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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^{c}) to an infectious PrP^{Sc} isoform. TSEs include scrapie, bovine spongiform encephalopathy, transmissible mink encephalopathy, Creutzfeldt-Jakob disease (CJD), Gerstmann–Sträussler–Scheinker syndrome, fatal familial insomnia, and kuru. The PrP^{Sc} isoform is more proteinase K-resistant than the PrP^{c}. Detection of PrP^{Sc} in the central nervous tissue of CJD patients by immunohistochemistry and confirmation of protease-resistant PrP^{Sc} 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 EAAT-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-polycrylammide 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 (PrP^{Sc}) 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 8489; E-mail: tenksy@korea.kr

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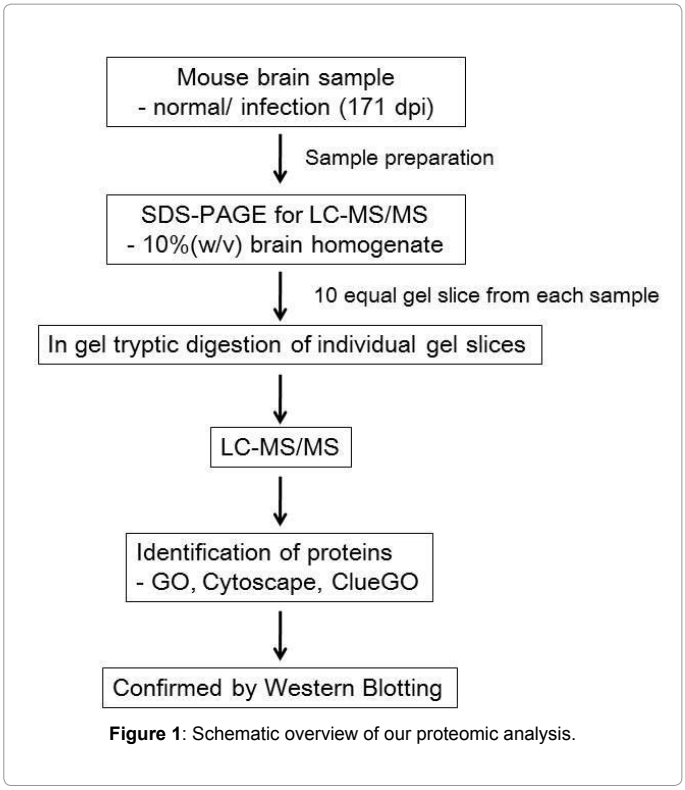

Figure 1: Schematic overview of our proteomic analysis.

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; 87min, 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 H_2O 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 mice)-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 ME7scrapie-infected and control mouse brain homogenates were separated by SDS-PAGE. There were no visual differences in the protein band patterns between control and ME7scrapie-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 ME7scrapie-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: 0051234, p-value < 0.05), cellular component organization and biogenesis (GO:0016043, p-value < 0.05), and vesicle-mediated transport (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,

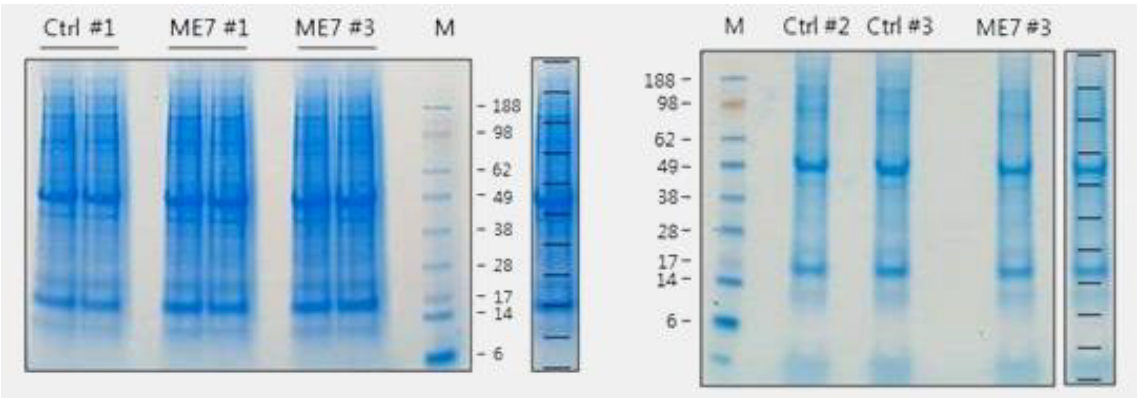

Figure 2: SDS-PAGE analysis control and ME7 infected mouse brain (Ctrl, Control: three (#1~#3); ME7, infected: three (#1~#3). Marked ten gel lanes excised into gel slices from three control and infected groups used in gel digestion on right side, respectively.

p-value < 0.05), transport (GO:0006810, p-value < 0.05), establishment of localization (GO:0051234, p-value < 0.05), localization (GO:0051179, p-value < 0.05), cellular metabolic process (GO:0044237, p-value < 0.05), carboxylic acid metabolic process (GO:0019752, p-value < 0.05), organic acid metabolic process (GO:0006082, p-value < 0.05), and metabolic process (GO:0008152, p-value < 0.05).The four major categories in biological process ontology were cellular process (GO: 0009987, p-value < 0.05), metabolic process, cellular metabolic process, and primary metabolic process (GO: 0044238, p-value < 0.05).In addition, several specific neuronal processes were enriched.

On the basis of the analysis with ClueGO [20], 2-fold up protein clusters involved three major biological processes such as glutamine family amino acid catabolic process, which included five proteins (i.e., argininosuccinate synthetase 1; glutamate oxaloacetate transaminase 2, mitochondrial; AU RNA binding protein/enoyl-coenzyme A hydratase; glutaminase; and aldehyde dehydrogenase family 6, subfamily A1); complement activation, a classical pathway which included three proteins (i.e., complement component 1, q subcomponent, alpha polypeptide; complement component 1, q subcomponent, beta polypeptide; and complement component 1, q subcomponent, C chain); and ATP biosynthetic process, which included six proteins (i.e., ATPase, Na+ / K+ transporting, alpha 2 polypeptide; ATPase, Ca++ transporting, plasma membrane 3; ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1; ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit; ATPase, H+ transporting, lysosomal V1 subunit A; and ATPase, H+ transporting, lysosomal V1 subunit B2). The same procedure revealed 17 2-folds down clusters such as regulation of neurotransmitter levels, exocytosis, and vesicle docking during exocytosis, and contained proteins such as syntaxin 1A (brain), reticulon 4, neurofibromatosis 1, and neurofilament, medium polypeptide. A total of 317 differentially expressed proteins with p-values less than 0.05 were selected. 165 proteins showed only to control group and defined increasing more than twice comparing infected group (down), 152 proteins showed only to infected group and defined increasing more than twice comparing control group (up). The identification of Gfap, Hspa5, Vim, chaperonin, Ywhaz, and Syn1 was highly significant because they are known to interact with PrP and are involved in disease progression (Tables 1 and 2).

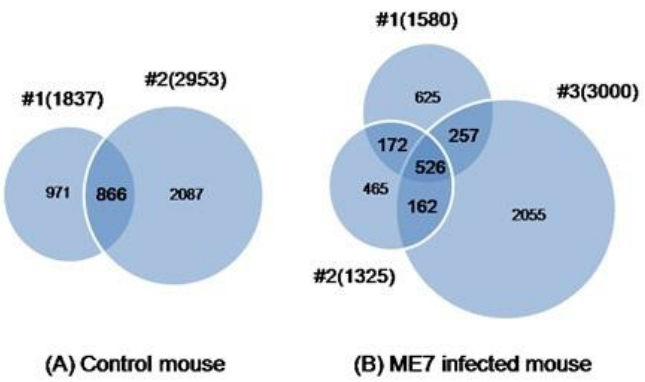

Figure 3: Venn diagram showing the number of unique and shared proteins from each individual mouse. Venn diagram of the proteins identified by one-dimensional gel electrophoresis-liquid chromatography-tandem mass spectrometry (1D-Gel-LC-MS/MS) in ME7 scrapie-infected and control mice. (A) Two control mice (#1, #2). (B) Three ME7 scrapie-infected mice (#1, #2, #3).

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 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, ATPase, Na+ / K+ transporting, alpha 2 polypeptide, glutaminase isoform 1, myosin VI, and synapsin II isoform IIb, were upregulated, while sepiapterinreductase 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

GI_number	Protein name	Average		Control #1 (control 11-20)		Control #2 (control 21-30)	
		Score 11-30	Spectral count11-30	Score 11-20	Spectral count 11-20	Score 21-30	Spectral count 21-30
6671565	adaptor-related protein complex 3, delta 1 subunit	20.20	2.5	20.21	2	20.19	3
6671600	solute carrier family 7 (cationic amino acid transporter, y+ system), member 3	10.16	1.5	10.15	1	10.16	2
6671684	catenin (cadherin associated protein), beta 1, 88 kDa	10.16	1	10.18	1	10.14	1
6678551	vesicle-associated membrane protein 2	15.21	1.5	10.22	1	20.20	2
6678553	vesicle-associated membrane protein 3	15.24	2.5	10.25	1	20.24	4
6679337	phosphatidylinositol transfer protein, alpha	40.22	5	40.18	4	40.27	6
6679379	phospholipase D2	10.12	1	10.12	1	10.12	1
6679597	kinesin family member 20A	10.13	1	10.15	1	10.12	1
6680095	glutamate receptor, ionotropic, NMDA1 (zeta 1)	10.13	1	10.13	1	10.12	1
6680560	kinesin family member 2A	10.12	1	10.12	1	10.12	1
6680722	ADP-ribosylation factor 5	30.14	5.5	30.15	7	30.14	4
6681233	solute carrier family 26 (sulfate transporter), member 2	10.14	1	10.13	1	10.14	1
6753144	ATPase, H+ transporting, lysosomal V0 subunit C	25.18	2.5	10.18	1	40.19	4
6753172	B-cell leukemia / lymphoma 6	10.13	1	10.12	1	10.14	1

6753912	ferritin heavy chain 1	15.18	1.5	20.19	2	10.17	1
6754426	potassium inwardly rectifying channel, subfamily J, member 11	10.11	2	10.10	2	10.13	2
6755040	profilin 1	10.15	1	10.18	1	10.12	1
6755448	SEC22 vesicle trafficking protein-like 1	30.19	3	40.23	4	20.15	2
7106303	EH-domain containing 1	15.14	2	10.12	1	20.17	3
7710086	RAB10, member RAS oncogene family	15.17	2	10.17	1	20.17	3
8394392	synaptotagmin V	15.14	2	20.14	2	10.14	2
8567336	chloride channel calcium activated 3	10.13	1.5	10.14	2	10.12	1
9506971	profilin 2	25.16	5	20.15	3	30.16	7
9790237	SMC1 structural maintenance of chromosomes 1-like 1	10.12	1	10.12	1	10.12	1
9910164	Double cortin-like kinase 1 isoform 1	10.20	1.5	10.16	2	10.25	1
9937988	secretory carrier membrane protein 5	10.17	1	10.17	1	10.17	1
12963633	NADH dehydrogenase (ubiquinone) 1 alpha sub-complex, 13	15.15	2	20.16	2	10.14	2
13385006	cytochrome c-1	10.18	1.5	10.18	1	10.18	2
13385054	NADH dehydrogenase (ubiquinone) 1 beta sub-complex 3	15.12	2	20.12	2	10.12	2
13385492	NADH dehydrogenase (ubiquinone) 1 alpha sub-complex, 6 (B14)	20.15	3.5	20.17	4	20.14	3
13386054	actin related protein 2 / 3 complex, subunit 4	20.16	2.5	30.18	3	10.14	2
13470090	RAB3C, member RAS oncogene family	15.23	2	10.24	1	20.22	3
15011853	syntaxin 1A (brain)	25.16	2.5	30.17	3	20.15	2
16716499	sideroflexin 3	30.22	3	30.20	3	30.24	3
19526968	tubulin, gamma complex associated protein 2	10.15	1.5	10.12	1	10.18	2
19527256	DEAD (Asp-Glu-Ala-Asp) box polypeptide 1	10.13	1	10.13	1	10.12	1
21312950	NADH dehydrogenase (ubiquinone) Fe-S protein 7	10.15	2	10.18	3	10.12	1
21313226	anti-silencing function 1B	10.15	1	10.17	1	10.12	1
21314824	ATPase, H+ transporting, lysosomal V1 subunit F	10.13	1	10.14	1	10.12	1
21361250	ATPase, H+ transporting, lysosomal accessory protein 2	10.17	1.5	10.17	2	10.18	1
22550098	AGP7	10.13	2	10.13	1	10.14	3
27228985	13kDa differentiation-associated protein	15.13	1.5	20.14	2	10.13	1
27229051	NECAP endocytosis associated 1	10.16	1.5	10.14	1	10.19	2
28202057	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 19	15.14	2	10.10	1	20.18	3
28316750	histone cluster 1, H2ba	15.18	6.5	20.17	10	10.18	3
28916667	cytochrome b5 reductase 4	10.15	1	10.15	1	10.14	1
29244298	transient receptor potential cation channel, subfamily A, member 1	10.13	1	10.13	1	10.13	1
29789343	eukaryotic translation initiation factor 3, subunit 9	10.11	1	10.12	1	10.11	1
30061401	histone cluster 2, H3c1 isoform 2	40.13	12	40.12	13	40.14	11
30410760	collagen, type XVI, alpha 1	10.15	1.5	10.15	2	10.16	1
30520173	BCSC-1	10.12	1	10.12	1	10.12	1
31324569	neurocalcin delta	10.16	1	10.17	1	10.14	1
31543971	YKT6 v-SNARE protein	25.19	2.5	20.21	2	30.17	3
31980729	RAS protein activator like 1 (GAP1 like)	10.15	1	10.12	1	10.18	1
31981304	ATPase, H+ transporting, V0 subunit D isoform 1	50.20	5.5	40.15	5	60.25	6
31981754	glycine receptor, beta subunit	10.16	1.5	10.15	2	10.16	1
31981826	electron transferring flavoprotein, alpha polypeptide	10.18	1.5	10.19	2	10.18	1
31982223	laminin, beta 2	25.18	2.5	30.20	3	20.16	2
31982236	integrin alpha 6	10.16	1	10.14	1	10.18	1
31982300	hemoglobin, beta adult major chain	55.22	16	70.23	23	40.20	9
31982452	collagen, type IX, alpha 1	10.18	1.5	10.17	1	10.18	2
32401469	kinesin family member 27	10.12	1	10.12	1	10.12	1
33859827	solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 7	10.15	2	10.16	1	10.14	3
34328045	procollagen, type IV, alpha 4	10.15	1.5	10.14	1	10.17	2
34328138	kinesin family member 3A	10.12	1	10.12	1	10.13	1
34328158	wingless-related MMTV integration site 6	10.14	1.5	10.13	1	10.16	2
34538601	cytochrome c oxidase subunit II	10.14	1	10.12	1	10.15	1
34878892	neurofibromin	35.17	6.5	30.15	4	40.20	9
38604071	exportin 1, CRM1 homolog	15.13	2	10.12	2	20.14	2

40254228	solute carrier family 39 (zinc transporter), member 10	10.15	1	10.15	1	10.14	1
40254514	GTPase activating protein and VPS9 domains 1	10.16	1.5	10.18	1	10.14	2
40254624	platelet-activating factor acetylhydrolase, isoform 1b, alpha2 subunit	10.14	1.5	10.15	2	10.14	1
40353214	suppressor of zeste 12 homolog	10.13	1.5	10.13	1	10.13	2
41054950	collagen, type VIII, alpha 2	10.14	1	10.11	1	10.16	1
41281837	chloride channel 3 isoform c	10.17	1	10.13	1	10.20	1
41281852	amphiphysin	30.21	3.5	20.21	2	40.21	5
41350312	nascent polypeptide-associated complex alpha subunit isoform b	15.23	2	10.24	2	20.23	2
45476581	sterol carrier protein 2, liver	10.14	1	10.15	1	10.13	1
45597447	superoxide dismutase 1, soluble	15.14	1.5	20.15	2	10.13	1
46195430	NADH dehydrogenase (ubiquinone) Fe-S protein 8	10.17	1	10.19	1	10.15	1
46559412	protein kinase C and casein kinase substrate in neurons 1	10.17	1	10.16	1	10.17	1
46849724	VPS9-ankyrin repeat-containing protein isoform 2	10.13	1	10.14	1	10.12	1
47604978	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 13	10.12	1	10.12	1	10.12	1
51491850	Rho guanine nucleotide exchange factor (GEF) 11	10.12	1	10.12	1	10.12	1
58037109	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10	15.15	2	10.14	1	20.16	3
58037145	small nuclear ribonucleoprotein D2	10.21	2.5	10.24	3	10.18	2
60460915	regulating synaptic membrane exocytosis 1 isoform 2	10.12	1	10.12	1	10.12	1
61102728	inositol 1,4,5-triphosphate receptor 3	15.17	2	20.18	3	10.15	1
62990166	structural maintenance of chromosomes 2-like 1	10.13	1	10.12	1	10.13	1
68341935	microtubule-associated protein 2 isoform 2	15.16	2.5	10.20	3	20.12	2
70906474	Ca < 2+_dependent activator protein for secretion isoform 1	10.22	1	10.19	1	10.25	1
70906477	Calcium / calmodulin-dependent protein kinase II, delta isoform 2	35.20	5	40.17	6	30.24	4
73622271	absent, small, or homeotic discs 1	10.18	2	10.16	1	10.21	3
77812697	titin isoform N2-A	10.16	1.5	10.12	1	10.20	2
78000173	sorbin and SH3 domain containing 1 isoform 2	10.16	1	10.14	1	10.17	1
82533043	skeletal muscle receptor tyrosine kinase isoform 1 precursor	10.15	1	10.13	1	10.18	1
83523732	slit homolog 3	10.12	1.5	10.11	1	10.13	2
84781802	ATPase, aminophospholipid transporter (APLT), class I, type 8A, member 1 isoform a	10.13	1	10.14	1	10.12	1
85540453	pleckstrin and Sec7 domain containing 3 isoform 2	10.15	1.5	10.12	1	10.17	2
90903233	glutathione peroxidase 4 isoform 1 precursor	15.13	1.5	20.14	2	10.13	1
94398520	PREDICTED: similar to vacuolar protein sorting 13B isoform 5	10.14	1	10.12	1	10.17	1
106507168	RAB12, member RAS oncogene family	10.17	1	10.20	1	10.14	1
110225337	ATPase, H+ / K+ exchanging, gastric, alpha polypeptide	10.14	1	10.16	1	10.12	1
110625645	sortilin-related receptor, LDLR class A repeats-containing	10.14	1	10.16	1	10.13	1
110625819	hypothetical protein LOC268739	10.14	4	10.13	3	10.15	5
111955376	lysosomal trafficking regulator	10.12	1	10.12	1	10.12	1
112363107	neurofilament 3, medium	80.24	30	80.24	25	80.24	35
114155137	dynein, axonemal, heavy chain 5	10.13	1	10.13	1	10.13	1
114158695	ATP / GTP binding protein 1 isoform 1	10.14	1	10.14	1	10.13	1
115648153	cadherin EGF LAG seven-pass G-type receptor 1 precursor	10.12	1.5	10.12	1	10.12	2
116089327	a disintegrin and metalloprotease domain 2 (fertilin beta)	10.13	1	10.13	1	10.14	1
116089329	Snap-25-interacting protein	15.21	2.5	10.15	1	20.27	4
116174793	spectrin beta 4	10.16	2	10.15	1	10.16	3
116256491	ankyrin 3, epithelial isoform a	35.18	5	30.18	4	40.18	6
117414174	sodium channel, voltage-gated, type VIII, alpha	10.12	1	10.12	1	10.12	1
117956385	RUN and TBC1 domain containing 1	10.13	1	10.13	1	10.13	1
118403316	hypothetical protein LOC235461	10.13	1	10.12	1	10.14	1
119433657	histone cluster 2, H2ab	10.12	1	10.12	1	10.12	1
120587003	TBC1 domain family, member 1	10.13	1	10.13	1	10.13	1

121583653	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 18	10.17	3.5	10.20	3	10.14	4
122114537	vacuolar protein sorting 13C	20.13	2.5	30.15	3	10.12	2
124486650	SLIT-ROBO Rho GTPase activating protein 1	10.13	1.5	10.14	2	10.13	1
124486692	integrin, alpha 10	10.14	1	10.12	1	10.15	1
124487141	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 3	10.14	1.5	10.12	1	10.15	2
124487229	breakpoint cluster region homolog	15.15	1.5	10.12	1	20.18	2
124487372	low density lipoprotein receptor-related protein 2	15.17	2	20.14	2	10.19	2
124487493	signal-induced proliferation-associated 1 like 2	10.12	1	10.13	1	10.11	1
130502228	hypothetical protein LOC320484	10.14	1	10.12	1	10.15	1
131889984	sodium channel, voltage-gated, type X, alpha	10.13	2	10.13	3	10.13	1
133505571	M-phase phosphoprotein 1	10.14	1.5	10.14	1	10.14	2
134031976	leucine-rich PPR motif-containing protein	55.24	6.5	40.25	5	70.22	8
134053947	bridging integrator 1 isoform 2	10.13	1.5	10.12	1	10.15	2
144922716	common-site lymphoma / leukemia guanine nucleotide exchange factor isoform a	10.13	1	10.14	1	10.12	1
145275191	ATP-binding cassette, sub-family C, member 6	10.14	1	10.13	1	10.14	1
145587082	triple functional domain (PTPRF interacting)	10.12	1	10.13	1	10.12	1
146231960	chondroitin sulfate proteoglycan 4	10.12	1	10.13	1	10.12	1
146231996	HECT, UBA and WWE domain containing 1	10.13	2	10.14	3	10.12	1
147902443	kinesin family member 26A	10.14	1	10.13	1	10.14	1
148747179	lymphocyte antigen 75	10.12	1	10.12	1	10.12	1
149254026	PREDICTED: SEC31-like 1	10.14	1	10.15	1	10.13	1
149254379	PREDICTED: similar to hCG1811879	15.15	3	20.15	3	10.15	3
149255369	PREDICTED: IQ motif and Sec7 domain 1	10.12	1	10.12	1	10.12	1
149255409	PREDICTED: jumonji, AT rich interactive domain 1A (Rbp2 like) isoform 1	15.12	1.5	10.12	1	20.12	2
149260924	PREDICTED: jumonji domain containing 1C	10.14	1	10.14	1	10.13	1
153792247	low density lipoprotein-related protein 1B	10.18	2	10.18	1	10.17	3
154146254	insulin related protein 2 (islet 2)	10.13	2	10.14	1	10.13	3
157823695	kinesin family member 21A isoform 3	10.22	1	10.22	1	10.22	1
157909825	synaptic vesicle glycoprotein 2 b	15.13	2	20.13	3	10.13	1
158711686	solute carrier family 12, member 5	20.15	3.5	10.15	2	30.15	5
159110614	FYVE and coiled-coil domain containing 1	10.13	1	10.14	1	10.13	1
159110663	integrin alpha 2b	10.15	1	10.19	1	10.12	1
160333276	intersectin 1 isoform 2	10.17	1	10.14	1	10.19	1
160333789	sepiapterin reductase	45.20	4.5	40.15	4	50.24	5
160333877	kinesin family member 1A isoform b	20.18	2	20.19	2	20.17	2
160358856	integrin beta 3 precursor	20.15	2.5	20.14	2	20.16	3
160707901	synapsin I isoform a	95.25	34	100.25	24	90.24	44
160707911	ankyrin 1, erythroid isoform 2	35.19	4	50.18	5	20.21	3
160707971	adaptor-related protein complex 3, sigma 2 subunit	20.19	2	20.19	2	20.18	2
161016797	non-SMC condensin II complex, subunit D3	10.13	1	10.14	1	10.12	1
161016826	receptor accessory protein 5	15.14	3	20.16	4	10.13	2
161086984	adaptor-related protein complex 2, sigma 1 subunit	30.18	6.5	20.19	5	40.17	8
161353508	chromobox homolog 4	10.13	1.5	10.12	1	10.14	2
161702988	apolipoprotein B precursor	10.16	3.5	10.13	2	10.19	5
162139829	myelin protein zero	15.13	3	10.12	1	20.13	5
163965444	centrosomal protein 290	10.14	1.5	10.13	1	10.15	2

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.

GI_number	Protein name	Average		Infected #1 (1-10)		Infected #2 (11-20)		Infected #3 (21-30)	
		Score 1-30	Spectral count 1-30	Score 1-10	Spectral count 1-10	Score 11-20	Spectral count 11-20	Score 21-30	Spectral count 21-30
6671539	aldolase 1, A isoform	16.85	2	30.18	3	10.13	1	10.24	1
6671549	peroxiredoxin 6	16.83	2	20.16	2	10.13	1	20.20	2
6671664	calnexin	23.52	2	30.20	3	20.15	2	20.21	2
6671702	chaperonin subunit 5 (epsilon)	30.24	3	40.26	4	40.25	4	10.20	1
6677809	ribosomal protein S6	20.17	2	30.23	3	20.14	3	10.13	1

6678047	synuclein, alpha	10.15	1	10.11	1	10.16	1	10.17	1
6678483	ubiquitin-activating enzyme E1	110.24	16	130.23	19	120.22	14	80.26	14
6679243	phosphodiesterase 1B, Ca^{2+} - calmodulin dependent	10.17	1	10.16	1	10.20	1	10.16	1
6679345	protein kinase C, beta 1	23.48	2	40.17	4	10.12	1	20.15	2
6679935	growth associated protein 43	10.17	1	10.16	1	10.15	1	10.19	2
6680345	isocitrate dehydrogenase 3 (NAD+), gamma	20.19	2	30.22	3	20.15	2	10.20	1
6680720	ADP-ribosylation factor 4	13.47	2	20.14	2	10.13	1	10.15	2
6681069	cysteine and glycine-rich protein	36.88	6	50.23	7	20.17	3	40.23	7
6753220	complement component 1, q subcomponent, B chain	26.86	3	40.21	5	10.17	1	30.21	4
6753280	caspase 14	10.17	1	10.18	1	10.14	1	10.20	1
6753320	chaperonin subunit 3 (gamma)	40.16	4	70.20	8	30.17	3	20.12	2
6753322	chaperonin subunit 4 (delta)	36.87	5	40.17	6	50.21	6	20.22	2
6753324	chaperonin subunit 6a (zeta)	13.50	2	10.22	1	10.12	1	20.14	3
6753992	gap junction protein, alpha 1	26.86	7	30.19	10	20.18	5	30.23	5
6754004	guanine nucleotide binding protein, alpha 11	10.15	3	10.18	6	10.13	2	10.14	1
6754036	glutamate oxaloacetate transaminase 2, mitochondrial	43.52	5	60.23	6	40.18	4	30.14	4
6754086	glutathione S-transferase, mu 5	13.49	2	10.16	2	20.14	2	10.17	1
6754206	hexokinase 1	50.17	5	80.20	8	50.19	5	20.13	2
6754624	mitogen activated protein kinase kinase 5	10.17	7	10.16	7	10.18	9	10.18	4
6755002	programmed cell death 6 interacting protein	13.51	1	10.18	1	20.15	2	10.20	1
6755080	protein kinase C, gamma	73.59	10	120.26	16	40.21	6	60.29	9
6755256	muscle glycogen phosphorylase	20.15	2	20.15	2	30.12	3	10.17	1
6755372	ribosomal protein S3	46.82	6	60.18	9	40.15	6	40.13	4
6755588	synaptosomal-associated protein 25	13.48	1	10.16	1	10.12	1	20.16	2
6755809	talin 1	16.85	2	10.13	2	20.21	2	20.22	2
6755863	tumor rejection antigen gp96	60.20	10	80.20	13	60.19	10	40.22	7
6755911	thioredoxin 1	13.54	2	10.21	1	10.20	1	20.22	3
6755967	voltage-dependent anion channel 3	23.51	3	40.21	5	20.19	3	10.12	1
6996911	argininosuccinate synthetase	20.16	2	20.14	2	30.20	3	10.15	1
7305029	erythrocyte protein band 4.1-like 1 isoform a	16.88	3	20.22	3	10.20	2	20.22	3
8392847	ARP1 actin-related protein 1 homolog A	16.90	2	30.24	3	10.23	1	10.23	1
8393544	heterogeneous nuclear ribonucleoprotein C	16.83	2	30.19	3	10.13	1	10.16	1
8567410	synapsin II isoform IIb	36.90	7	70.28	14	20.18	3	20.24	5
9507023	Rab geranylgeranyl transferase, a subunit	10.15	2	10.14	2	10.13	3	10.19	1
9789991	hydroxysteroid (17-beta) dehydrogenase 12 protein	16.84	2	30.22	3	10.16	1	10.13	1
9790051	phosphofructokinase, platelet	50.24	6	60.25	9	50.25	6	40.22	4
9790055	mitochondrial carrier homolog 2	23.50	2	50.23	5	10.16	1	10.12	1
10946940	RAB2, member RAS oncogene family	16.82	2	30.19	3	10.12	1	10.14	1
11230802	actinin alpha 4	63.55	8	80.22	10	70.21	9	40.23	6
12963511	ribosomal protein S19	10.14	2	10.11	1	10.15	3	10.15	2
12963737	chromosome segregation 1-like	23.51	3	40.24	4	20.15	2	10.14	2
13173473	prion protein	66.86	14	70.26	19	60.16	9	70.17	15
13385998	TNF receptor-associated protein 1	20.21	2	30.24	3	10.16	1	20.22	2
13928670	vacuolar protein sorting 35	23.53	2	30.20	3	30.19	3	10.20	1
14149645	methyl CpG binding protein 2 isoform 2	13.47	2	10.15	2	20.12	3	10.13	1
16716465	acyl-CoA synthetase bubblegum family member 1	70.20	7	90.23	9	60.18	7	60.19	6

18079339	aconitase 2, mitochondrial	63.55	7	100.24	11	40.21	5	50.21	5
18266680	3-oxoacid CoA transferase 1	13.50	1	20.21	2	10.17	1	10.13	1
19705578	vacuolar H+ ATPase B2	36.84	6	40.17	6	60.19	10	10.16	2
21311845	solute carrier family 25 (mitochondrial carrier, glutamate), member 22	23.53	3	30.20	3	10.16	1	30.22	4
21313308	heterogeneous nuclear ribonucleoprotein M isoform a	10.14	1	10.14	2	10.13	1	10.16	1
21426889	ribosomal protein S11	13.45	1	20.12	2	10.10	1	10.14	1
21704020	NADH dehydrogenase (ubiquinone) Fe-S protein 1	30.22	3	50.25	5	20.21	2	20.20	3
21704162	ubiquitin-conjugating enzyme E2M	23.47	2	40.15	4	20.13	2	10.12	1
21704242	CaM kinase-like vesicle-associated	50.22	7	100.25	13	30.22	3	20.19	4
21746161	tubulin, beta	10.18	2	10.19	2	10.17	2	10.18	3
22267442	ubiquinol cytochrome c reductase core protein 2	20.19	2	30.22	3	20.22	2	10.13	1
23943838	solute carrier family 25, member 1	13.48	1	20.18	2	10.14	1	10.13	1
24233554	solute carrier family 1 (glial high affinity glutamate transporter), member 3	10.21	2	10.26	2	10.21	2	10.15	2
24418919	brain glycogen phosphorylase	60.22	6	70.23	8	80.23	8	30.20	3
24429570	exportin 5	10.13	1	10.12	1	10.14	2	10.13	1
25742730	ribosomal protein L32	10.22	1	10.23	1	10.22	1	10.21	1
27369581	solute carrier family 25 (mitochondrial carrier, Aralar), member 12	120.24	14	160.26	19	110.22	13	90.22	10
27369896	transcriptional regulating factor 1 isoform 2	10.16	1	10.14	1	10.13	1	10.21	1
29336026	myosin, heavy polypeptide 14	20.20	3	20.16	4	20.18	2	20.24	4
29789191	asparaginyl-tRNA synthetase	30.18	3	60.24	6	20.16	2	10.13	1
29789199	RAN binding protein 5	20.17	3	20.16	3	10.15	1	30.20	5
30409956	ATPase, Na+ / K+ transporting, alpha 2 polypeptide	106.88	41	140.23	54	100.19	40	80.21	28
30425158	zinc finger protein 526	10.13	1	10.14	1	10.13	1	10.13	1
31542608	elongation protein 4 homolog	10.13	1	10.14	1	10.13	1	10.12	1
31543797	synaptotagmin II	26.86	3	50.19	5	20.16	3	10.21	1
31560433	sorting nexin 3	10.15	1	10.16	1	10.14	1	10.15	1
31560731	ATPase, H+ transporting, lysosomal V1 subunit A	83.56	11	160.27	20	80.20	9	10.20	3
31980648	ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit	106.86	22	120.21	24	120.21	26	80.17	16
31980844	Dehydrogenase / reductase (SDR family) member 1	36.88	4	60.26	7	10.14	1	40.25	4
31981185	phosphofructokinase, muscle	86.93	11	110.29	12	80.23	13	70.26	7
31981237	thimet oligopeptidase 1	10.16	1	10.17	1	10.12	2	10.17	1
31981722	heat shock protein 5	36.85	4	70.21	7	20.14	2	20.21	2
31981769	glycerol phosphate dehydrogenase 2, mitochondrial	10.15	1	10.19	1	10.12	1	10.12	2
31982178	malate dehydrogenase 1, NAD (soluble)	33.50	3	60.17	6	20.12	2	20.19	2
31982186	malate dehydrogenase 2, NAD (mitochondrial)	90.22	14	90.20	12	110.17	18	70.28	11
31982332	glutamine synthetase	20.17	2	30.20	3	10.14	1	20.16	2
31982497	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c, isoform 1	10.11	3	10.11	3	10.11	3	10.10	2
31982664	inosine triphosphatase	20.15	2	30.17	3	20.14	2	10.13	1
31982755	vimentin	106.88	24	110.24	26	140.22	23	70.17	24
31982856	dihydrolipoamide dehydrogenase	13.50	1	10.17	1	20.17	2	10.15	1
32567788	phosphatidylinositol-binding clathrin assembly protein	20.18	3	30.17	5	20.17	3	10.20	2
33859474	complement receptor 2	10.12	2	10.11	1	10.12	4	10.12	1

33859811	mitochondrial trifunctional protein, alpha subunit	73.55	8	100.24	11	70.19	8	50.22	6
34740335	tubulin, alpha 1B	13.56	7	10.15	8	20.18	5	10.35	8
36031132	ATPase, Ca^{++} transporting, fast twitch 1	16.83	3	20.17	3	20.19	3	10.14	3
37202121	4-aminobutyrate aminotransferase	30.20	4	30.19	4	50.20	6	10.20	1
38372907	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39	20.14	3	30.15	3	10.14	1	20.12	4
39930477	septin 8	20.19	2	30.23	3	20.20	2	10.16	1
40254635	kinesin family member 5A	23.56	3	20.25	2	30.20	3	20.23	3
52353955	3-phosphoglycerate dehydrogenase	46.87	5	40.17	4	50.22	6	50.22	6
56699478	plasma membrane calcium ATPase 3	20.17	2	10.12	1	10.16	1	40.23	4
58037481	sec1 family domain containing 1	10.17	1	10.16	1	10.16	2	10.19	1
61097906	actinin, alpha 1	56.87	6	60.24	6	70.19	8	40.20	4
61888842	partitioning-defective protein 3 homolog isoform 2	10.16	2	10.15	1	10.16	2	10.17	3
67763830	zinc finger protein 62 isoform 1	10.12	1	10.13	1	10.12	1	10.12	1
71725385	DIRAS family, GTP-binding RAS-like 2	13.52	1	10.21	1	20.16	2	10.19	1
71774133	peptidylprolyl isomerase B	20.20	2	30.20	3	10.21	1	20.18	2
75992915	acyl-CoA synthetase long-chain family member 6 isoform 3	70.23	7	90.24	9	50.23	5	70.23	8
75992920	acyl-CoA synthetase long-chain family member 3	23.50	2	50.21	5	10.13	1	10.16	1
82891441	PREDICTED: similar to DnaJ (Hsp40) homolog, subfamily B, member 14 isoform 8	10.13	1	10.12	1	10.17	1	10.11	1
83776571	protein kinase C-binding protein NELL1	10.17	1	10.16	1	10.15	1	10.19	1
83816893	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	20.24	3	40.24	4	10.17	2	10.30	2
83921618	villin 2	16.84	2	20.18	3	20.19	2	10.14	1
84000448	glial fibrillary acidic protein	236.90	116	280.25	141	250.22	99	180.23	108
85861218	guanine monphosphate synthetase	16.85	2	10.17	1	10.18	1	30.20	4
87298845	calmodulin binding protein 1	10.13	2	10.12	2	10.13	2	10.14	1
88196800	myosin VI	30.21	4	20.20	3	20.21	3	50.22	5
88853578	adaptor protein complex AP-1, beta 1 subunit	90.24	20	100.26	23	110.21	26	60.24	10
91992157	AP2 associated kinase 1 isoform 1	10.15	1	10.12	1	10.19	2	10.14	1
93102409	fatty acid synthase	150.22	18	140.21	14	150.21	17	160.24	22
93102417	glycyl-tRNA synthetase	13.53	1	20.17	2	10.17	1	10.25	1
110625886	alpha isoform of regulatory subunit B55, protein phosphatase 2	10.18	3	10.18	3	10.19	3	10.16	2
110625979	eukaryotic translation elongation factor 1 gamma	23.51	3	40.22	5	20.17	3	10.12	1
111185930	transmembrane protease, serine 13	10.13	1	10.13	1	10.11	1	10.15	1
112734861	importin 9	10.15	1	10.19	1	10.13	2	10.14	1
113680120	complement component 1, q subcomponent, gamma polypeptide	30.17	3	30.20	3	20.12	2	40.18	4
113865903	hypothetical protein LOC216976	10.16	2	10.19	3	10.17	1	10.13	1
114155155	mediator of RNA polymerase II transcription, subunit 8 homolog isoform 1	10.13	2	10.13	2	10.13	1	10.13	2
116256510	adaptor protein complex AP-2, alpha 1 subunit isoform b	136.89	23	170.21	30	140.22	23	100.24	15
116268115	AU RNA-binding enoyl-coenzyme A hydratase	23.53	3	30.30	4	30.15	4	10.13	1
117606277	excitatory amino acid transporter 2 isoform 1	13.47	1	20.18	2	10.12	1	10.12	1
118136297	chapsyn-110	10.13	1	10.14	1	10.12	1	10.13	1
118918400	nuclear receptor-binding SET-domain protein 1	10.15	1	10.18	1	10.12	1	10.16	2

120586994	homeobox D13	10.12	1	10.12	1	10.13	1	10.13	1
124486670	solute carrier family 25 (mitochondrial carrier), member 18	20.13	3	20.13	4	20.12	2	20.14	3
124487037	myosin IA	10.14	1	10.15	1	10.16	2	10.12	1
124487313	glutaminase isoform 1	40.20	5	70.23	9	30.21	3	20.15	3
124494256	low density lipoprotein receptor-related protein 1	40.20	6	30.17	3	20.20	2	70.22	12
125347376	filamin, alpha	20.14	2	40.17	4	10.12	1	10.13	1
126090749	inositol polyphosphate-4-phosphatase, type I isoform 2	13.50	2	20.19	2	10.14	1	10.18	2
130505929	potassium inwardly-rectifying channel J16	10.11	2	10.10	2	10.11	2	10.12	1
147899467	plakophilin 4 isoform 2	10.12	3	10.11	3	10.11	2	10.13	4
149268231	PREDICTED: similar to synaptojanin	20.17	3	30.18	3	20.20	3	10.14	2
153792586	SPEG complex locus isoform	10.13	1	10.12	2	10.12	1	10.15	1
157951604	CAP, adenylate cyclase-associated protein 1	40.21	5	70.22	8	40.24	5	10.17	1
157951686	ATPase, class V, type 10A	10.13	1	10.16	1	10.12	1	10.12	1
157951727	catenin (cadherin associated protein), alpha 2 isoform 1	23.51	3	50.16	5	10.13	2	10.24	1
158508501	septin 5	36.83	4	50.20	5	20.14	2	40.16	4
160333829	sorting nexin 12 isoform 3	10.16	1	10.22	1	10.12	1	10.12	1
162461907	heat shock protein 9	36.89	4	70.26	7	20.19	2	20.23	2
164698474	cytoplasmic FMR1 interacting protein 1	13.50	1	10.21	1	10.12	1	20.17	2

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

Gi_number	Protein name	Gene symbols	Protein MW	Control (C)		ME7 (M)		ratio
				Score	Spectral count	Score	Spectral count	(c/m)
up-regulated proteins								
31981185	phosphofructokinase, muscle	Pfkm	85248.63			86.93	11	
75992915	acyl-CoAsynthetaselong-chainfamilymember6isoform3	Acsl6	77967.26			70.23	7	
16716465	acyl-CoAsynthetasebubblegumfamilymember1	Acsbg1	80374.56			70.2	7	
11230802	actininalpha4	Actn4	104911.4			63.55	8	
61097906	actinin,alpha1	Actn1	103003.6			56.87	6	
21704242	CaMkinase-likevesicle-associated	Camkv	54785.59			50.22	7	
6754206	hexokinase1	Hk1	105506.5			50.17	5	
6754036	glutamateoxaloacetatetransaminase2,mitochondrial	Got2	47381.25			43.52	5	
124487313	glutaminaseisoform1	Gls	73916.26			40.2	5	
6753320	chaperoninsubunit3(gamma)	Cct3	60591.46			40.16	4	
8567410	synapsinIIisoformIIb	Syn2	52418.46			36.9	7	
162461907	heatshockprotein9	Hspa9	73415.7			36.89	4	
31980844	Dehydrogenase / reductase (SDRfamily) member1	Dhrs1	33983.37			36.88	4	
31981722	heatshockprotein5	Hspa5	72378.49			36.85	4	
6671702	chaperoninsubunit5 (epsilon)	Cct5	59586.04			30.24	3	
88196800	myosinVI	Myo6	145641.3			30.21	4	
37202121	4-aminobutyrateaminotransferase	Abat	56415.68			30.2	4	
29789191	asparaginyl-tRNAsynthetase	Nars	63025.58			30.18	3	
113680120	complementcomponent1, qsubcomponent,gammapolypeptide	C1qc	25974.87			30.17	3	
84000448	glialfibrillaryacidicprotein	Gfap	49869.61	45.18	10	236.9	116	12.2
13173473	prionprotein	PrnP	27959.5	20.18	2	66.86	14	7.2
31982755	vimentin	Vim	53655.16	20.16	4	106.88	24	7
24418919	brainglycogenphosphorylase	Pygb	96668.61	10.13	1	60.22	6	6.3
18079339	aconitase2,mitochondrial	Aco2	85410.13	15.18	2	63.55	7	4.7
9790051	phosphofructokinase,platelet	Pfkp	85400.41	15.19	2	50.24	6	4.2

31980648	ATPsynthase, H+ transportingmitochondrialF1complex, betasubunit	Atp5b	56265.58	35.16	7	106.86	22	3.4
6755863	tumorrejectionantigengp96	Hsp90b1	92418.14	20.15	3	60.2	10	3.3
27369581	solutecarrierfamily25 (mitochondrialcarrier, Aralar), member12	Slc25a12	74522.94	40.22	5	120.24	14	2.8
31560731	ATPase, H+ transporting, lysosomalV1subunitA	Atp6v1a	68282.63	40.17	4	83.56	11	2.7
6678483	ubiquitin-activatingenzymeE1	Uba1	117734.1	45.2	6	110.24	16	2.6
6755080	ProteinkinaseC, gamma	Prkcg	78307.24	25.2	4	73.59	10	2.6
116256510	adaptorproteincomplexAP-2,alpha1subunitisoformb	Ap2a1	105413.1	60.18	10	136.89	23	2.4
93102409	fattyacidsynthase	Fasn	272254.3	60.25	8	150.22	18	2.4
31982186	malatedehydrogenase2,NAD(mitochondrial)	Mdh2	35588.76	50.19	6	90.22	14	2.3
30409956	ATPase, Na+ / K+ transporting,alpha2polypeptide	Atp1a2	112145.6	100.24	20	106.88	41	2.1
88853578	adaptorproteincomplexAP-1,beta1subunit	Ap1b1	103869.1	50.17	10	90.24	20	2.1
down-regulated proteins								
31982300	hemoglobin, betaadultmajorchain	Hbb_bt	15738.15	55.22	16	40.18	5	3.2
112363107	neurofilament3,medium	Nefm	95883.48	80.24	30	43.52	10.3	2.9
161086984	adaptor-relatedproteincomplex2,sigma1subunit	Ap2s1	17006.7	30.18	6.5	16.81	2.3	2.8
6680722	ADP-ribosylationfactor5	Arf5	20516.58	30.14	5.5	20.17	2	2.8
160333789	sepiapterinreductase	Spr	27910.39	45.2	4.5	16.84	1.7	2.7
116256491	ankyrin3,epithelialisoforma	Ank3	188126	35.18	5	10.17	2	2.5
28316750	histonecluster1,H2ba	Th2b	14105	15.1	6.5	20.15	3	2.2

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.

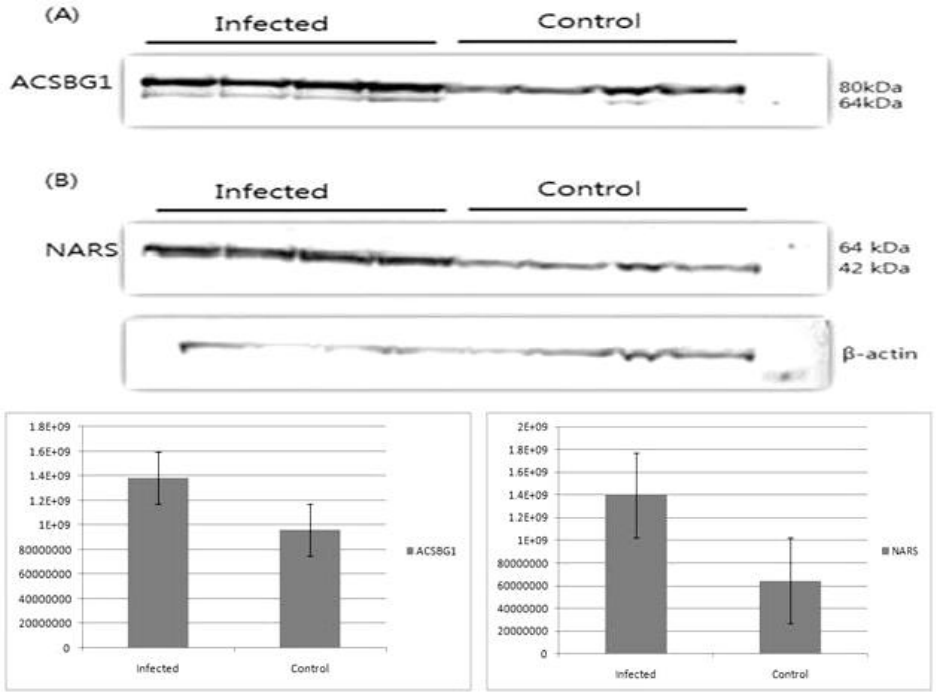

Figure 4: Confirmation of acyl-CoA synthetase bubblegum family member 1 (ACSBG1) (A) and asparaginyl-tRNAsynthetase (NARS) (B) expressed by western blotting including corresponding bar graphs of them below. Brain homogenates were analyzed with antibodies against ACSBG1 (1:200) and NARS (1:2,000) based on the total loading amount. Expression levels were normalized to β-actin levels.

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 were17, 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

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-tRNAsynthetase (NARS)

Our proteomics results indicated that ACSBG1 and NARS are significantly increased in ME7scrapie-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 ME7scrapie-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 ME7scrapie-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 included19 proteins such as Sptbn4, Cadps, Slc17a7, Grin1, Musk, Ctnnb1, Glrb, Agtpbp1, Vamp2, Nf1, 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 isoform 3; ACGSBG1; 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., dehydrogenase / reductase [SDR family] member 1and fatty acid synthase) and asparaginyl-tRNAaminoacylation 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 (Ywhab) and is an important paralog ofacyl-CoA synthetase long-chain family member 6 isoform 3. The lipidosin 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 adrenoleukodystrophyis associated with accumulation of very-long-chain fatty acid, and is related to metabolism and myelinogenesis attributed to reduced peroxisomal β-oxidation. Asparaginyl-tRNAsynthetase 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 PrP^{Sc} 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 as well as further investigations 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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