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Analysis of Cerebrospinal Fluid Proteins Reveals Association of Calbindin 1 Concentrations with Neurological Outcomes in Patients Resuscitated from Out-of-Hospital Cardiac Arrest: A Proteomics-Based Pilot Study

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Abstract The purpose of this study is to establish an analytical platform for identifying a major subset of cerebrospinal fluid (CSF) proteins in patients resuscitated from out-of-hospital cardiac arrest and evaluating their associations with neurological outcomes. Thirteen patients, who were resuscitated from cardiac arrest and survived for more than 48 hours, were enrolled and their CSF was obtained. Patients were classified into favorable (group F) and unfavorable (group U) outcomes based on the Glasgow Outcome Scale at 6 months after the return of spontaneous circulation. Control CSF was also obtained from 8 subjects with no obvious neurological disorders (group N). Protein identification was performed on one individual from each group by liquid chromatography-tandem mass spectrometry and verified on all 21 individuals by western blotting. In total, 107 proteins were identified in CSF. Of those, 40 proteins were identified only in the group U patients, including brain-specific proteins, such as enolase 2 and calbindin 1. The levels of calbindin 1 in individual CSF were significantly higher in group U than in group N or in group F, and associated with the Glasgow Outcome Scale scores. A combination of liquid chromatography-tandem mass spectrometry and western blotting is useful to analyze CSF from critical care unit patients.

Key words: cerebrospinal fluid, proteomic analysis, cardiopulmonary arrest, calbindin 1, biomarker

Introduction

Outcomes of patients resuscitated from out-of-hospital cardiac arrest (OHCA) are poor largely due to neuronal injury after global cerebral ischemia.[1] Several biomarkers, such as neuron specific enolase (NSE),[2] a protein soluble in 100% ammonium sulfate (S-100),[3] interleukin-8 (IL-8)[4,5] and high mobility group box 1 (HMGB1)[6] have been reported as prognostic neurological markers in resuscitated patients. Despite a large body of research in this area, the timing and optimal approach to prognostication using these biomarkers is controversial[7]. Therefore, more sensitive and informative biomarkers are still needed for the better clinical practices. Antibody-based methods are frequently employed in biomarker discovery to identify and validate certain protein biomarkers because of their excellent sensitivity and selectivity. However, antibodies possessing both excellent sensitivity and specificity are not always available and, in most cases, commercially available reagents are expensive. The antibody-based methods, therefore, may be best suited for studies targeting only a limited number of "likely candidates", but not for comprehensive biomarker discovery.

Proteomics approaches based on mass spectrometry analysis of biological fluids or tissue specimens may provide the novel classes of biomarker candidates for a disease. Such novel disease-specific biomarkers could not only serve as a prognosis marker, but also provide better understanding of the disease process. Cerebrospinal fluid (CSF) is an appropriate source of biomarkers involved in the disease process of the central nervous system disorders. Therefore, proteomic analyses have been applied to CSF samples from patients with stroke,[8] Alzheimer's disease,[9] and multiple sclerosis[10] in recent studies.

There is currently no report regarding proteomic study on CSF from patients resuscitated from OHCA. The purpose of this study is to establish an analytical platform for identifying CSF proteins in patients resuscitated from OHCA by mass spectrometry-based analysis and evaluating their associations with patients' neurological outcomes.

Materials and methods

Subjects

This was designed as a prospective clinical observational study and it was carried out from September 2000 to April 2005. Subjects enrolled in this study were identical to those in the previous report.[6] The protocol was approved by the institutional review board of Yamaguchi University Hospital. Informed consent for participation in this study was sought from next of kin after each patient' s arrival at our hospital. Patients with a successful return of spontaneous circulation (ROSC) from OHCA were consecutively admitted to our intensive care unit (ICU). The exclusion criteria were extracorporeal resuscitation, cardiac arrest resulting from severe stroke, neurological diseases before OHCA, multiple trauma, renal dysfunction with dialysis, heparinization after percutaneous transluminal coronary angioplasty, and terminal illness because of either severe cardiac dysfunction, refractory hypotension, or bleeding tendency. Patients who died within 48 hours from ROSC were also excluded or withdrawn.

Patient's core body temperature was maintained between 36-37 ℃ for at least 72 hours after ROSC using an external cooling device (Blanketrol™, Cincinnati Sub-Zero, Cincinnati, OH, USA). We administered a sedative (midazolam, 0.1-0.2 mg/kg/hour), an analgesic (butorphanol tartrate, 0.01-0.02 mg/kg/hour), and a muscle relaxant (vecuronium bromide, 0.05 mg/kg/hour), and the doses of these agents were adjusted as needed. Then, we stopped administering these drugs, and observed whether or not the patients were able to recover consciousness. The patients who regained consciousness were extubated, but those who remained deeply comatose underwent a tracheostomy and were discharged from the ICU. They were followed up for at least 6 months.

The neurological outcome at 6 months after the ROSC was assessed using the Glasgow Outcome Scale (GOS) score, which provides 5 categories.[11] A favorable neurological outcome (group F) was defined as a good recovery and a moderate disability, while an unfavorable outcome (group U) was defined

as a severe disability, a persistent vegetative state, and death.

CSF sampling

The CSF was taken from the patients, who had not been administered heparin for at least 24 hours and who was not suspected of an intracranial hypertension by brain computed tomography. The CSF sample was centrifuged at 3,000 rpm for 10 minutes at 4 ℃ and the supernatant was stored in a -80 ℃ freezer until analysis. CSF from control subjects (n = 8), who were neurologically normal, was taken by a lumber puncture at spinal anesthesia prior to an orthopedic surgery (group N). Three men and five women were included in the group N. Their median age was 64 years old (interquartile range: 45-80 years old).

Sample preparation

Figure 1 represents the protein identification procedure of the CSF samples. Total of 3 CSF samples, taken from a single individual from each group, were processed as previously described with slight modifications.[12] Briefly, the aliquot of CSF was filtered through a 0.22 μm filter, precipitated and rinsed with cold acetone, dried, and solubilized with 8 M urea in 0.1 M Tris-HCl (pH 8.8). Protein samples were reduced with 2 mM dithiothreitol, alkylated with 5 mM iodoacetamide, and diluted 4-fold with distilled water. Protein samples were digested for 16 hours with TPCK-treated trypsin (Promega, WI, USA), and the peptide mixtures were subjected to nanoflow liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Additionally, CSF samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by in-gel tryptic digestion as previously described.[13]

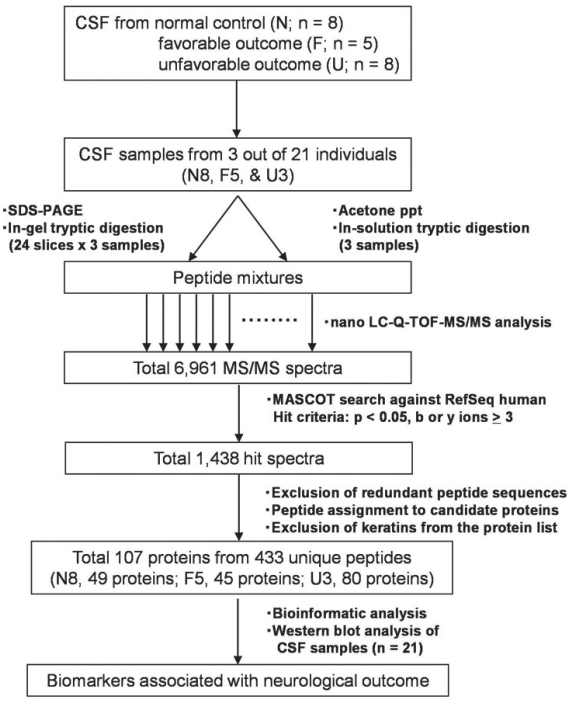

Figure 1. Schematic representation of the procedure of protein identification.

Nanoflow LC-MS/MS analysis

The peptide mixtures were analyzed on an automated nanoflow LC-MS/MS system using reversed phase chromatography as described previously.[14] Briefly, the peptide mixture was captured on a trap column (Mightysil C18, 0.5 mm internal diameter × 1 mm long, 3 μm particles; Kanto Chemicals, Tokyo, Japan) for desalting, and then separated on a Mightysil C18 column (0.15 mm internal diameter × 40 mm long, 3 μm particles; Kanto Chemicals) using a linear acetonitrile gradient (0-80%, 35 minutes) at a flow rate of 100 nl/minutes. The eluted peptides were sprayed directly into a high-resolution quadrupole time-of-flight hybrid mass spectrometer (Q-Tof Ultima, Micromass, Manchester, UK).

Protein identification by database search

The MS/MS signals were acquired by the MassLynx and converted to text files by the ProteinLynx software (Micromass). The database search was performed by the MASCOT (Matrix Science Ltd., London, UK) against the Refseq human database (National Center for Biotechnology Information, NCBI, USA) with following parameters: fixed modification, carbamoylmethylation (Cys); variable modifications, oxidation (Met) and acetylation (protein N-terminal); maximum miss cleavages, 2; peptide mass tolerance, 500 ppm; and MS/MS tolerance, 0.5 Da.

For protein identification, the search results were processed using the STEM software as follows.[15] (i) All the datasets derived from 3 CSF samples were combined. (ii) The candidate peptide sequences were screened

with the probability-based MOWSE (molecular weight search) scores that exceeded their thresholds ($p < 0.05$) and with MS/MS signals for y- or b-ions ≥ 3. (iii) Redundant peptide sequences were removed. (iv) Each peptide sequence was assigned to a protein that gave the maximal number of peptide assignments among the candidates. (v) Finally, the rule of peptide assignment was consistently applied to the individual datasets. Potential contaminants (i.e., keratin, porcine trypsin) were excluded from the final list of identified proteins. The CSF proteins identified in this study were categorized based on gene ontology (GO) categories using the Expression Analysis System Explorer (EASE) software.[16] Relative gene expression levels in each organ were calculated using the Expressed Sequence Tag (EST) Profile Viewer on UniGene database (NCBI).

Electrophoresis and immunoblotting

Two microliters of 20-fold diluted CSF were resolved by SDS-PAGE, and protein bands were electrotransferred to a polyvinylidene difluoride membrane. The blots were blocked with 5% skim milk and incubated with a primary antibody, anti-calbindin 1 (Abcam, MA, USA), anti-aldolase C (Abcam), or anti-enolase 2 (Abcam) followed by an appropriate peroxidase-labeled secondary antibody. The blots were developed by ECL Plus Western Blotting Detection System (GE Healthcare Biosciences) and visualized by exposure to a X-ray film. The band intensity was quantified by the densitometric analysis using the ImageJ software (National Institutes of Health, MD, USA).

Statistical analysis

The statistical analysis was performed using the statistical software program SPSS, version 11.0 (SPSS, Inc., Chicago, IL, USA). The continuous variables such as each protein level in the CSF and the cardiopulmonary resuscitation (CPR) interval, which were not normally distributed, were reported as medians and interquartile ranges. Statistical testing for comparison among the 3 groups was done by the Kruskal-Wallis H test. Ad-hoc comparisons between 2 groups were done by Mann-Whitney U test. The p values were corrected for multiple testing by the Bonferroni method. The categorical variables, such as gender and number of bystander CPR, were reported as counts and percentages. Spearman rank correlation coefficients were calculated between GOS scores and each protein level in the CSF. A value of $p < 0.05$ was considered statistically significant.

Results

Patients' characteristics

One hundred and thirty of 430 patients with OHCA achieved ROSC. Ninety-four in 130 patients were admitted to our ICU and were assessed for their eligibility in this study over a period of 56 months. After the exclusion criteria were applied, 14 patients were enrolled in this study. However, we did not obtain a sufficient amount of CSF sample from one patient. Consequently, samples from 13 patients were analyzed in this study. The clinical characteristics of the enrolled patients are described in Table 1. Five patients achieved favorable neurological outcomes with GOS of good recovery (n = 2) and moderate disability (n = 3). The etiology of OHCA was cardiogenic in 4 of the 5 patients and the initial rhythm was ventricular fibrillation in 2 of the 4 patients. Eight patients had unfavorable neurological outcomes with persistent vegetative state (n = 3) or death (n = 5). The etiology of OHCA was cardiogenic in 2 of the 8 patients and the initial rhythm was ventricular fibrillation in 1 of the 2 patients. The cause of death in the 5 patients was severe cerebral dysfunction, but this was neither cardiogenic nor infectious. They had been in a vegetative state before they died.

CSF protein profile in patients with OHCA

The consecutive nanoflow LC-MS/MS analyses of in-gel or in-solution prepared tryptic digests of CSF proteins from 3 individuals generated a total of 6,961 MS/MS spectra. These spectra were assigned to 433 unique peptides that could be attributed to a total of 107 unique proteins. Major annotations assigned to these CSF proteins were "*extracellular*" (57.9%) or "*cytoplasm*" (15.9%) in cellular components, "*binding*" (29.0%) or "*transporter*" (16.8%) in molecular function,

Table 1. Characteristics of the patients investigated in this study.

	Patients (n = 13)
Age, yrs	
median	50
interquartile range	44 - 68
Gender	
Male, n (%)	11 (85)
Arrest witnessed, n (%)	7 (54)
Bystander CPR[a], n (%)	7 (54)
Collapse-to-start of CPR[a] interval, minute	
median	8
interquartile range	5 - 12
Start of CPR[a]-to-ROSC[b] interval, minute	
median	32
interquartile range	26 - 37
Cardiac etiology, n (%)	6 (46)
Initial rhythm, n (%)	
VF / VT[c]	3 (23)
PEA[d]	2 (15)
Asystole	8 (62)
Glasgow outcome scale, n (%)	
Good recovery	2 (15)
Moderate disability	3 (23)
Severe disability	0 (0)
Persistent vegetative state	3 (23)
Death	5 (38)

[a]CPR: cardiopulmonary resuscitation.
[b]ROSC: return of spontaneous circulation.
[c]VF: ventricular fibrillation; VT: ventricular tachycardia.
[d]PEA: pulseless electrical activity.

and "*transport*" (15%) or "*metabolism*" (10.3%) in biological process, indicating typical characteristics of body fluid proteins as expected from the nature of CSF (Figure 2A). Therefore, the list of total proteins identified was assumed to include a major subset of CSF proteins. The rule of peptide assignment between 433 peptide sequences and 107 proteins were consistently applied to the individual datasets, resulting in unambiguous identification of 49 proteins in a subject (N8) of group N, 45 proteins in a patient (F5) of group F, and 80 proteins in a patient (U3) of group U. Comparing annotations of identified proteins among 3 individuals, we observed noticeable differences in the cellular component category (Figure 2B). The number of identified proteins classified as "*extracellular*" or "*cytoplasm*" was greater in patient U3 (49 proteins and 17 proteins, respectively) than that in patient F5 (36 proteins and none, respectively) or subject N8 (36 proteins and 1 protein, respectively). When the cellular component category was simply divided into "*extracellular*" and "*intracellular*", the percentage of "*intracellular*" was greater in patient U3 (33%) than that in patient F5 (13%) or subject N8 (22%) (Figure 2B). Among 107 proteins, 23 proteins were identified in all 3 individuals, including known major CSF proteins, such as albumin and apolipoprotein E (Figure 2C). On the other hand, 63 proteins were found in only one of the three individuals (subject N8, 12 proteins; patient F5, 11 proteins; patient U3, 40 proteins) (Figure 2C). In contrast to the commonly identified proteins, 40 proteins identified only in patient U3 belonged to intracellular proteins in a greater

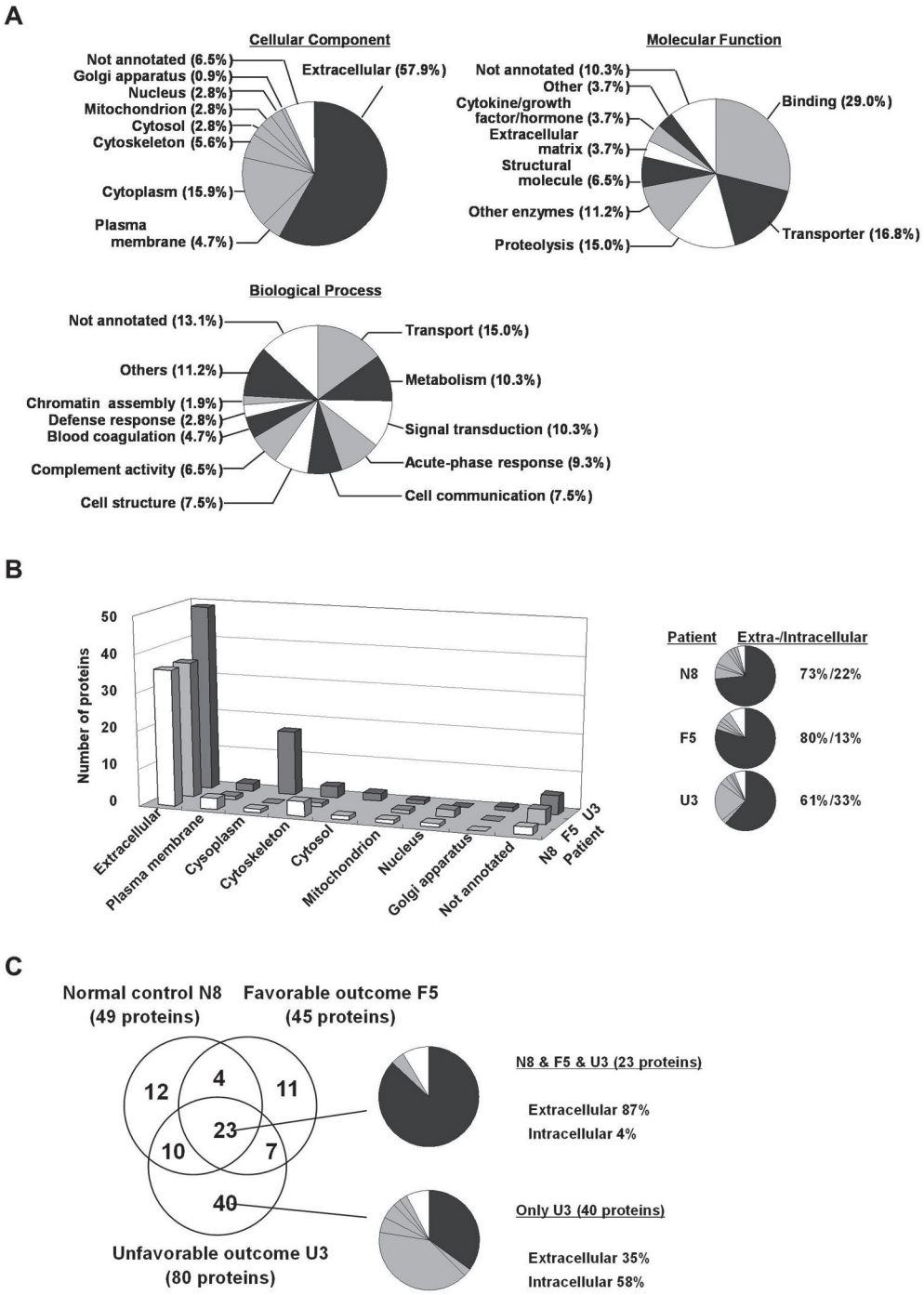

Figure 2. Bioinformatic characterization of the CSF proteins identified from 3 individuals exhibiting distinct neurological outcomes.

A total of 107 proteins identified from 3 individuals (N8, F5, and U3) were classified according to the gene ontology categories (A). Subcellular distribution (left), and the percentage of extracellular and intracellular proteins (right) were compared among 3 individuals (B). Venn diagram indicating the number of CSF proteins identified in each individual (C). The largest number (40 proteins) was found in the subset of proteins identified only in the group U patient (U3).

percentage (4% vs. 58%) (Figure 2C). Furthermore, these proteins specific to patient U3 included a number of biomarker candidates, such as acute-phase proteins, common metabolic enzymes, and brain-specific proteins (Table 2). To validate these results, aldolase C, calbindin 1 and enolase 2 that showed both higher number of unique peptide (≥ 2) and higher brain EST levels (≥ 0.1) were selected as candidates of group U-specific proteins.

Table 2. List of proteins specifically identified in the patient with unfavorable outcome.

Accession	Protein name	Protein mass (Da)	Unique peptides	Total spectra	Cellular component	Molecular function	Biological process	Brain EST level	Liver EST level
gi|4885063	aldolase C, fructose-bisphosphate	39,830	11	15	Mitochondrion	enzyme	metabolism	0.234	0.01
gi|4826655	calbindin 1	30,291	6	6	Cytoplasm	binding	metabolism	0.11	0.053
gi|21464101	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide	28,456	4	4	Cytoplasm	binding	signal transduction	0.063	0.012
gi|4557032	lactate dehydrogenase B	36,900	4	4	Cytoplasm	enzyme	metabolism	0.017	0.015
gi|21735625	tyrosine 3/tryptophan 5 -monooxygenase activation protein, zeta polypeptide	27,899	3	3	Cytoplasm	binding	signal transduction	0.021	0.021
gi|4504349	beta globin	16,102	3	3	Cytoplasm	transporter activity	transport	0.004	0.005
gi|5803225	tyrosine 3/tryptophan 5 -monooxygenase activation protein, epsilon polypeptide	29,326	3	3	Cytoplasm	binding	signal transduction	0.032	0.032
gi|16418467	leucine-rich alpha-2-glycoprotein 1	38,382	2	2	Extracellular	not annotated	not annotated	0.003	0.212
gi|4501887	actin, gamma 1 propeptide	42,108	2	2	Cytoskeleton	structural molecule	cell structure	0.021	0.013
gi|4504345	alpha 2 globin	15,305	2	2	Cytoplasm	transporter activity	transport	0	0
gi|4507951	tyrosine 3/tryptophan 5 -monooxygenase activation protein, eta polypeptide	28,372	2	2	Cytoplasm	binding	signal transduction	0.04	0.018
gi|5803011	enolase 2	47,581	2	2	Cytoplasm	enzyme	metabolism	0.196	0.003
gi|51460659	PREDICTED: similar to Ig kappa variable region	16,693	1	2	Not annotated	not annotated	not annotated	0	0
gi|10835095	serum amyloid A4, constitutive	14,854	1	1	Extracellular	transporter activity	acute-phase response	0	0
gi|11038662	complement component 1, q subcomponent, beta polypeptide precursor	26,915	1	1	Extracellular	binding	complement activity	0.015	0.014
gi|11056061	thymosin, beta 4	5,050	1	1	Cytoplasm	binding	cell structure	0.011	0.022
gi|13325072	phosphatidylinositol polyphosphate 5-phosphatase isoform a	105,392	1	1	Golgi apparatus	enzyme	cell structure	0.025	0.01
gi|13775198	SH3 domain binding glutamic acid-rich protein like 3	10,488	1	1	Cytoplasm	not annotated	not annotated	0.012	0.017
gi|17136078	VGF nerve growth factor inducible precursor	67,275	1	1	Extracellular	transporter activity	not annotated	N/A	N/A
gi|19557645	secretogranin III	52,973	1	1	Extracellular	not annotated	not annotated	0.208	0.005
gi|19923362	Thy-1 cell surface antigen	18,151	1	1	Plasma membrane	binding	not annotated	0	0
gi|21071030	alpha 1B-glycoprotein	54,790	1	1	Extracellular	not annotated	not annotated	0.017	0.489
gi|21071039	carnosinase 1	56,770	1	1	Cytosol	proteolysis	proteolysis and peptidolysis	0.389	0
gi|21359871	SPARC-like 1	76,057	1	1	Extracellular	binding	cell communication	0.091	0.001
gi|21361091	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	25,151	1	1	Cytoplasm	proteolysis	protein catabolism	0.06	0.017
gi|31542984	inter-alpha (globulin) inhibitor H4 (plasma Kallikrein-sensitive glycoprotein)	103,521	1	1	Extracellular	proteolysis	acute-phase response	0.009	0.551
gi|32490572	erythrocyte membrane protein band 4.1-like 3	121,458	1	1	Cytoplasm	structural molecule	cell structure	0.051	0
gi|40316910	serum amyloid A1 isoform 2	13,581	1	1	Extracellular	transporter activity	acute-phase response	0	0
gi|41058276	PREDICTED: similar to Triosephosphate isomerase (TIM)	27,211	1	1	Not annotated	not annotated	not annotated	0	0
gi|4502397	complement factor B preproprotein	86,819	1	1	Extracellular	proteolysis	complement activity	0.005	0.196
gi|4502511	complement component 9	64,615	1	1	Extracellular	binding	complement activity	0	0
gi|4502805	chromogranin A	50,829	1	1	Extracellular	binding	negative modulator of the neuroendocrine system	0.035	0
gi|4504067	aspartate aminotransferase 1	46,447	1	1	Cytoplasm	enzyme	metabolism	0.057	0.035
gi|4505763	phosphoglycerate kinase 1	44,985	1	1	Cytoplasm	enzyme	metabolism	0.013	0.042
gi|4506155	kallikrein 6 preproprotein	27,523	1	1	Extracellular	proteolysis	protein metabolism; protease	0.025	0
gi|4507509	tissue inhibitor of metalloproteinase 1 precursor	23,840	1	1	Extracellular	proteolysis	negative regulation of proteolysis	0.005	0.012
gi|45580727	microtubule-associated protein 1A	293,155	1	1	Cytoskeleton	structural molecule	microtubule stabilization	0.181	0.006
gi|51464757	PREDICTED: similar to Keratin, type I cytoskeletal 18 (Cytokeratin 18)	47,176	1	1	Not annotated	not annotated	not annotated	0	0
gi|5174539	cytosolic malate dehydrogenase	36,631	1	1	Cytosol	enzyme	metabolism	0.02	0.016
gi|5803227	tyrosine 3/tryptophan 5 -monooxygenase activation protein, theta polypeptide	28,032	1	1	Cytoplasm	binding	signal transduction	0.024	0.015

N/A: Not assigned.

Quantitative screening of biomarker candidates and their relationship with GOS scores

To verify the levels of calbindin 1, aldolase C and enolase 2, CSF samples from 8 subjects in group N, 5 patients in group F, and 8 patients in group U were examined by western blot analysis. Strong band signals for calbindin 1 were observed in group U, but not in group N or group F (Figure 3A). This tendency was also apparent for aldolase C and enolase 2 (Figure 3A). The median level of calbindin 1 in CSF was significantly higher in group U (75% of U3) than in group N (0% of U3) ($p = 0.001$) or in group F (2% of U3) ($p = 0.005$), and also higher in group F than in group N ($p = 0.003$) (Figure 3B). The median level of enolase 2 in CSF was significantly higher in group U (154% of U3) than in group N (2% of U3) ($p = 0.008$) or in group F (0%) ($p = 0.003$) (Figure 3D). The median levels of aldolase C and total protein were higher in group U (91% for aldolase C and 1,414 μg/ml for total protein) than in group N (22% for aldolase C and 437 μg/ml for total protein) or group F (23% for aldolase C and 554 μg/ml for total protein) (Figures 3C and 3E). However, these trends were not statistically significant. The GOS scores at 6 months after the ROSC were significantly correlated with the levels of calbindin 1 ($r_s = 0.73$, $p = 0.005$) and enolase 2 ($r_s = 0.851$, $p < 0.001$), but were not correlated with the levels of aldolase C in the OHCA patients (Figures 4A and 4B).

Discussion

This pilot study demonstrated the application of LC-MS/MS-based proteomic analysis to identify neurological prognostic markers in CSF from patients resuscitated from OHCA. First, we performed direct nanoflow LC-MS/MS analysis of CSF proteins on 3 individuals having distinct neurological outcomes, and identified total 107 proteins, including 40 proteins specific to one patient (U3) having unfavorable outcome. To obtain promising biomarker candidates, 3 proteins were then narrowed down from the 40 proteins based on their peptide hit numbers and expression levels in brain. The results were verified quantitatively by western blotting on all 21 individuals enrolled in this study, resulting in identification of calbindin 1 as a potential early predictor of poor neurological outcomes. To our knowledge, this is the first study to investigate CSF proteins in patients resuscitated from OHCA using a LC-MS/MS-based approach. The analytical platform established in this study appeared to have several advantages. First, the direct nanoflow LC-MS/MS system used in this study was equipped with a fritless microbore capillary column and a splitless nanoflow gradient elution system,[14] enabling rapid, high-resolution, and sensitive detection of early prognostic biomarkers, i.e., brain-derived proteins in CSF 48 hours after ROSC. Second, peptide hit quantification method, which is much simpler to implement than quantification by conventional 2-dimensional gel electrophoresis method,[17] provided useful information on protein abundance for the candidates selection from the list of identified proteins in this study. Third, although a small number of CSF samples were examined by the proteomic approach, bioinformatic analyses of identified proteins with respect to their gene annotations and organ distributions allowed efficient selection of biomarker candidates for the following quantitative screening.

We identified 40 proteins that were specific to the CSF of one patient who exhibited neurologically poor outcome. Among these proteins, alpha 1B-glycoprotein, SPARC-like 1, kallikrein 6 preprotein, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (also known as 14-3-3 protein), and ubiquitin C-terminal hydrolase-L1 have been already reported as biomarker candidates in CSF for several types of neurological diseases, such as Alzheimer's disease, multiple sclerosis, Parkinson's disease, and Creutzfeldt-Jakob disease.[18-20] Although these disease-related proteins appeared to be reasonable biomarker candidates for prognosis of OHCA, we did not verify them further because these proteins were identified with a single peptide hit or their expression levels in brain were not predominant (Table 2). When pooled CSF from the normal control subjects was analyzed in triplicate by the LC-MS/MS system, overlap of identified proteins between analyses was in the range of 75% ~ 81%, and 6% ~ 14% were newly identified in

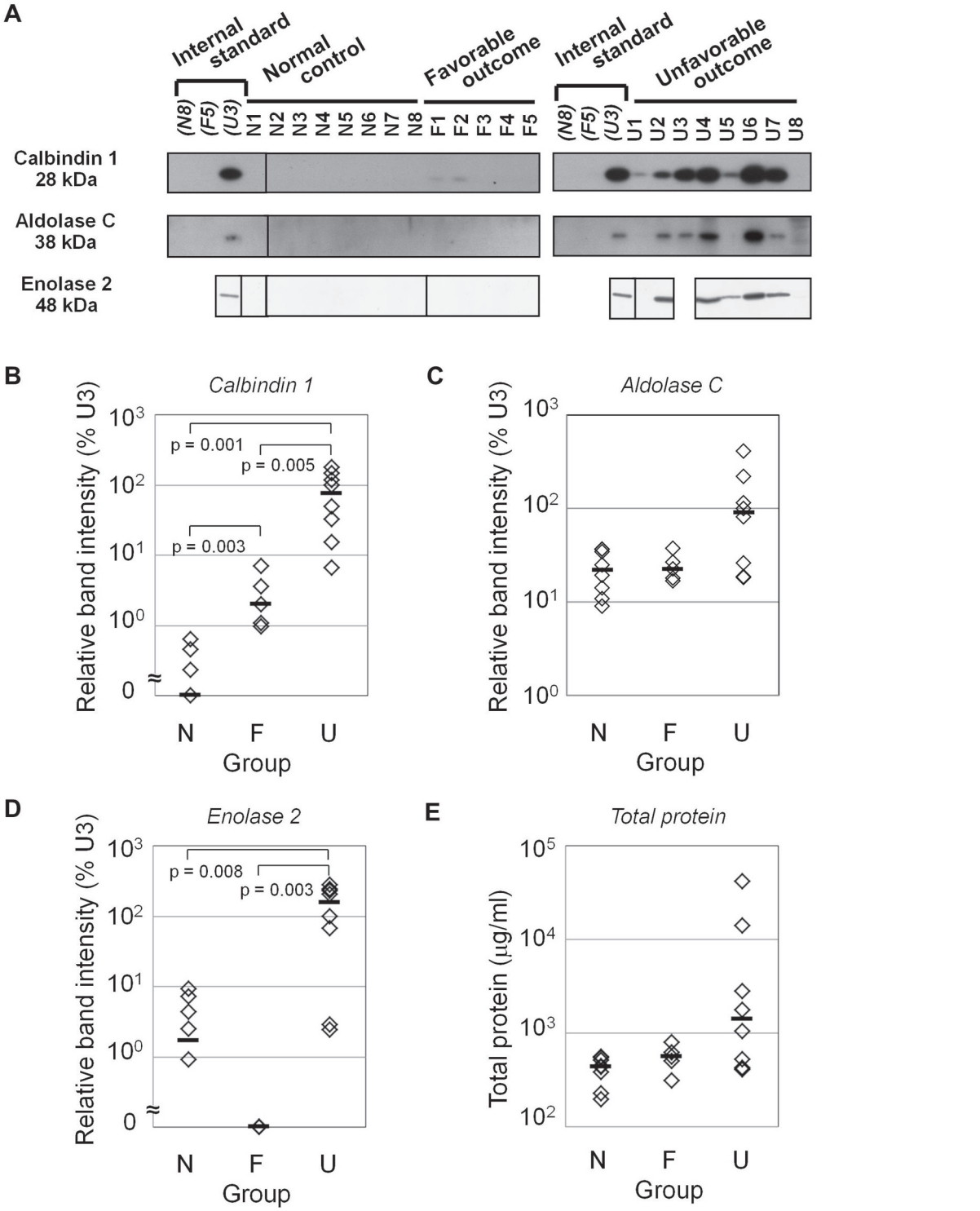

Figure 3. Verification of calbindin 1, aldolase C, and enolase 2 levels in CSF by western blot analysis.

Western blot analysis was performed with CSF samples from 8 subjects in group N, 5 patients in group F and 8 patients in group U (A). Blots were arranged in order of subject identifiers to compare the results directly. For enolase 2, U3 was included only in the lane for the internal standard. The levels of calbindin 1 (B), aldolase C (C), and enolase 2 (D) in individual subjects were plotted as relative band intensities (% of U3). Total protein levels were measured by the Bradford assay (E). Median values for individual groups were represented by horizontal bar and *p* values were determined by Mann-Whitney U test with Bonferroni correction (B-E).

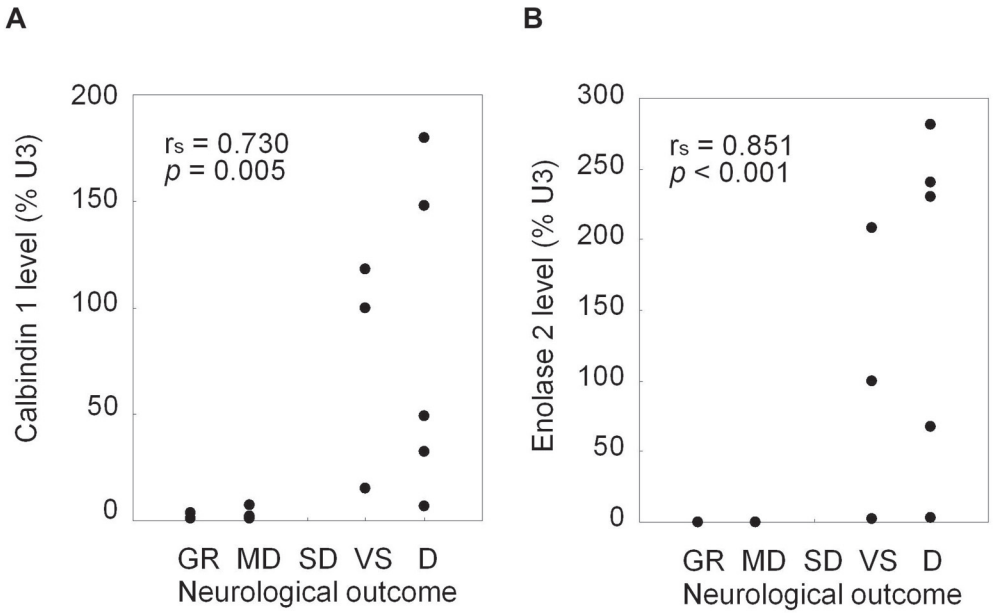

Figure 4. The association between the GOS scores and the calbindin 1 levels (A) or enolase 2 levels (B) in CSF in all 13 patients resuscitated from OHCA. The GOS scores were significantly and positively associated with the calbindin 1 levels and enolase 2 levels (Spearman rank correlation coefficient: $r_s = 0.730$, $p = 0.005$, and $r_s = 0.851$, $p < 0.001$, respectively). GR, good recovery; MD, moderate disability; SD, severe disability; VS, persistent vegetative state; D, death.

each analysis (data not shown). Thus, we did not exclude the possibility that some of low abundance proteins specific to one patient could be detectable in another patient. In contrast, proteins that were identified with multiple peptide hits, i.e., calbindin 1, aldolase C, and enolase 2, were also detectable from only one of three individuals by western blotting, confirming the results of protein identification by the LC-MS/MS analysis. From the reasons above, we selected calbindin 1, aldolase C, and enolase 2, as promising biomarker candidates for verification by quantitative screening.

Calbindin 1 is a calcium-binding protein involved in the regulation of intracellular calcium in the central nervous system.[21] Our study showed that higher calbindin 1 levels in CSF were associated with worse neurological outcomes in patients resuscitated from OHCA. In addition, proteomic comparisons between antemortem and postmortem specimens have showed a significant increase of calbindin 1 in the postmortem CSF.[22] Therefore, the increased level of calbindin 1 in CSF may reflect the progression of ischemic neuronal cell death resulting from OHCA. The expression level of calbindin 1 in the cerebellum is approximately 10 times higher than that in other CNS regions,[23] and is known to be useful in estimating the damage to Purkinje cells in cerebellar diseases.[24] Since enolase 2 is expressed predominantly in neurons and neuroendocrine cells,[25] a combination of calbindin 1 and enolase 2 levels in CSF might be applicable to estimating the region of the brain damage caused by OHCA. Aldolase C was also identified exclusively in the group U patient (U3) although the statistical significance was not demonstrated in the western blot analyses possibly due to low signal (or high background) intensities. Therefore, association of aldolase C levels with unfavorable outcomes should be re-examined by using an alternative antibody with higher sensitivity in the future.

This pilot study revealed a couple of issues regarding proteomics-based biomarker discovery in patients resuscitated from OHCA. First, protein composition in CSF seems to be quite different among the 3 individuals. Thus, biomarker candidates identified by

analyzing CSF from patients with OHCA should be verified extensively to exclude possible individual variations. Second, total protein concentrations of the CSF samples obtained from the group U patients were distributed over an extremely wide range (up to 100-fold), despite no obvious blood contamination. Higher protein concentrations can affect the efficiency of tryptic digestion, deteriorate resolution in electrophoretic or chromatographic separation, and often reduce the number of identified proteins. Considering the diversity of etiologies in patients with OHCA, these might be unavoidable issues in proteomics-based biomarker discovery. To overcome these issues, the extensive analyses of a well-characterized pooled sample appear to be more productive than analyzing individual samples. Repeated analyses of a pooled sample can compensate the low reproducibility of current MS technology and minimize the high variability of protein profiles in biological samples of individual patients. By combining the analytical platform with the quantitative approach using isobaric tag,[26] the mass spectrometry-based proteomic analysis will be valuable in the characterization of CSF proteins in patients resuscitated from OHCA.

Conclusion

The analytical platform using a combination of nanoflow LC-MS/MS and western blot screening is applicable to analyzing CSF samples from critical care patients. Calbindin 1 identified in this study would be a good candidate for the prognostic marker of unfavorable outcomes in patients resuscitated from OHCA. The achievement of this pilot study will contribute to future full-scale comparative studies on CSF proteomes derived from patients with OHCA, thereby further promoting identification of valuable molecules associated with and/or influencing on patients' neurological outcomes.

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Conflict of Interest

Yuki Akiyoshi is an employee of Nano-Solution, Inc. and Shin Nippon Biomedical Laboratories, Ltd. The other authors state no conflict of interest.

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