Proteomics-based Development of Biomarkers for Prion Diseases
Bo-Yeong Choi, Yeong Seon Lee, Young Ran Ju, Chi-Kyeong Kim and Su Yeon Kim*
Division of Zoonoses, Center for Immunology and Pathology, Korea National Institute of Health, Centers for Disease Control and Prevention, Cheongju-si, Chungcheongbuk-do, 28159, Republic of Korea

Abstract
We analyzed the proteomic profile of ME7 scrapie-infected mouse brains, and the interactions and functions of selected differentially expressed proteins to identify potential new biomarkers to be applicable for the diagnosis of Prion diseases. Mice were intracerebrally inoculated with 10% homogenate of ME7 scrapie-infected mouse brains, and monitored for neurological symptoms. We screened for proteins specifically expressed in infected brain samples using one-dimensional gel electrophoresis and liquid chromatography-mass spectrometry. 317 proteins based on their peptide scores and ratio values were selected. The major biological processes identified were cellular and metabolic processes, localization, and transport. Selected proteins had functions related to neurological processes, including cell-cell signaling, transmission of nerve impulse, and synaptic transmission. We analyzed infected host cells using experimental and computational methods, and found many significant protein expression changes. We identified 43 candidate proteins with high peptide scores and ratio values. Of these, 36 potential candidate proteins were related to up regulated biological processes, and 7 to down regulated biological processes. We confirmed the presence of two of these differentially expressed candidate proteins using immunoblotting. Keywords: Proteomic; Biomarker; Diagnosis; Prion disease

Introduction
Transmissible spongiform encephalopathies (TSEs) are rare fatal neurodegenerative diseases that occur in animals and humans through conformational conversion of normal prion protein (PrP) to an infectious PrPSc isoform. TSEs include scrapie, bovine spongiform encephalopathy, transmissible mink encephalopathy, Creutzfeldt-Jakob disease (CJD), Gerstmann–Sträussler–Scheinker syndrome, familial fatal insomnia, and kuru. The PrPSc isoform is more protease K-resistant than the PrP. Detection of PrPSc in the central nervous tissue of CJD patients by immunohistochemistry and confirmation of protease-resistant PrPSc has diagnostic values of definite human TSE. However, definite diagnoses of prion diseases are limited because these analyses require neuropathological confirmation by brain biopsy or post-mortem examination. Several protein markers, including 14-3-3 protein [1,2], Tau [3], astrocytic protein S-100 [4], apolipoprotein E [5], neuron-specific enolase [6], and cystatin C [7] have been reported in the cerebrospinal fluid of patients showing clinical symptoms of CJD. The assay for 14-3-3 protein that has been used in the laboratory diagnosis of CJD has high false-positive rates. Diverse potential biomarkers have been identified through proteomic approaches, and many research groups have attempted to identify more sensitive and specific markers for use in the diagnosis of CJD and other neurodegenerative diseases [8,9]. However, the effectiveness of these markers, except the 14-3-3 protein has never been fully specified in prion diseases. We need to verify their availability in biological to apply them biochemically for pre-mortem diagnosis, understand their application and identify as surrogate markers. The development of molecular alternative biomarkers have been demanded to define the cause for occurrence, mechanism and pathological phenotypes of related diseases. Several significant genes have been identified through proteomic approach using microarray and quantitative real time PCR tools, and gene expression for up-regulated and down-regulated proteins especially in model of scrapie infected mouse brain [10-12]. Clq beta polypeptide, Cathepsin D, Cystatin C, Glial fibrillary acidic protein (GFAP), Clusterin, Peroxiredoxin-6 and EAA-2, and S-Acetyltransferase, Syaptotagmin 1 or 5, Ubiquitin-conjugating enzyme were reported as up-regulated proteins and down-regulated proteins, respectively [13-16]. Mass spectrometry (MS)-based proteomics is a powerful tool for large-scale identification of peptides in protein complexes from cell lysates and tissue extracts. In addition, liquid chromatography-tandem MS (LC-MS/MS) is a high-throughput, highly sensitive method that requires only very low sample volumes [17], which enables its application to systems biology approaches such as in the investigation of signaling pathways and interrelationships among proteins [18]. Although 2D-polyacrylamide gel electrophoresis (PAGE) has been widely used in the past to compare the relative abundances of proteins, this method has limitations, including low sensitivity and reduced resolution of proteins with extreme molecular weights and or pI values. We profiled the proteome of ME7 scrapie-infected mouse brains and analyzed the interaction and function of selected proteins to develop their potential novel biomarkers for the differential diagnosis in prion diseases. In this study, we analyzed candidate protein biomarkers expressed in the brains of control and ME7 scrapie-infected mice using 1D-Gel-LC-MS / MS analysis (Figure 1).

This approach may be useful for application of development biomarkers for diagnosis of human prion diseases through the specifying and confirmatory assay using body fluid as well as tissue. We also anticipate that these proteins could be used for ante-mortem diagnosis, prognosis, and therapeutic development.

Materials and Methods
Prion (PrPSc) inoculations
Four-week-old male C57BL / 6 mice (n = 3) were inoculated

*Corresponding author: Su Yeon Kim, Division of Zoonoses, Center for Immunology and Pathology, Korea National Institute of Health, Centers for Disease Control and Prevention, Cheongju-si, Chungcheongbuk-do, 28159, Republic of Korea, Tel: +82 43 719 8463; Fax: +82 43 719 8459; E-mail: tenksy@korea.kr
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intracerebrally with 20 µL of 10% brain homogenate from an ME7 scrapie-infected mouse. Age- and gender-matched control male mice (n = 3) were inoculated intracerebrally with 20 µL of 10% brain homogenate from a normal mouse. Mice were monitored for clinical symptoms for up to 171 days post inoculation (dpi). All animal experiments were performed in the bio safety 2 level facility. Legal compulsory education is required for all researchers and users annually (more than 6 h/year). Animals were investigated twice per week after inoculation until the appearance of abdominal behavior and then examined daily.

Preparation of mouse brain samples

Brain homogenates from three infected mice and three control mice were prepared using a Precellys® 24 homogenizer (Bertin Technologies, Rockville, MD, USA) at 6,500 rpm for 35 sec (twice) in phosphate-buffered saline (PBS). Individual samples were diluted to 10% (w/v) with PBS and stored at -80°C until use. The 10% homogenates were lysed in 500 µL of radioimmunoprecipitation assay buffer (Thermo Scientific, Rockford, IL, USA) using a Precellys® 24 homogenizer at 6,500 rpm for 35 sec (twice). The homogenates were centrifuged at 14,000 rpm for 3 h at 4°C, the supernatant was discarded, and the pellet was resuspended in 2x lithium dodecyl sulfate sample loading buffer (Invitrogen, Carlsbad, CA, USA) for analysis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for LC-MS / MS

SDS-PAGE was performed using NuPAGE® Novex 4% – 12% Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) with 2-(N-morpholino)ethanesulfonic acid running buffer at 100mA until the tracking dye reached the bottom of the gel. The proteins in the gel were visualized using the GelCode™ Blue Stain Reagent (Thermo Scientific).

In-gel digestion

Individual gel lanes were excised into 10 gel slices, so digestion was performed to total 60 slices of each 10 gel slices from three individual of control and infected groups, respectively. The gel slices were destained with 50% acetonitrile (ACN) in 50 mM ammonium bicarbonate buffer (pH 7.8) and washed with 100% ACN. The proteins in the gel slices were reduced with 10 mM dithiothreitol for 45 min, and then alkylated with 55 mM iodoacetamide for 30 min. Trypsin digestion was performed with 500 ng of Sequencing Grade Modified Trypsin (Promega, Madison, WI, USA) in 50 mM ammonium bicarbonate buffer overnight at 37°C. The digested peptides were extracted using 5% formic acid in ACN, and the extract was dried in a Speed Vac. For mass analysis, the dried peptides were dissolved in 6 µL of solubilization buffer containing 5% ACN, 0.4% formic acid, and 0.1% trifluoroacetic acid (final concentrations). After desalting with a zip-tip (Millipore, Billerica, MA, USA), the digested peptides were loaded onto a fused silica microcapillary C18 column (75 µm × 150 mm).

Liquid chromatography-tandem mass spectrometry analysis and protein identification

LC (UltiMate, nano flow LC, Dionex) was conducted with a linear gradient as follows: 0 min, 3% B; 5 min, 3% B; 72 min, 40% B; 77 min, 90% B; 87 min, 90% B; 92 min, 3% B; 120 min, 3% B. The initial solvent was 3% solvent B and the flow rate was 200 nL/min. Solvent A was 0.1% formic acid in H₂O and solvent B was 0.1% formic acid in ACN. The separated peptides were subsequently analyzed on a linear trap quadrupole ion-trap mass spectrometer (Thermo Fisher, San Jose, CA, USA). The electrospray voltage was set at 2.0 kV, and the threshold for switching from MS to MS/MS was 250. The normalized collision energy for MS / MS was 35% of the main radiofrequency amplitude and the duration of activation was 30 ms. All spectra were acquired in data-dependent mode. Each full MS scan was followed by three MS / MS scans corresponding to from the most intense peak to the third intense peak of the full MS scan. The repeat count of the peaks for dynamic exclusion was 1, and its repeat duration was 30 sec. The dynamic exclusion duration was set at 180s, and the exclusion mass width was ± 1.5 Da. The list size for dynamic exclusion was 50.

Statistical tests

Data was analyzed for statistical significance using two-tailed unpaired Mann-Whitney t-test 95% confidence interval.

Database searching and validation

All MS / MS spectra recorded were searched on mice protein database downloaded from the National Center for Biotechnology Information (NCBI, on January 21st, 2008; 35129 entries). SEQUEST was used as the peptide-searching program, and dynamic modifications of oxidized methionine (+16 Da) and carboxyamidomethylated cysteine (+57 Da) were permitted. SEQUEST criteria for peptide selection were Xcorr, which must be greater than 1.8, 2.3, and 3.5 for +1, +2, and +3 charge state peptides, respectively, and Cn above 0.1. The criterion for protein selection was a consensus score above 10.1. Functional groupings, such as gene ontology (GO) mapping, and protein-protein interactions of the identified proteins were analyzed using the web-based programs DAVID (http://david.abcc.ncifcrf.gov/) and Cytoscape (http://cytoscape.org/).

The acquired LC-electron spry ionization-MS/MS fragment spectra were searched against the NCBI (http://www.ncbi.nlm.nih.gov/)
non-redundant mouse database in the BioWorks Browser (version Rev. 3.3.1 SP1, Thermo Fisher Scientific, Inc., CA, USA) using the SEQUEST search engines. The search parameters included trypsin enzyme specificity, up to two permissible missed cleavages, peptide tolerance of ± 2 amu, mass error of ± 1 amu on fragment ions, and fixed modifications of carbamidomethylation of cysteine (+57 Da) and oxidation (+16 Da) of methionine residues. We performed the experiments in triplicate technically as three times running of MS / MS and three times repeatedly of analysis for the three sets of control and infected mice, and selected the proteins that were identified more than twice in the experiments. The two groups of proteins were then compared to identify control or ME7-infected specific proteins. To identify sample (control or ME7-infected mouse)-specific proteins, we utilized a label-free protein quantification method [19], and a protein was considered specific to a certain sample if its quantity was more than twice that in the other sample (in our quantification method, Rsc value > 1.0).

**Verification using western blot analysis**

The 10% brain homogenates (20 µL) were lysed in 1mL of radio immunoprecipitation assay buffer using a Precellys® 24 homogenizer at 6,500 rpm for 35 sec (twice). The homogenates were centrifuged at 14,000 rpm for 3 h at 4°C. The supernatant was discarded, and the pellet was resuspended in 2x lithium dodecyl sulfate sample loading buffer for analysis. The samples were boiled for 10min and separated by SDS-PAGE on NuPAGE Novex 4% – 12% Bis-Tris gels. Proteins were transferred to polyvinylidene fluoride membranes using iBlot® Gel Transfer stacks on an iBlot™ Gel Transfer system (Invitrogen, Carlsbad, CA, USA). The membranes were blocked with 5% nonfat milk in PBS containing 0.001% Tween-20 (PBST), and then incubated with specific antibodies against ACSBG1 (sc-130090, rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA, USA); NARS (14882-1-AP, rabbit polyclonal; ProteinTechGroup, Inc., Chicago, IL); and β-actin (#4967, rabbit polyclonal; Cell Signaling Technology) in PBST containing 0.5% skim milk for 2 h at room temperature. After three washes in PBST, membranes were incubated with a horseradishperoxidase-conjugated secondary antibody (#7074; Cell Signaling Technology) in PBST. After three washes in PBST, membranes were developed with SuperSignal® West FemtoChemiluminescent Substrate (Pierce, Rockford, IL, USA) and visualized using ChemDoc XRS (Bio-Rad).

**Results and Discussion**

We used 1D-Gel-LC-MS / MS to identify differentially expressed proteins in brains obtained from mice with neurological symptoms. All infected mice showed neurodegenerative symptoms approximately 120 dpi – 150 dpi; moving impairment and loss their direction showing circulation behavior repeatedly in cage. Control mice did not show prion-associated clinical symptoms even beyond 171dpi.

**Brain protein separation by 1D electrophoresis**

In the initial step, the proteins in ME7-scrapie-infected and control mouse brain homogenates were separated by SDS-PAGE. There were no visual differences in the protein band patterns between control and ME7-scrapie-infected mouse brain samples (Figure 2).

**LC-MS / MS and protein identification**

In a subsequent step, we digested the proteins in the separated brain samples with trypsin, and analyzed the tryptic peptides using LC-MS / MS. The proteins from LC-MS / MS were identified by comparing them with those in a peptide mass database, and were bioinformatically annotated based on their molecular weight, peptide score, spectral count, and other parameters in the NCBI and GO databases. In the control and infected samples, 3,924 (1,837 and 2,953 for control #1 and control #2, respectively; #3 was not used protein identification for experiment error.) proteins and 4,262 (1,580, 1,325, and 3,000 for ME7 #1, ME7 #2, and ME7 #3, respectively) proteins were identified, respectively. Many changes in protein levels were observed in ME7 scrapie-infected samples (Figure 3).

**Functional categorization by gene ontology**

We compared the protein expression profiles of infected and uninfected mouse brains using two controls and three ME7-scrapie-infected mice. First, we investigated the biological processes using protein identification data. Proteins identified in the control samples (with peptide scores in control samples 2-fold higher than that in infected samples and being detected only in control samples) were generally associated with localization (GO: 0051179, p-value < 0.05), transport (GO: 0006810, p-value < 0.05), establishment of localization (GO:0016192, p-value < 0.05). The three major categories in biological process ontology were localization, transport, and establishment of localization. Proteins identified in the infected samples (with peptide scores in infected samples 2-fold higher than that in control samples and being detected only in infected samples) were generally associated with generation of precursor metabolites and energy (GO: 0006091,
Differentially regulated brain proteins

Seven proteins with average scores greater than 20.0 and spectral counts of 3.0 among the proteins expressed in control mice with and with spectral count ratios greater than 2.0 among the infected mice were selected and classified as downregulated. We selected 36 proteins that were remarkably upregulated in the ME7-infected samples (Table 3). Among these, we found six proteins associated with neuronal processes such as transmission of nerve impulse, synaptic transmission, regulation of neurotransmitter levels, cell-cell signaling, neurotransmitter transport, neurotransmitter uptake, and neurological control of breathing. Proteins involved in neurological processes, including 4-aminobutyrate aminotransferase, ATPas, Na+ / K+ transporting, alpha 2 polypeptide, glutaminase isoform 1, myosin VI, and synapsin II isoform IIb, were upregulated, while sepiapterin reductase was downregulated.

These results showed that significantly more proteins were identified in the infected mouse brains than in the controls.

Neurodegenerative diseases associated with proteins

The 43 differentially regulated proteins was uploaded into Michigan Molecular interactions Cytoscape plug-in and a network was generated.

### Table 1: Protein Identification

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Table 1: The 165 differentially expressed proteins selected preferentially over score 10.1 and increased by more than 2-fold compared with infected mice based on the Gene Ontology and Cytoscape with p-values less than 0.05.

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Table 2: The 152 differentially expressed proteins selected preferentially over score 10.2 and increased by more than 2-fold compared with control based on the Gene Ontology and Cytoscape with p-values less than 0.05.
using the “Query genes + nearest neighbors” option. These networks consisted of 241 nodes and 4085 edges. We focused on neurodegenerative diseases associated with proteins of the Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/) pathway: Alzheimer’s disease [mmu05010], Parkinson’s disease [mmu05012], amyotrophic lateral sclerosis [mmu05014], Huntington’s disease [mmu05016], and prion disease [mmu05020]. The proteins related to neurodegenerative diseases were 17, such as Gfap, Apoe, Ncor1, Prnp, Bcl2, Calm3, Grb2, Apbb1, Calm1, Calm2, Uba1, Hspa5, Aplp1, Crebbp, Ppp3ca, Cat, and Trp53. Among these, the seeds proteins were Gfap, Prnp, Uba1, and

**Table 3:** Summary of the biological processes associated with the differentially regulated proteins in ME7 scrapie-infected mouse brains. On the basis of the host response to the scrapie agent, we selected 36 up-regulated and 7 down-regulated proteins as potential biomarkers.

**Figure 4:** Confirmation of acyl-CoA synthetase bubblegum family member 1 (ACSBG1) (A) and asparaginyl-tRNA synthetase (NARS) (B) expressed by western blotting including corresponding bar graphs of them below. Brain homogenates were analyzed with antibodies against ACSBG1 (1:200) and NARS (1:2,000) based on the total loading amount. Expression levels were normalized to β-actin levels.
Hspa5. PRNP, which consisted of 241 nodes, was one of the interaction networks and included 20 proteins such as Syn2 acting direct interaction (network distance=1).

**Western blotting of acyl-CoA synthetase bubblegum family member 1 (ACSBG1) and asparaginyl-tRNA synthetase (NARS)**

Our proteomics results indicated that ACSBG1 and NARS are significantly increased in ME7-scrapie-infected mice. We conducted biological validation of ACSBG1 and NARS, which were not reported to interact with prion protein among 43 candidates. To validate our proteomic results, we analyzed their protein levels in mouse brain homogenates by western blotting. The predicted molecular size of ACSBG1 was approximately 80kDa. Western blotting showed that the levels of ACSBG1 were significantly increased in ME7-scrapie-infected mice. NARS, a member of the class II aminoacyl-tRNAsynthetases, was observed as a distinct band at the appropriate molecular weight of 63kDa in ME7-scrapie-infected mouse brain (Figure 4).

**Conclusion**

Proteomics is a powerful method for the study of protein expression pattern and protein interactions in the blood, in particular the discovery and development of novel biomarkers for diagnosis of disease. We used proteomics to study the correlation between differentially expressed genes and their GO in scrapie-infected mice brains. Each gene was involved in one or more biological processes, and most candidate biomarkers were associated with cellular process, metabolic process, or cellular metabolic process. Many of these proteins are associated with neural processes, including cell-cell signaling, transmission of nerve impulse, and synaptic transmission. The control group included 19 proteins such as Sptbn4, Cadps, Slc17a7, Grin1, Musk, Ctnnb1, Glrb, Agtbp1, Vamp2, Nfl, Stx1a, Slc12a5, Sod1, Spr, Sv2b, Syn1, Celsr1, Cyb5r4, and Wnt6. The ME7-scrapie-infected group contained 12 proteins such as Snca, Gls, Myo6, Snap25, Abat, Dlg2, Syn2, Gna11, dac3, Atp1a2, Gja1, and Synj.

The differentially expressed proteins identified in this study that have not been previously reported as related to human prion diseases were those involved in metabolic processes (i.e., phosphofructokinase, muscle; acyl-CoA synthetase long-chain family member 6 isofrom 3; ACSBG1; glutamate oxaloacetate transaminase 2, mitochondrial; aconitase 2, mitochondrial; and ATP synthase, H+ transporting, mitochondrial F1 complex, beta subunit), glycolysis (i.e., hexokinase 1 and phosphofructokinase, platelet), glutamine catabolic process (i.e., glutaminase isoform 1), oxidation reduction processes (i.e., glutathione reductase [SRD family] member 1, and fatty acid synthase) and asparaginyl-tRNA aminocacylation and generic transcription (i.e., NARS), which is related to asparagine tRNA ligase activity and nucleic acid binding, and is affected in diseases such as inclusion conjunctivitis and filariasis.

Proteins with several major functions were identified in this study. Acyl-CoA synthetase is related to metabolic pathways and participates in gene expression. It may also play a role in aerobic respiration as an energy producer, and in the mitochondrial matrix as an electron carrier. ACSBG1 interacts with 14-3-3 beta (Yehab) and is an important paralog of acyl-CoA synthetase long-chain family member 6 isofrom 3. The lipidosis mouse homologue ACSBG1, which has long chain acyl-CoA synthetase activity, is exclusively expressed in the brain, adrenal gland, and testis, and is a key enzyme in the initial step of very-long-chain fatty acid β-oxidation. ACSBG1 is affected in neurodegenerative disorders such as human X-linked adrenoleukodystrophy [21], and in tuberculosis. X-linked adrenoleukodystrophy is associated with accumulation of very-long-chain fatty acids, and is related to metabolism and myelogenesis attributed to reduced peroxosomal β-oxidation. Asparaginyl-tRNA synthetase is classified as class-II aminoacyl-tRNAsynthetases, and is responsible for catalyzing the ligation of amino acids to their cognate tRNAs [22,23]. The etiology of various human diseases, including neuronal diseases, cancer, autoimmune diseases, and diabetes is connected to specific class-II aminoacyl-tRNAsynthetases, particularly affects neuronal diseases such as Charcot–Marie–Tooth disease, ataxia, amyotrophic lateral sclerosis, leukoencephalopathy and Parkinson’s disease [24].

Therefore, we suggest that ACSBG1 and NARS, which are related to metabolic and neurodegenerative disorders, can be applied as candidate biomarkers in the pre-mortem diagnosis for neuronal disorders.

In conclusion, we expect that our data may provide comprehensive information for significant proteins that are consistently differentially expressed in Prion diseases caused by amplification and aggregation of PrPSc though our study has the limitation in that numerous differentially expressed proteins were identified in the scrapie-infected mouse brain. The candidate biomarkers identified in this study might be useful for correlating responsiveness to experimental therapeutic or diagnostic regimens. Thus, clinical validation will be further investigated using quantitative assay and immunohistochemistry with high diagnostic specificity and sensitivity is necessary to confirm the expression of these candidate biomarkers to apply human prion diseases’ diagnosis. Moreover, the current approach will provide us new insight and help defining the change of physiological mechanism through understand of the protein interaction or function and to develop therapeutic strategies in the field of neurodegenerative diseases.

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**References**