Proteomics-based Development of Biomarkers for Prion Diseases

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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 humans and animals 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, fatal familial insomnia, and kuru. The PrPSc isoform is more proteinase K-resistant than the PrPc. Detection of PrPSc in the central nervous system 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]. CqI 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-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

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Received November 28, 2015; Accepted March 15, 2016; Published March 23, 2016

Citation: Bo-Yeong C, Lee YS, Ju YR, Chi-Kyeong K, Kim SY (2016) Proteomics-based Development of Biomarkers for Prion Diseases. J Proteomics Bioinform 9: 087-100. doi: 10.4172/jpb.1000394

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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 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 permmissible 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 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: 0016043, p-value < 0.05), establishment of localization (GO: 0051179, p-value < 0.05), transport (GO: 0006810, p-value < 0.05), and vesicle-mediated transport (GO: 0006810, p-value < 0.05), 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).

**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:0031179, 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:0006802, p-value < 0.05), and metabolic 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., arginosuccinate synthetase 1; glutamate oxaloacetate transaminase 2, mitochondrial; AU RNA binding protein/enolyl-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), reticulin 4, neurofibromatosis 1, and neuropilin, 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).

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.
| Protein Name | Description |
|--------------|-------------|
| ferritin heavy chain 1 | |
| potassium inwardly rectifying channel, subfamily J, member 11 | |
| profilin 1 | |
| SECC2 vesicle trafficking protein-like 1 | |
| EH-domain containing 1 | |
| RAB10, member RAS oncogene family | |
| synaptojan V | |
| chloride channel calcium activated 3 | |
| profilin 2 | |
| SMC1 structural maintenance of chromosomes 1-like 1 | |
| double Cortin-like kinase 1 isoform 1 | |
| secretory carrier membrane protein 5 | |
| NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 13 | |
| cytochrome c-1 | |
| NADH dehydrogenase (ubiquinone) 1 beta subcomplex 3 | |
| actin related protein 2 / 3 complex, subunit 4 | |
| RAB3C, member RAS oncogene family | |
| syntaxin 1A (brain) | |
| sidefexin 3 | |
| tubulin, gamma complex associated protein 2 | |
| DEAD (Asp-Glu-Ala-Asp) box polypeptide 1 | |
| NADH dehydrogenase (ubiquinone) Fe-S protein 7 | |
| anti-silencing function 1B | |
| ATPase, H+ transporting, lysosomal V1 subunit F | |
| ATPase, H+ transporting, lysosomal accessory protein 2 | |
| AGP7 | |
| 13kDa differentiation-associated protein | |
| NECAP endocytosis associated 1 | |
| a disintegrin-like and metallopeptase (reprolysin type) with thrombospondin type 1 motif, 19 | |
| histone cluster 1, H2ba | |
| cytochrome b5 reductase 4 | |
| transient receptor potential cation channel, subfamily A, member 1 | |
| eukaryotic translation initiation factor 3, subunit 9 | |
| histone cluster 2, H3c1 isoform 2 | |
| collagen, type XVI, alpha 1 | |
| neurocalcin delta | |
| YKT6 v-SNARE protein | |
| RAS protein activator like 1 (GAP1 like) | |
| ATPase, H+ transporting, V0 subunit D isoform 1 | |
| glycine receptor, beta subunit | |
| electron transferring flavoprotein, alpha polypeptide | |
| laminin, beta 2 | |
| integrin alpha 6 | |
| hemoglobin, beta adult major chain | |
| collagen, type IX, alpha 1 | |
| kinesin family member 27 | |
| solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 7 | |
| procollagen, type IV, alpha 4 | |
| kinesin family member 3A | |
| wingless-related MMTV integration site 6 | |
| cytochrome c oxidase subunit II | |
| neurofilamin | |
| exportin 1, CRM1 homolog | |
solute carrier family 39 (zinc transporter), member 10
10.15
1
10.15
1
10.14
1

GTPase activating protein and VPS9 domains 1
10.16
1.5
10.16
1
10.14
2

platelet-activating factor acetylhydrolase, isoform 1b, alpha2 subunit
10.14
1.5
10.15
2
10.14
1

suppressor of zeste 12 homolog
10.13
1.5
10.13
1
10.13
2

collagen, type VIII, alpha 2
10.14
1
10.11
1
10.16
1

chloride channel 3 isoform c
10.17
1
10.13
1
10.20
1

amphiphysin
30.21
3.5
20.21
2
40.21
5

nascent polypeptide-associated complex alpha subunit isoform b
15.23
2
10.24
2
20.23
2

sterol carrier protein 2, liver
10.14
1
10.15
1
10.13
1

superoxide dismutase 1, soluble
15.14
1.5
20.15
2
10.13
1

NADH dehydrogenase (ubiquinone) Fe-S protein 8
30.18
1
1
10.14
1

lysosomal trafficking regulator
10.20
2

Calcium / calmodulin-dependent protein kinase II, delta
10.11
1

platelet-activating factor acetylhydrolase, isoform 1b, alpha2 subunit
10.16
1.5
10.16
1
10.14
2

ATP / GTP binding protein 1 isoform 1
10.15
10.17
10.13
1

dynein, axonemal, heavy chain 5
30.24
1
1
10.14
1

a disintegrin-like and metalloprotease (reprolysin type)
10.13
1
10.14
1
10.13
1

sortilin-related receptor, LDLR class A repeats-
1.5

neurofilament 3, medium
10.15
1
10.13
1
10.12
1

Ca < 2+ dependent activator protein for secretion
10.14
1

microtubule-associated protein 2 isoform 2
2.5
20.15
1
10.12
2

superoxide dismutase 1, soluble
10.12
1
10.14
1
10.12
1

Calcium / calmodulin-dependent protein kinase II, delta isoform 2
35.20
5
40.17
6
30.24
4

absent, small, or homoeotypic discs 1
10.18
2
10.16
1
10.21
3

titin isoform N2-A
10.16
1.5
10.12
1
10.20
2

structural maintenance of chromosomes 2-like 1
3.5
1
10.13
1
10.12
1

microtubule-associated protein 2 isoform 1
10.15
1
10.13
1
10.18
1

skeletal muscle receptor tyrosine kinase isoform 1 precursor
83523732

pleckstrin and Sec7 domain containing 3 isoform 2
15.20
10.15
1.5
10.12
1
10.17
2

glutathione peroxidase 4 isoform 1 precursor
15.13
1.5
20.14
2
10.13
1

PREDICTED: similar to vacuolar protein sorting 13B isoform 5
10.14
1
10.12
1
10.17
1

RAB12, member RAS oncogene family
10.17
1
10.20
1
10.14
1

ATPase, H+/K+ exchaging, gastric, alpha polypeptide
10.14
1
10.16
1
10.12
1

sortilin-related receptor, LDLR class A repeats-containing
110625665

lysosomal trafficking regulator
11195376

neurofilament 3, medium
112363107

dynin, axonemal, heavy chain 5
114155137

ATP / GTP binding protein 1 isoform 1
114158695

cadherin EGF LAG seven-pass G-type receptor 1 precursor
115648153

a disintegrin and metalloprotease domain 2 (fertilin beta)
110689327

Snap-25-interacting protein
116089329

spectrin beta 4
116174793

ankyrin 3, epithelial isoform a
116256891

sodium channel, voltage-gated, type VIII, alpha
117414174

RUN and TBC1 domain containing 1
117956385

hypothetical protein LOC235461
118403316

histone cluster 2, H2ab
119436597

TBC1 domain family, member 1
120587003

Microarray Proteomics-based Development of Biomarkers for Prion Diseases. J Proteomics Bioinform 9: 087-100. doi:10.4172/jpb.1000934
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.

### Table 1

| GI_number | Protein name | Average | 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 isomorf | 16.85   | 30.18      | 10.13               | 10.24      | 1                   | 10.14      | 4                   |
| 6671549   | peroxiredoxin 6 | 16.83   | 20.16      | 10.13               | 10.20      | 1                   | 10.14      | 4                   |
| 6671664   | cathepsin D | 23.52   | 30.20      | 20.15               | 20.21      | 2                   | 10.14      | 4                   |
| 6671702   | chaperonin subunit 5 (epsilon) | 30.24  | 40.26      | 40.25               | 40.21      | 4                   | 10.20      | 1                   |
| 6677809   | ribosomal protein S6 | 20.17   | 30.23      | 20.14               | 10.13      | 1                   | 10.14      | 4                   |
| Gene Name | Description | Protein ID | Protein ID | Protein ID | Protein ID | Protein ID |
|-----------|-------------|------------|------------|------------|------------|------------|
| synuclein, alpha | 6678047 | 1.01 | 1 | 1.011 | 1 | 1.016 | 1 | 1 | 1.017 | 1 |
| ubiquitin-activating enzyme E1 | 668483 | 110.24 | 16 | 130.23 | 19 | 120.22 | 14 | 80.26 | 14 |
| phosphodiesterase 1B, Ca<sup>2+</sup> - calmodulin dependent | 6679243 | 10.17 | 1 | 1.016 | 1 | 1.20 | 1 | 1.016 | 1 |
| protein kinase C, beta 1 | 6679345 | 23.48 | 2 | 40.17 | 4 | 10.12 | 1 | 20.15 | 2 |
| growth associated protein 43 | 6679935 | 10.17 | 1 | 1.016 | 1 | 1.015 | 1 | 1.019 | 2 |
| isocitrate dehydrogenase 3 (NAD+), gamma | 6680345 | 20.19 | 2 | 30.22 | 3 | 20.15 | 2 | 10.20 | 1 |
| ADP-ribosylation factor 4 | 6680720 | 13.47 | 2 | 20.14 | 2 | 10.13 | 1 | 10.15 | 2 |
| cysteine and glycine-rich protein | 6681069 | 36.88 | 6 | 50.23 | 7 | 20.17 | 3 | 40.23 | 7 |
| complement component 1, q subcomponent, B chain | 6753220 | 26.86 | 3 | 40.21 | 5 | 10.17 | 1 | 30.21 | 4 |
| caspase 14 | 6753280 | 10.17 | 1 | 1.016 | 1 | 1.014 | 1 | 1.20 | 1 |
| chaperonin subunit 3 (gamma) | 6753320 | 40.16 | 4 | 70.20 | 8 | 30.17 | 3 | 20.12 | 2 |
| chaperonin subunit 4 (delta) | 6753322 | 36.87 | 5 | 40.17 | 6 | 50.21 | 6 | 20.22 | 2 |
| chaperonin subunit 6a (zeta) | 6753324 | 13.50 | 2 | 10.22 | 1 | 10.12 | 1 | 20.14 | 3 |
| gap junction protein, alpha 1 | 6753992 | 26.86 | 7 | 30.19 | 10 | 20.18 | 5 | 30.23 | 5 |
| guanine nucleotide binding protein, alpha 11 | 6754004 | 10.15 | 3 | 10.18 | 6 | 10.13 | 2 | 10.14 | 1 |
| glutamate oxaloacetate transaminase 2, mitochondrial | 6754036 | 43.52 | 5 | 60.23 | 6 | 40.18 | 4 | 30.14 | 4 |
| glutathione S-transferase, mu 5 | 6754086 | 13.49 | 2 | 10.16 | 2 | 20.14 | 2 | 10.17 | 1 |
| hexokinase 1 | 6754206 | 50.17 | 5 | 80.20 | 8 | 50.19 | 5 | 20.13 | 2 |
| mitogen activated protein kinase | 6754624 | 10.17 | 7 | 10.16 | 7 | 10.18 | 9 | 10.18 | 4 |
| programmed cell death 6 interacting protein | 6755002 | 13.51 | 1 | 10.18 | 1 | 20.15 | 2 | 10.20 | 1 |
| protein kinase C, gamma | 6755080 | 73.59 | 10 | 120.26 | 16 | 40.21 | 6 | 60.29 | 9 |
| muscle glycogen phosphorylase | 6755256 | 20.15 | 2 | 20.15 | 2 | 30.12 | 3 | 10.17 | 1 |
| ribosomal protein S3 | 6755372 | 46.82 | 6 | 60.18 | 9 | 40.15 | 6 | 40.13 | 4 |
| synaptosomal-associated protein 25 | 6755588 | 13.48 | 1 | 10.16 | 1 | 10.12 | 1 | 20.16 | 2 |
| talin 1 | 6755809 | 16.85 | 2 | 10.13 | 2 | 20.21 | 2 | 20.22 | 2 |
| tumor rejection antigen gp96 | 6755863 | 60.20 | 10 | 80.20 | 13 | 60.19 | 10 | 40.22 | 7 |
| thioredoxin 1 | 6755911 | 13.54 | 2 | 10.21 | 1 | 10.20 | 1 | 20.22 | 3 |
| voltage-dependent anion channel 3 | 6755967 | 23.51 | 3 | 40.21 | 5 | 20.19 | 3 | 10.12 | 1 |
| arginosuccinate synthetase | 6996911 | 20.16 | 2 | 20.14 | 2 | 30.20 | 3 | 10.15 | 1 |
| erythrocyte protein band 4.1-like 1 isoform a | 7305029 | 16.88 | 3 | 20.22 | 3 | 10.20 | 2 | 20.22 | 3 |
| ARFI actin-related protein 1 homolog A | 8392847 | 16.83 | 2 | 30.24 | 3 | 10.23 | 1 | 10.23 | 1 |
| heterogeneous nuclear ribonucleoprotein C | 8393544 | 16.83 | 2 | 30.19 | 3 | 10.13 | 1 | 10.16 | 1 |
| synapsin II isoform Ib | 8567410 | 36.90 | 7 | 70.28 | 14 | 20.18 | 3 | 20.24 | 5 |
| Rab geranylgeranyl transferase, a subunit | 9507023 | 10.15 | 2 | 10.14 | 2 | 10.13 | 3 | 10.19 | 1 |
| hydroxysteroid (17-beta) dehydrogenase 12 protein | 9789991 | 16.84 | 2 | 30.22 | 3 | 10.16 | 1 | 10.13 | 1 |
| phosphofructokinase, platelet | 9790051 | 50.24 | 6 | 60.25 | 9 | 50.25 | 6 | 40.22 | 4 |
| mitochonrdial carrier homolog 2 | 9790055 | 23.50 | 2 | 50.23 | 5 | 10.16 | 1 | 10.12 | 1 |
| RAB2, member RAS oncogene family | 10946940 | 16.82 | 2 | 30.19 | 3 | 10.12 | 1 | 10.14 | 1 |
| actinin alpha 4 | 11230802 | 63.55 | 8 | 80.22 | 10 | 70.21 | 9 | 40.23 | 6 |
| ribosomal protein S19 | 12963511 | 10.14 | 2 | 10.11 | 1 | 10.15 | 3 | 10.15 | 2 |
| chromosome segregation 1-like | 12963737 | 23.51 | 3 | 40.24 | 4 | 20.15 | 2 | 10.14 | 2 |
| prion protein | 13173473 | 66.86 | 14 | 70.26 | 19 | 60.16 | 9 | 70.17 | 15 |
| TNF receptor-associated protein 1 | 13385998 | 20.21 | 2 | 30.24 | 3 | 10.16 | 1 | 20.22 | 2 |
| vacuolar protein sorting 35 | 13928670 | 23.53 | 2 | 30.20 | 3 | 30.19 | 3 | 10.20 | 1 |
| methyl CpG binding protein 2 isoform 2 | 14149645 | 13.47 | 2 | 10.15 | 2 | 20.12 | 3 | 10.13 | 1 |
| acyl-CoA synthetase bubblegum family member 1 | 16716465 | 70.20 | 7 | 90.23 | 9 | 60.18 | 7 | 60.19 | 6 |
| Gene Name                                      | Protein Function                                                                 |
|-----------------------------------------------|----------------------------------------------------------------------------------|
| aconitase 2, mitochondrial                    |                                                                                   |
| 3-oxoacid CoA transferase 1                   |                                                                                   |
| vacuolar H+ ATPase B2                         |                                                                                   |
| solute carrier family 25 (mitochondrial carrier, glutamate), member 22 |                                                                                   |
| heterogeneous nuclear ribonucleoprotein M isoform a |                                                                                   |
| ribosomal protein S11                        |                                                                                   |
| NADH dehydrogenase (ubiquinone) Fe-S protein 1|                                                                                   |
| ubiquitin-conjugating enzyme E2M              |                                                                                   |
| CaM kinase-like vesicle-associated            |                                                                                   |
| tubulin, beta                                |                                                                                   |
| ubiquinol cytochrome c reductase core protein 2|                                                                                   |
| solute carrier family 25, member 1            |                                                                                   |
| solute carrier family 1 (glial high affinity glutamate transporter), member 3 |                                                                                   |
| brain glycogen phosphorylase                 |                                                                                   |
| exportin 5                                    |                                                                                   |
| ribosomal protein L32                        |                                                                                   |
| solute carrier family 25 (mitochondrial carrier, Avarlar), member 12 |                                                                                   |
| transcriptional regulating factor 1 isoform 2 |                                                                                   |
| myosin, heavy polypeptide 14                  |                                                                                   |
| asparaginyl-tRNA synthetase                   |                                                                                   |
| RAN binding protein 5                         |                                                                                   |
| ATPase, Na+/K+ transporting, alpha 2 polypeptide|                                                                                   |
| zinc finger protein 526                      |                                                                                   |
| elongation protein 4 homolog                 |                                                                                   |
| synaptotagmin II                             |                                                                                   |
| sorting nexin 3                              |                                                                                   |
| ATPase, H+ transporting, lysosomal V1 subunit A |                                                                                   |
| ATP synthase, H+ transporting, mitochondrial F1 complex, beta subunit |                                                                                   |
| Dehydrogenase / reductase (SDR family) member 1 |                                                                                   |
| phosphofructokinase, muscle                  |                                                                                   |
| thimet oligopeptidase 1                      |                                                                                   |
| heat shock protein 5                          |                                                                                   |
| glycerol phosphate dehydrogenase 2, mitochondrial |                                                                                   |
| malate dehydrogenase 1, NAD (soluble)         |                                                                                   |
| malate dehydrogenase 2, NAD (mitochondrial)   |                                                                                   |
| glutamine synthetase                         |                                                                                   |
| ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c, isoform 1 |                                                                                   |
| inosine triphosphatase                       |                                                                                   |
| vimentin                                      |                                                                                   |
| dihydroxoisomide dehydrogenase               |                                                                                   |
| phosphodiynositol-binding calirhin assembly protein |                                                                                   |
| complement receptor 2                        |                                                                                   |
| Gene Name | Description | Accession |
|-----------|-------------|-----------|
| 33859811 | mitochondrial trifunctional protein, alpha subunit | 73.55 |
| 34740335 | tubulin, alpha 1B | 13.56 |
| 36031132 | ATPase, Ca**-transporting, fast twitch 1 | 16.83 |
| 37202121 | 4-aminobutyrate aminotransferase | 30.20 |
| 38372907 | DEAD (Asp-Glu-Ala-Asp) box polypeptide 39 | 20.14 |
| 39930477 | septin 8 | 20.19 |
| 40254635 | kinesin family member 5A | 23.56 |
| 52353955 | 3-phosphoglycerate dehydrogenase | 46.87 |
| 56699478 | plasma membrane calcium ATPase 3 | 20.17 |
| 58037481 | sec1 family domain containing 1 | 10.17 |
| 61097906 | actinin, alpha 1 | 56.87 |
| 61888842 | partitioning-defective protein 3 homolog isoform 2 | 10.16 |
| 67763380 | zinc finger protein 62 isoform 1 | 10.12 |
| 71725385 | DDIRAS family, GTP-binding RAS-like 2 | 13.52 |
| 71774133 | peptidylprolyl isomerase B | 20.20 |
| 75992915 | acyl-CoA synthetase long-chain family member 6 isoform 3 | 70.23 |
| 75992920 | acyl-CoA synthetase long-chain family member 3 | 23.50 |
| 82891441 | PREDICTED: similar to DnaJ (Hsp40) homolog, subfamily B, member 14 isoform 8 | 10.13 |
| 83776571 | protein kinase C-binding protein NELL1 | 10.17 |
| 83816893 | DEAD (Asp-Glu-Ala-Asp) box polypeptide 5 | 20.24 |
| 83921618 | villin 2 | 16.84 |
| 84000448 | glial fibrillary acidic protein | 236.90 |
| 85861218 | guanine monophosphate synthetase | 16.85 |
| 87298845 | calmodulin binding protein 1 | 10.13 |
| 88196800 | myosin VI | 30.21 |
| 88853578 | adaptor protein complex AP-1, beta 1 subunit | 90.24 |
| 91992157 | AP2 associated kinase 1 isoform 1 | 10.15 |
| 93102409 | fatty acid synthase | 150.22 |
| 93102417 | glycol-1RNA synthetase | 13.53 |
| 110625886 | alpha isoform of regulatory subunit B55, protein phosphatase 2 | 10.18 |
| 110625979 | eukaryotic translation elongation factor 1 gamma | 23.51 |
| 111185930 | transmembrane protease, serine 13 | 10.13 |
| 112734861 | importin 9 | 10.15 |
| 113680120 | complement component 1, q subcomponent, gamma polypeptide | 30.17 |
| 113865903 | hypothetical protein LOC216976 | 10.16 |
| 114155155 | mediator of RNA polymerase II transcription, subunit 8 homolog isoform 1 | 10.13 |
| 116256510 | adaptor protein complex AP-2, alpha 1 subunit isoform b | 136.89 |
| 116268115 | AU RNA-binding enol-coenzyme A hydratase | 23.53 |
| 117606277 | excitatory amino acid transporter 2 isoform 1 | 13.47 |
| 118136297 | chapsyn-110 | 10.13 |
| 118918400 | nuclear receptor-binding SET-domain protein 1 | 10.15 |
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) Score | MET (M) Spectral count | ratio (c/m) |
|--------------------|-------------------------------------------------------------------------------|--------------|------------|-------------------|------------------------|-------------|
| 125501190          | phosphofructokinase, muscle                                                  | Pfkm         | 85248.63   | 86.93             | 11                     |             |
| 75959215           | acyl-CoA synthetase-long-chain family member isoform 3                        | AcsL6        | 77967.26   | 70.23             | 7                      |             |
| 16716465           | acyl-CoA synthetase bubblegum family member 1                                 | Acsbg1       | 80374.56   | 70.2             | 2                      |             |
| 11230802           | actinin, alpha 4                                                             | Actn4        | 104911.4   | 63.55             | 8                      |             |
| 60197096           | actinin, alpha 1                                                             | Actn1        | 103003.6   | 56.87             | 6                      |             |
| 21704242           | CalM kinase-like/vesicle-associated                                           | CamkIV       | 57485.59   | 50.22             | 7                      |             |
| 6754206            | hexokinase 1                                                                  | Hk1          | 105506.5   | 50.17             | 5                      |             |
| 6754036            | glutamate oxidase/acetate/transferase isomerase 2, mitochondrial               | Gots2        | 47381.25   | 43.52             | 5                      |             |
| 124487313          | glutaminase isomerase 1                                                        | Gls          | 73916.26   | 40.2              | 5                      |             |
| 6753320            | chaperonin subunit 3 (gamma)                                                  | Cct3         | 60591.46   | 40.16             | 4                      |             |
| 8587410            | synapsin III isoform 1                                                        | Syn2         | 52418.46   | 36.9              | 7                      |             |
| 162461907          | heat shock protein 9                                                           | Hsp9a        | 73415.7    | 36.89             | 4                      |             |
| 31980844           | dehydrogenase / reductase (SDRFamily) member 1                                | Dhrs1        | 33983.37   | 36.88             | 4                      |             |
| 31981722           | heat shock protein 5                                                           | Hsp5         | 72378.49   | 36.85             | 4                      |             |
| 6671702            | chaperonin subunit 5 (eplion)                                                 | Cct5         | 59586.04   | 30.24             | 3                      |             |
| 88196800           | myosin VI                                                                    | Myo6         | 145641.3   | 30.21             | 4                      |             |
| 37202121           | 4-Aminobutyrateaminotransferase                                               | Abat         | 56415.68   | 30.2              | 4                      |             |
| 29789191           | asparaginyl-IRNA synthetase                                                    | Nars         | 63025.58   | 30.18             | 3                      |             |
| 113680120          | complement component 1, ubiquitin component, gammapoly peptide                 | C1q      | 25974.87   | 30.17             | 3                      |             |
| 84000448           | gliadin fibrillary acidic protein                                             | Glap         | 49866.61   | 45.18             | 10                     | 116.2       |
| 13173473           | prion protein                                                                 | PnnP         | 27959.5    | 20.18             | 2                      | 68.8        | 7.2         |
| 31982755           | vimentin                                                                     | Vlm          | 53655.16   | 20.16             | 4                      | 106.8       | 24          |
| 24418919           | brain glycogen phosphorylase                                                   | Pygb         | 96668.81   | 10.13             | 1                      | 60.22       | 6.3         |
| 18079339           | aconitase 2, mitochondrial                                                    | Acoc2        | 85410.13   | 15.18             | 2                      | 63.55       | 7.4         |
| 9790051            | phosphofructokinase, platelet                                                | PfKp         | 85400.41   | 15.19             | 2                      | 50.24       | 6.4         |
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.

| Entry       | Description                                                                 | UniProt ID | Score | FoldChange | p-Val  |
|-------------|-----------------------------------------------------------------------------|------------|-------|------------|--------|
| 3198200     | hemoglobin, betaadultmajorchain                                             | Hbb_bt     | 15738.15 | 52.22      | 40.18  |
| 112363107   | neurofilament3,middle                                                       | Nefm       | 95863.48 | 80.24      | 43.52  |
| 6680722     | ADP-riboseylationfactor5                                                    | Arf5       | 20516.58 | 30.14      | 20.17  |
| 16033789    | sepiapterinreductase                                                        | Spr        | 27910.39 | 45.2       | 16.84  |
| 116256491   | ankyrin3,epithelialisiform                                                 | Ank3       | 188126  | 35.18      | 10.17  |
| 28316750    | histonecluster1,H2ba                                                        | Thz2       | 14105   | 15.1       | 20.15  |

**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 (1:2000) 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 proteomic 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 included19 proteins such as Sptbn4, Cadps, Slc17a7, Grin1, Musk, Ctnnb1, Glrb, Atp1a2, Vamp2, Nf1, Stx1a, Slc12a5, Sod1, Spr, Svt2, 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; 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., glutamate isoform 1), oxidation reduction processes (i.e., dehydrogenase / 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 (Ywhab) and is an important paralog of acyl-CoA synthetase long-chain family member 6 isoform 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 acid, and is related to metabolism and myelinogenesis attributed to reduced peroxisomal β-oxidation. Asparaginyl-tRNA synthetase is classified as class-II aminoacyl-tRNA synthetases, 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-tRNA synthetases, 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 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 regimes. 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 understanding of the protein interaction or function and to develop therapeutic strategies in the field of neurodegenerative diseases.

Acknowledgement

We thank to Dr. Hyeong Soon Park and Dr. Soo Jae Lee (DIATECH Korea Co., Ltd. South Korea) for contribution of valuable comments for analysis mass data and interpretation. This study was supported by grants from the Korea Centers for Disease Control and Prevention (Funding no. 4800-4847-311-210-13 and 2014-NG52001-00).

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