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.

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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 KH_2PO_4 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-conjgated 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

[a]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|P02768|
Complement C4-A	1.0317	sp|P0C0L4|
Complement C3	1.0003	sp|P01024|
Transthyretin	1.0717	sp|P02766|
α-1-antitrypsin	0.7250	sp|P01009|
α-2-macroglobulin	0.9938	sp|P01023|
Serotransferrin	0.8150	sp|P02787|
Fibronectin	1.0084	sp|P02751|
Apolipoprotein A1	1.0930	sp|P02647|
Ig γ1 chain C region	0.9304	sp|P01857|
Apolipoprotein E	1.1323	sp|P02649|
Gelsolin	1.0509	sp|P06396|
Apolipoprotein A-IV	1.1446	sp|P06727|
Clusterin	1.0969	sp|P10909|
Cystatin C	1.0671	sp|P01034|
Vitamin D-binding protein	0.8710	sp|P02774|
Contactin-1	1.0430	sp|Q12860|
Complement factor	1.0036	sp|P08603|
Pigment epithelium-derived factor	0.9803	sp|P36955|
Secretogranin-1	1.0670	sp|P05060|
Ceruloplasmin	0.8720	sp|P00450|
Serum albumin	1.0588	sp|P51693|
Haptoglobin	0.6926	sp|P00738|
Secretogranin-3	1.1640	sp|Q8WXD2|
Antithrombin-III	0.8452	sp|P01008|
Chromogranin-A	1.0098	sp|P010645|
α-1-B glycoprotein	0.9835	sp|P04217|
β-Ala-His dipeptidase	1.1591	sp|Q96KN2|
Neuronal cell adhesion molecule	1.0097	sp|Q92823|
Ig γ2 chain C region	1.0383	sp|P01859|
Monocyte differentiation antigen CD14	0.8775	sp|P08571|
Fibrinogen α chain	1.0375	sp|P02671|
α-1-antichymotrypsin	0.9855	sp|P01011|
Neurosecretory protein VGF	1.0510	sp|015240|
α-2-HS-glycoprotein	1.0036	sp|P02765|
Angiotensinogen	1.0014	sp|P01019|
Ig α1 chain C region	1.0096	sp|P01876|
Collagen α-1(I) chain	1.0412	sp|P02452|
Plasminogen	0.8738	sp|P00747|
Kininogen-1	0.8529	sp|P01042|
Fibulin-1	0.9324	sp|P23142|

Table II. Continued.

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*-acetylglucosaminyltransferase	1.0294	sp|O43505|
Neuronal pentraxin receptor	1.0815	sp|O95502|
Hemopexin	0.8432	sp|P02790|
Retinol-binding protein 4	0.9796	sp|P02753|
Apolipoprotein D	0.9616	sp|P05090|
Ectonucleotide pyrophosphatase/phosphodiesterase family member 2	0.9689	sp|Q13822|
β-2-glycoprotein 1	0.9413	sp|P02749|
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|P04004|
Nucleobindin-1	1.0513	sp|Q02818|
Ig μ chain C region	0.8467	sp|P01871|
Ig κ chain C region	1.0135	sp|P01834|
Ig γ3 chain C region	0.9289	sp|P01860|
Extracellular superoxide dismutase (Cu-Zn)	1.0356	sp|P08294|
Cathepsin D	0.9478	sp|P07339|
Afamin	1.0176	sp|P43652|
Complement component C7	0.9460	sp|P10643|
Apolipoprotein A-II	1.2524	sp|P02652|
Contactin-2	1.0433	sp|Q02246|
Inter-α-trypsin inhibitor heavy chain	1.0549	sp|Q14624|
Neural cell adhesion molecule 1	1.0091	sp|P13591|
EGF-containing fibulin-like extracellular matrix protein	0.9392	sp|Q12805|
Ig λ chain C regions	1.0045	sp|P01842|
Complement component C9	0.7597	sp|P02748|
Neural cell adhesion molecule L1-like protein	1.0405	sp|O00533|
Procollagen C-endopeptidase enhancer 1	1.0410	sp|Q15113|
Mimecan	0.9845	sp|P20774|
Fibrinogen β chain	1.0713	sp|P02675|
Hemoglobin subunit α	1.5451	sp|P69905|
ProSAAS	1.0492	sp|Q9UHG2|
Neuronal pentraxin-1	1.1167	sp|Q15818|
β-2-microglobulin	1.0138	sp|P61769|
Collagen α-1(VI) chain	1.0602	sp|P12109|
Neural cell adhesion molecule 2	0.9561	sp|O15394|
Leucine-rich α-2-glycoprotein	0.6430	sp|P02750|
Insulin-like growth factor-binding protein 2	0.9574	sp|P18065|
Insulin-like growth factor-binding protein 6	0.9883	sp|P24592|
Protein kinase C-binding protein NELL2	0.9929	sp|Q99435|
Keratin, type II cytoskeletal 1	0.9729	sp|P04264|
Dickkopf-related protein 3	1.0396	sp|Q9UBP4|
Ig κ chain V-III region	0.9945	sp|P01623|
Complement C1r subcomponent	0.9240	sp|P00736|
Prothrombin	0.9113	sp|P00734|
Dystroglycan	1.0292	sp|Q14118|
Tetranectin	0.9282	sp|P05452|
α-2-antiplasmin	0.9126	sp|P08697|
Complement factor B	0.8143	sp|P00751|

Table II. Continued.

Protein name	iTRAQ ratio (ALS/NC)	Accession no.
Cartilage acidic protein 1	1.0590	sp|Q9NQ79|
Peptidylglycine α-amidating monooxygenase	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 $\alpha_2\delta$-1	0.9343	sp|P54289|
Pyruvate kinase isozymes 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|P02679|
Extracellular matrix protein 1	1.0849	sp|Q16610|
Collagen α-1(XVIII) chain	1.0000	sp|P39060|
Cadherin-2	1.0560	sp|P19022|
Semaphorin 7A	0.9433	sp|O75326|
Ig κ chain V-II region GM607	0.9526	sp|P06309|
Ig λ chain V-III region LOI	0.7060	sp|P01617|
Transmembrane protein 132A	1.1680	sp|Q24JP5|
Metalloproteinase 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|P60709|
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|P04075|
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|P11021|
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 Fc-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|Q8N126|
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/calmodulin-dependent protein kinase type II α chain	1.1677	sp|Q9UQM7|
Fibrillin-1	0.2204	sp|P35555|
Complement C2	0.9405	sp|P06681|
Cell growth regulator with EF hand domain protein 1	1.3740	sp|Q99674|
Myopalladin	0.6801	sp|Q86TC9|
Neuronal growth regulator 1	1.0667	sp|Q7Z3B1|
Serum amyloid A-4 protein	1.0645	sp|P35542|
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|O94772|
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|P11047|
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|Q14112|
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|Q9BRK5|
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|
Phosphatidylethanolamine-binding protein 1	1.1293	sp|P30086|
Thy-1 membrane glycoprotein	0.7535	sp|P04216|
Cell adhesion molecule 4	0.9868	sp|Q8NFZ8|
Sjoegren 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|02747|
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|

Table II. Continued.

Protein name	iTRAQ ratio (ALS/NC)	Accession no.
Cysteine-rich with EGF-like domain protein 1	1.0219	sp|Q96HD1|
Putative α-1-antitrypsin-related protein	0.8877	sp|P20848|
Scrapie-responsive protein 1	1.0576	sp|O75711|

[a]Not 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 α1	0.7250	sp|P01009|
Haptoglobin	0.6926	sp|P00738|
Complement component 9	0.7597	sp|P02748|
Leucine-rich α-2-glycoprotein	0.6430	sp|P02750|
Zinc-α-2-glycoprotein	0.7912	sp|P25311|
Sex hormone-binding globulin	0.6051	sp|P04278|
Chorionic somatomammotropin hormone 1	0.5234	sp|P01243|
Ribonuclease pancreatic	0.7527	sp|P07998|
Protein AMBP	0.7950	sp|P02760|
α-1-acid glycoprotein 1	0.7355	sp|P02763|
Fibrillin-1	0.2204	sp|P35555|
Myopalladin	0.6801	sp|Q86TC9|
Insulin-like growth factor II	0.4053	sp|P01344|
Pregnancy-specific β-1-glycoprotein 1	0.7522	sp|P43251|
Cell surface glycoprotein MUC18	0.7681	sp|P43121|
Serum amyloid A protein	0.6516	sp|P02735|
Thy-1 membrane glycoprotein	0.7535	sp|P04216|
Dermcidin	0.7257	sp|P81605|
Ig λ chain V-III region LOI	0.7060	sp|P01617|
Ig κ chain V-II region RPMI 6410	0.7960	sp|P06310|
Tumor protein 63	0.7444	sp|Q9H3D4|

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 ± 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.

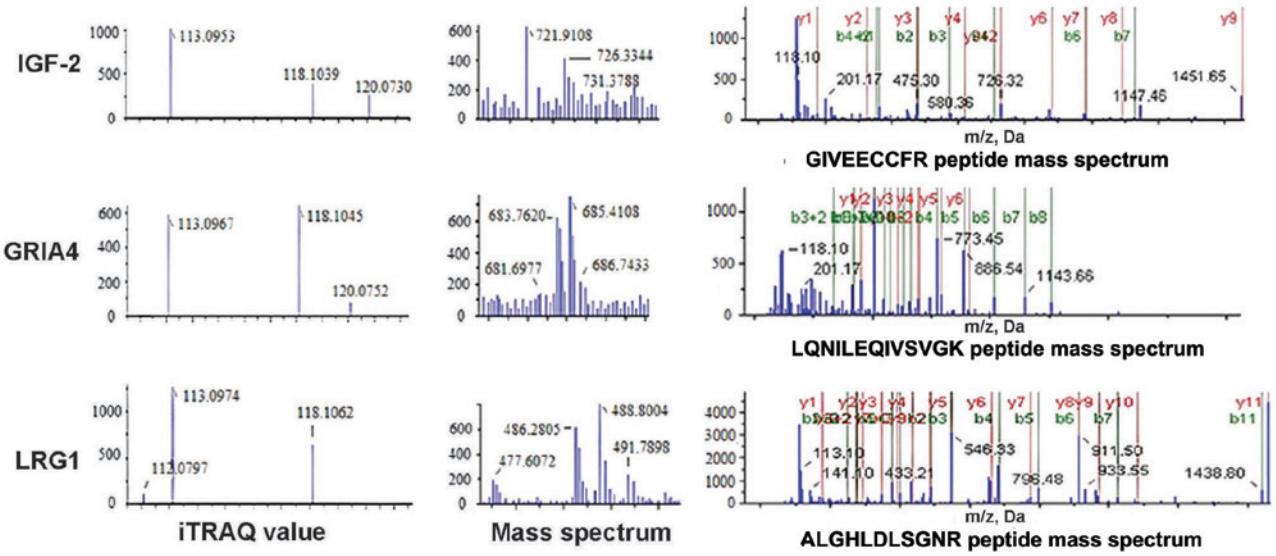

Figure 1. Sample data of 3 differentially-expressed proteins. GIVEECCFR, ALGHLDLSGNR and LQNILEQIVSVGK are enzyme-specific peptides. IGF-2, insulin-like growth factor II; GRIA4, glutamate receptor 4; LRG1, leucine-rich α-2-glycoprotein 1; iTRAQ, isobaric tags for relative and absolute quantitation.

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

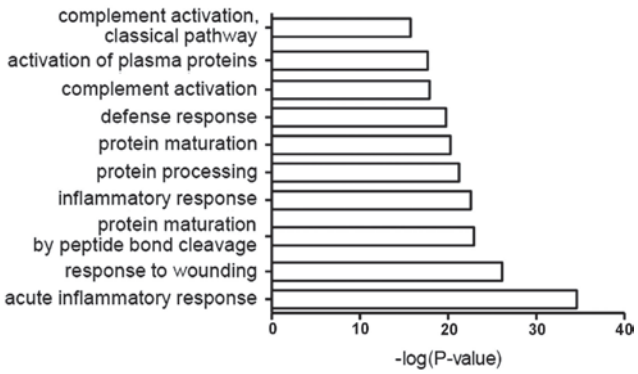

Figure 2. Identified cerebrospinal fluid proteins, classified by the biological processes that they are involved in. Activation of plasma proteins refers to this process in the acute inflammatory response.

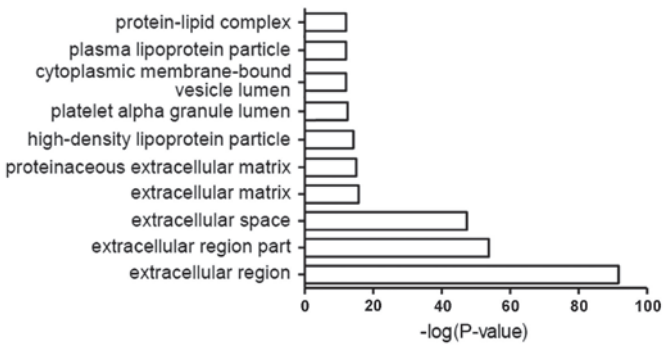

Figure 3. Identified cerebrospinal fluid proteins, classified by their cellular localization. Extracellular region refers to the space external to the outermost structure of the cell, indicating gene products that are not attached to the cell surface. Extracellular region part refers to any constituent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

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).

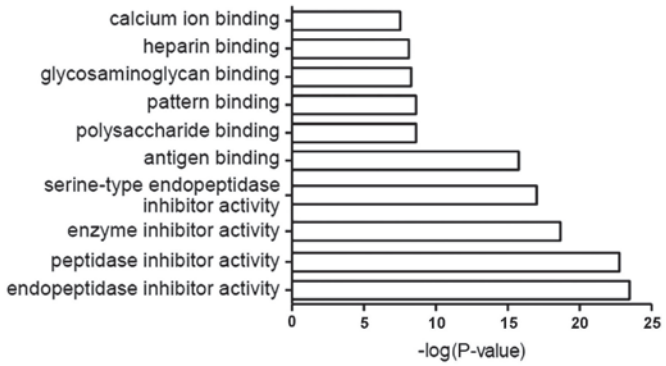

Figure 4. Identified cerebrospinal fluid proteins, classified by their molecular function.

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

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. [a]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[a]
GRIA4	608502±519012	200100±150810	0.002[a]
LRG1	1097255±961025	746070±703690	0.196

Data are presented as the mean ± standard deviation. [a]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.

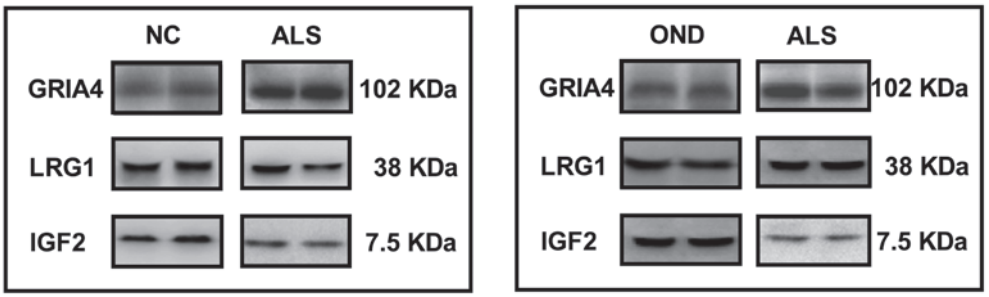

Figure 5. Western blot analysis of the three candidate proteins, glutamate receptor 4 (GRIA4), leucine-rich α-2-glycoprotein 1 (LRG1) and insulin-like growth factor II (IGF-2). NC, normal control; ALS, amyotrophic lateral sclerosis; OND, other neurological disease.

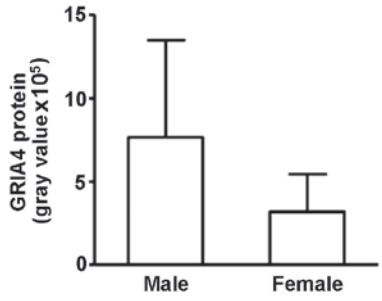

Figure 6. Correlation between GRIA4 and clinical features. GRIA4, glutamate receptor 4.

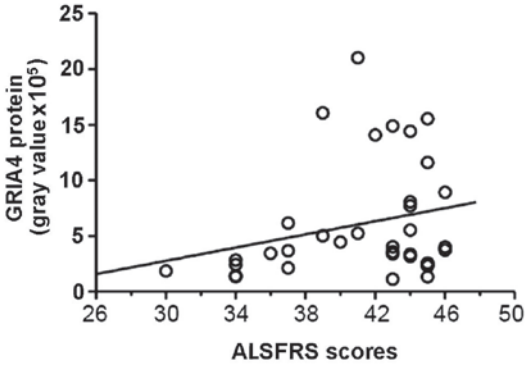

Figure 7. Correlation of ALS value with GRIA4. ALS, amyotrophic lateral sclerosis; GRIA4, glutamate receptor 4; ALSFRS, ALS functional rating scale.

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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