090|
| Ectonucleotide pyrophosphatase/phosphodiesterase family member 2 | 0.9689 | sp|Q13822|
| β-2-glycoprotein 1 | 0.9413 | sp|P02749i|
| Carboxypeptidase E | 1.0193 | sp|P16870|
| Collagen α-2(I) chain | 1.0000 | sp|P08123|
| Calsyntenin-1 | 1.1224 | sp|O94985|
| Vitronectin | 0.8401 | sp|P04004i|
| Nucleobindin-1 | 1.0513 | sp|Q02818|
| Ig µ chain C region | 0.8467 | sp|P01871i|
| Ig κ chain C region | 1.0135 | sp|P01834i|
| Ig γ3 chain C region | 0.9289 | sp|P01860i|
| Extracellular superoxide dismutase (Cu-Zn) | 1.0356 | sp|P08294|
| Cathepsin D | 0.9478 | sp|P07339|
| Afamin | 1.0176 | sp|P43652i|
| Complement component C7 | 0.9460 | sp|P10643i|
| Apolipoprotein A-II | 1.2524 | sp|P02652|
| Contactin-2 | 1.0433 | sp|Q02246i|
| Inter-α-trypsin inhibitor heavy chain | 1.0549 | sp|P13591i|
| Neural cell adhesion molecule 1 | 1.0091 | sp|P01842i|
| EGF-containing fibulin-like extracellular matrix protein | 0.9392 | sp|P01805i|
| Ig λ chain C regions | 1.0045 | sp|P01842i|
| Complement component C9 | 0.7597 | sp|P02748i|
| Neural cell adhesion molecule L1-like protein | 1.0405 | sp|P000533i|
| Procollagen C-endopeptidase enhancer 1 | 1.0410 | sp|Q15113i|
| Mimecan | 0.9845 | sp|P20774i|
| Fibrinogen β chain | 1.0713 | sp|P02675i|
| Hemoglobin subunit α | 1.5451 | sp|P69905i|
| ProSAAS | 1.0492 | sp|Q9UHG2i|
| Neuronal pentraxin-1 | 1.1167 | sp|Q15818i|
| β-2-microglobulin | 1.0138 | sp|P61769i|
| Collagen α-1(VI) chain | 1.0602 | sp|P12109i|
| Neural cell adhesion molecule 2 | 0.9561 | sp|P01842i|
| Leucine-rich α-2-glycoprotein | 0.6430 | sp|P02750i|
| Insulin-like growth factor-binding protein 2 | 0.9574 | sp|P18065i|
| Insulin-like growth factor-binding protein 6 | 0.9883 | sp|P24592i|
| Protein kinase C-binding protein NELL2 | 0.9929 | sp|Q99435i|
| Keratin, type II cytoskeletal 1 | 0.9729 | sp|P04264i|
| Dickkopf-related protein 3 | 1.0396 | sp|Q9UBP4i|
| Ig κ chain V-III region | 0.9945 | sp|P01623i|
| Complement C1r subcomponent | 0.9240 | sp|P00736i|
| Prothrombin | 0.9113 | sp|P00734i|
| Dystroglycan | 1.0292 | sp|Q14118i|
| Tetranectin | 0.9282 | sp|P05452i|
| α-2-antiplasmin | 0.9126 | sp|P08697i|
| Complement factor B | 0.8143 | sp|P00751i|
| Protein name                                      | iTRAQ ratio (ALS/NC) | Accession no.     |
|--------------------------------------------------|-----------------------|-------------------|
| Cartilage acidic protein 1                       | 1.0590                | sp|Q9NQ79| |
| Peptidylglycine α-amidating monoxygenase          | 0.8763                | sp|P19021| |
| Major prion protein                              | 1.0478                | sp|P04156| |
| Zinc-α-2-glycoprotein                            | 0.7912                | sp|P25311| |
| Neuroendocrine protein 7B2                       | 1.1447                | sp|P05408| |
| Multiple epidermal growth-factor-like domains 8  | 0.9706                | sp|Q7Z7M0| |
| Insulin-like growth factor-binding protein 7      | 1.0327                | sp|Q16270| |
| SPARC                                            | 0.8425                | sp|P09486| |
| Trypsin-1                                        | 1.2077                | sp|P07477| |
| Secretogranin-2                                  | 0.9307                | sp|P13521| |
| Voltage-dependent calcium channel subunit α2δ-1  | 0.9343                | sp|P54289| |
| Pyruvate kinase isoymes M1/M2                    | 1.0611                | sp|P14618| |
| Cadherin 13                                      | 1.0163                | sp|P55290| |
| GM2 Ganglioside activator                        | 1.0083                | sp|P17900| |
| Fibrinogen γ chain                               | 1.0925                | sp|P06390| |
| Extracellular matrix protein 1                   | 1.0849                | sp|P16610| |
| Collagen α-1(XVIII) chain                        | 1.0000                | sp|P39060| |
| Cadherin-2                                       | 1.0560                | sp|P19022| |
| Semaphorin 7A                                    | 0.9433                | sp|P07532| |
| Ig κ chain V-II region GM607                     | 0.9526                | sp|P06390| |
| Ig λ chain V-III region LOI                      | 0.7060                | sp|P01617| |
| Transmembrane protein 132A                       | 1.1680                | sp|Q24JP5| |
| Metalloprotease inhibitor 2                      | 0.9855                | sp|P16035| |
| Osteopontin                                      | 1.0354                | sp|P10451| |
| Kallikrein-6                                     | 0.9713                | sp|Q92876| |
| Sex hormone-binding globulin                     | 0.6051                | sp|P04278| |
| Actin, cytoplasmic 1                             | 0.8566                | sp|P06709| |
| Ig γ-4 chain C region                            | 1.1808                | sp|P01861| |
| Protein FAM3C                                    | 0.9182                | sp|Q92520| |
| Chorionic somatomammotropin hormone              | 0.5234                | sp|P01243| |
| Keratin, type I cytoskeletal 9                   | 0.9161                | sp|P35527| |
| Limbic system-associated membrane protein        | 0.9398                | sp|Q13449| |
| Phospholipid transfer protein                    | 1.1687                | sp|P55058| |
| Ig heavy chain V-III region BRO                  | 0.9650                | sp|P01766| |
| SPARC-like protein 1                             | 0.9325                | sp|Q14515| |
| Fructose-bisphosphate aldolase                   | 0.9490                | sp|Q14075| |
| N-acetylmuramoyl-L-alanine amidase               | 0.9820                | sp|Q96PD5| |
| Complement C1s subcomponent                     | 0.9598                | sp|P09871| |
| Ig κ chain V-IV region B17                       | 0.8581                | sp|P06314| |
| Lumican                                          | 1.0259                | sp|P51884| |
| Opioid-binding protein/cell adhesion molecule    | 0.8758                | sp|Q14982| |
| Ribonuclease pancreatic                          | 0.7527                | sp|P07998| |
| Ig κ chain V-III region CLL                      | 0.8486                | sp|P04207| |
| Immunoglobulin superfamily member 8             | 0.8751                | sp|Q969P0| |
| 78-kDa glucose-regulated protein                | 0.9751                | sp|P10211| |
| Protein AMBP                                     | 0.7950                | sp|P02760| |
| Coagulation factor V                             | 1.0938                | sp|P12259| |
| Histidine-rich glycoprotein                      | 0.9048                | sp|P04196| |
| Ig heavy chain V-III region KOL                  | 0.9839                | sp|P01772| |
| L-lactate dehydrogenase B chain                 | 0.9649                | sp|P07195| |
| Complement component C6                          | 0.9164                | sp|P13671| |
| Ephrin type-A receptor 4                         | 0.9178                | sp|P54764| |
Table II. Continued.

| Protein name                                                                 | iTRAQ ratio (ALS/NC) | Accession no.     |
|-------------------------------------------------------------------------------|----------------------|------------------|
| Cerebellin-3                                                                 | 1.0609               | sp|Q6UW01| |
| Proenkephalin A                                                              | 1.0079               | sp|P01210| |
| Insulin like growth factor binding protein 4                                 | 0.8461               | sp|P22692| |
| Apolipoprotein C-III                                                          | 1.1181               | sp|P02656| |
| Trypsin -3                                                                   | 1.1478               | sp|P35030| |
| Transforming growth factor-β-induced protein ig-h3                           | 1.0709               | sp|Q15582| |
| IgG Fe-binding protein                                                        | 1.0775               | sp|Q9Y6R7| |
| Plasma serine protease inhibitor                                              | 0.9604               | sp|P05154| |
| Coagulation factor XII                                                        | 0.9422               | sp|P00748| |
| Biotinidase                                                                  | 1.2970               | sp|P43251| |
| Ig κ chain V-III region VG (Fragment)                                         | 1.09987              | sp|P04433| |
| Collagen α-3(VI) chain                                                       | 0.9422               | sp|P00748| |
| Neuroserpin                                                                  | 1.0459               | sp|Q99574| |
| Keratin, type I cytoskeletal 10                                               | 0.8858               | sp|P13645| |
| Fibulin-5                                                                    | 0.9587               | sp|Q9UBX5| |
| Receptor-type tyrosine-protein phosphatase S                                  | 1.1670               | sp|Q13332| |
| Complement factor I                                                          | 0.8627               | sp|P05156| |
| Ig heavy chain V-III region TRO                                               | 1.1189               | sp|P01762| |
| Basement membrane-specific heparan sulfate proteoglycan core protein         | 0.9080               | sp|P98160| |
| α-1 acid glycoprotein 1                                                       | 0.7355               | sp|P02763| |
| Chitinase-3-like protein 1                                                    | 0.9904               | sp|P36222| |
| Cell adhesion molecule 3                                                      | 0.8572               | sp|Q08380| |
| Galectin-3-binding protein                                                   | 0.9876               | sp|Q08380| |
| Ig heavy chain V-III region POM                                               | 1.0712               | sp|P01774| |
| Endonuclease domain-containing 1 protein                                      | 1.0166               | sp|P01776| |
| Ig λ chain V-I region HA                                                     | 1.0838               | sp|P01779| |
| Complement C1q subcomponent subunit B                                        | 1.0301               | sp|P02746| |
| Leucine-rich repeat-containing protein 4B                                     | 1.0174               | sp|Q9NT99| |
| Peroxiredoxin-2                                                              | 1.6278               | sp|P32119| |
| Glyceraldehyde-3-phosphate dehydrogenase                                     | 1.2506               | sp|P04406| |
| Serum paraoxonase/arylesterase 1                                              | 0.8635               | sp|P27169| |
| Calcium/calcmodulin-dependent protein kinase type II α chain                 | 1.1677               | sp|Q9UQM7| |
| Fibrillin-1                                                                  | 0.2204               | sp|P35555| |
| Complement C2                                                                 | 0.9405               | sp|P00681| |
| Cell growth regulator with EF hand domain protein 1                           | 1.3740               | sp|P02746| |
| Myopalladin                                                                  | 0.6801               | sp|Q86TC9| |
| Neuronal growth regulator 1                                                   | 1.0667               | sp|P7Z3B1| |
| Serum amyloid A-4 protein                                                     | 1.0645               | sp|P43026| |
| Protocadherin Fat 2                                                           | 1.1409               | sp|Q9NYQ8| |
| Cathepsin F                                                                  | 1.1142               | sp|Q9UBX1| |
| DNA repair protein RAD50                                                       | 0.9463               | sp|Q92878| |
| α-enolase                                                                    | 1.1591               | sp|P06733| |
| Insulin-like growth factor II                                                 | 0.4053               | sp|P01344| |
| Ig λ chain V-III region SH                                                    | 1.0399               | sp|P01714| |
| Reelin                                                                       | 1.1149               | sp|P78509| |
| Pregnancy-specific β-1-glycoprotein 1                                         | 0.7522               | sp|P11464| |
| Retinoic acid receptor responder protein 2                                    | 1.0850               | sp|Q99969| |
| Lymphocyte antigen 6H                                                         | 1.0322               | sp|Q94772| |
| Receptor-type tyrosine-protein phosphatase N2                                 | 1.0020               | sp|Q92932| |
| Multimerin-2                                                                 | 1.0029               | sp|Q9H8L6| |
Table II. Continued.

| Protein name                                               | iTRAQ ratio (ALS/NC) | Accession no. |
|------------------------------------------------------------|----------------------|---------------|
| Apolipoprotein L1                                           | 0.9537               | sp|O14791| |
| Ig κ chain V-I region Roy                                   | a                    | sp|P01608| |
| Neurofascin                                                | 1.0305               | sp|O94856| |
| V-type proton ATPase                                        | 0.8780               | sp|Q15904| |
| Heparin cofactor 2                                          | 1.0087               | sp|P05546| |
| Plasma glutamate carboxypeptidase                          | 1.0663               | sp|Q9Y646| |
| Hypoxia upregulated protein 1                               | 1.0213               | sp|Q9Y4L1| |
| Ig κ chain V-I region Ka                                    | 0.9834               | sp|P01603| |
| Protein DJ-1                                                | 1.2886               | sp|Q99497| |
| Laminin subunit γ-1                                        | 0.8128               | sp|P1047| |
| Cell surface glycoprotein MUC18                             | 0.7681               | sp|P43121| |
| Neuroendocrine convertase 2                                 | 1.2290               | sp|P16519| |
| Inter-α-trypsin inhibitor heavy chain H5                   | 0.9165               | sp|Q86UX2| |
| Exostosin-like 2                                            | 0.9342               | sp|Q9UBQ6| |
| Metalloproteinase inhibitor 1                               | 1.0673               | sp|P01033| |
| Immunoglobulin J chain                                      | 1.0429               | sp|P01591| |
| Ig κ chain V-I region BAN                                    | a                    | sp|P04430| |
| Ig κ chain V-I region DEE                                   | 1.0241               | sp|P01597| |
| Ig κ chain V-I region Wes                                   | 0.8814               | sp|P01611| |
| Serum amyloid A-1 protein                                   | 0.6516               | sp|P02735| |
| Glutamate receptor 4                                        | 1.3098               | sp|P48058| |
| Amyloid β A4                                                | 1.0164               | sp|P05067| |
| Zinc finger protein                                         | 0.9751               | sp|B1APH4| |
| Nidogen-2                                                  | 1.0441               | sp|P14112| |
| 72-kDa type IV collagenase                                  | 0.8378               | sp|P08253| |
| WAP, kazal, immunoglobulin, Kunitz and NTR domain-containing protein 2 | 1.0204               | sp|Q8TEU8| |
| Kallistatin                                                 | 0.8933               | sp|P29622| |
| 45-kDa calcium-binding protein                              | 1.0575               | sp|Q9BKRK5| |
| Tissue α-L-fucosidase                                       | 1.1211               | sp|P04066| |
| protein Cut A                                               | 1.0521               | sp|O60888| |
| Ig heavy chain V-I region                                   | 0.9126               | sp|P06326| |
| Ig heavy chain V-I region                                   | 0.9126               | sp|P06326| |
| γ-glutamyl hydrolase                                       | 1.2209               | sp|Q92820| |
| Complement component C8 γ chain                             | 0.9202               | sp|P07360| |
| Phosphatidyethanolamine-binding protein 1                   | 1.1293               | sp|P30086| |
| Thy-1 membrane glycoprotein                                 | 0.7535               | sp|P04216| |
| Cell adhesion molecule 4                                    | 0.9868               | sp|Q8NFZ8| |
| Sjogren syndrome/scleroderma autoantigen 1                  | 0.9615               | sp|O60232| |
| Uncharacterized protein C6orf170                            | 1.1061               | sp|Q96NH3| |
| N-acetylglucosamine-1-phosphotransferase subunit γ          | 1.0938               | sp|Q9UJJ9| |
| Testican-2                                                  | 1.2140               | sp|Q92563| |
| Fructose-bisphosphate aldolase C                           | a                    | sp|P09972| |
| Lysozyme C                                                  | 0.8222               | sp|P61626| |
| V-type proton ATPase subunit D                              | 1.2915               | sp|Q9Y5K8| |
| Coagulation factor XI                                       | a                    | sp|P03951| |
| Complement C1q subcomponent subunit C                      | 0.8441               | sp|P02747| |
| Dermcidin                                                   | 0.7257               | sp|P81605| |
| Ig κ chain V-II region RPMI 6410                            | 0.7960               | sp|P06310| |
| Hemoglobin subunit δ                                       | a                    | sp|P06310| |
| Titin                                                      | 0.9960               | sp|Q8WZ42| |
| Tumor protein 63                                            | 0.7445               | sp|Q9H3D4| |
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 ± 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                   | sp|P32119| |
| Glutamate receptor 4                              | 1.3097                   | sp|P02735| |
| Apolipoprotein A-II                               | 1.2523                   | sp|P48058| |
| Hemoglobin subunit α                               | 1.5451                   | sp|P69905| |
| Trypsin-1                                          | 1.2076                   | sp|P69905| |
| Biotinidase                                        | 1.2970                   | sp|P43251| |
| Hemoglobin subunit β                               | 1.4623                   | sp|P68871| |
| Glyceraldehyde-3-phosphate dehydrogenase           | 1.2505                   | sp|P04406| |
| Cell growth regulator with EF hand domain protein 1| 1.3748                   | sp|Q99674| |
| Protein DJ-1                                       | 1.2886                   | sp|Q99497| |
| Neuroendocrine convertase 2                        | 1.2294                   | sp|P16519| |
| γ-glutamyl hydrolase                               | 1.2209                   | sp|Q92820| |
| Testican-2                                         | 1.2140                   | sp|Q92563| |
| V-type proton ATPase subunit D                     | 1.2915                   | sp|Q9Y5K8| |

ALS, amyotrophic lateral sclerosis.

Figure 1. Sample data of 3 differentially-expressed proteins. GIVEECFR, ALGLDLSGNR and LQNIQIIVSVGK 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
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±583,227 and 319,766±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 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
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

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*  |
| GRIA4   | 102                  | 715730±432220   | 305796±130600  | 0.016*  |
| LRG1    | 38                   | 1278000±702040  | 1807000±115500 | 0.224   |

Data are presented as the mean ± standard deviation. *P<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*  |
| GRIA4   | 608502±519012   | 200100±150810   | 0.002*  |
| LRG1    | 1097255±961025  | 746070±703690   | 0.196   |

Data are presented as the mean ± standard deviation. *P<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.
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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