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ORIGINAL ARTICLE

From Analysis of Ischemic Mouse Brain Proteome to Identification of Human Serum Clusterin as a Potential Biomarker for Severity of Acute Ischemic Stroke

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Abstract

Ischemic stroke is a devastating neurological disease that can cause permanent brain damage, but to date, few biomarkers are available to reliably assess the severity of injury during acute onset. In this study, quantitative proteomic analysis of ischemic mouse brain detected the increase in expression levels of clusterin (CLU) and cystatin C (CST3). Since CLU is a secretary protein, serum samples ($n = 70$) were obtained from acute ischemic stroke (AIS) patients within 24 h of stroke onset and together with 70 matched health controls. Analysis of CLU levels indicated significantly higher levels in AIS patients than healthy controls (14.91 ± 4.03 vs. 12.79 ± 2.22 ng/L; $P = 0.0004$). Analysis of serum CST3 also showed significant increase in AIS patients as compared with healthy controls (0.90 ± 0.19 vs. 0.84 ± 0.12 ng/L; $P = 0.0064$). The serum values of CLU were also positively correlated with the NIH Stroke Scale (NIHSS) scores, the time interval after stroke onset, as well as major stroke risk factors associated with lipid profile. These data demonstrate that elevated levels of serum CLU and CST3 are independently associated with AIS and may serve as peripheral biomarkers to aid clinical assessment of AIS and its severity. This pilot study thus contributes to progress toward preclinical proteomic screening by using animal models and allows translation of results from bench to bedside.

Keywords Biomarkers · Ischemic stroke · Clusterin · Cystatin C · Proteomics

Introduction

Stroke occurs upon sudden disruption of the brain's blood supply and is a leading cause of permanent disability in the aging population. Acute ischemic stroke (AIS) is a devastating neurological condition that accounts for approximately 87% of all stroke cases [1–3]. If adequate circulation is restored within a short period of time, the ischemic brain tissue in affected

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regions can be potentially salvageable. However, brain damage is permanent if the restoration of circulation is delayed [4]. Therefore, early detection of the severity of stroke is critical to allow better outcomes and improved care for AIS patients.

Biomarkers can be used to evaluate AIS, either by suggesting a prognosis or providing an explanation for a poor outcome [5]. Over recent decades, significant work has led to class I and II recommendations using low-density lipoprotein-cholesterol, hemoglobin A1c, and C-reactive protein as biomarkers for stroke prevention. Despite this effort, developing biomarkers that are both sufficient and specific for improving the diagnosis/prognosis and stratifying the severity for AIS remains challenging [6]. Despite that some biomarkers have been explored for patients with global brain ischemia [2, 7, 8], only a few potential serum biomarkers are related to the pathophysiology of ischemic stroke, including glial fibrillary acidic protein (GFAP) and S100 calcium-binding protein B [2]. Additionally, some of these candidate biomarkers have only been examined in a research setting, rather than in clinical practice. Therefore, there is an urgent need to identify potential biomarkers related to ischemic stroke pathophysiology. With careful validation, these biomarkers will be invaluable to early assessment and thus improving AIS patient care.

Proteomics is a valuable tool that can be used to improve the capabilities of biomarker discovery and evaluation [9]. There is significant interest in a systems biology approach using proteomic analysis to identify biomarkers related to pathogenesis of post-AIS brain damage. Quantitative proteomics enable accurate examination a variety of biospecimens for their protein content and can help to delineate global biochemical activity. Bioinformatic advances are also critical for meaningful interpretation of the large proteomic datasets. Over the past decade, quantitative proteomics and machine learning–based bioinformatic tools have been used to study cerebrovascular disease by evaluating a complex protein mixture to identify biological pathways [10–12]. Furthermore, proteomics has been successful in identification of potential and novel biomarkers for therapeutic and diagnostic use, including thrombospondin-1, cystatin A, and fatty acid binding protein [13–15].

In this study, an initial quantitative proteomic analysis, involving liquid chromatography/tandem mass spectrometry (LC-MS/MS) with deuterium isobaric amine reactive tag (DiART) labeling, was carried out to examine proteins in mouse brain subjected to global cerebral ischemia [16, 17]. Our analysis detected changes in expression levels of clusterin (CLU) and cystatin C (CST3). CLU is a secretory protein that inactivates members of the cathepsin family of cysteine proteases and has been identified associated with Alzheimer's disease [18–20]. CST3 is previously used as a serum marker of renal function and has been reported as a strong predictor of the risk of cardiovascular events and death in stroke [21–23]. However, the role of CLU and CST3 in AIS patients and their relationship with stroke severity have not been fully evaluated. In this study, we examined these two candidate marker proteins in serum samples from patients with AIS. Continued research in AIS biomarkers will help to develop effective diagnostic techniques and treatment for patients.

Methods

Additional details of the methods for proteomic and bioinformatic analysis are provided in the Supplemental Methods.

Animal Handling for the BCCAo Procedure

A total of 12 male C57Bl/6J mice (The Jackson Laboratory, Bar Harbor, ME) at 4 months of age were used in this study and housed under 12-h light/dark cycle ad libitum conditions. The bilateral common carotid artery occlusion (BCCAo) surgery was performed as previously described [24]. Surgical protocols were carried out in accordance with the MU-approved protocols for the Care and Use of Laboratory Animals. The rotarod behavioral test was used to assess motor coordination and balance alterations 24 h after ischemia, as described previously [24]. The rotarod data were used to guide us to select animals for quantitative proteomics analysis and to assist in identification of protein marker surrogates. Animal experiments were independently conducted by two investigators, and data were analyzed in a double-blind manner.

Quantitative Proteomic Method

Protein sample preparation was performed as previously described [25]. For protein isolation, 100 mg (wet weight) of cortex, striatum, and hippocampus was homogenized on ice, respectively, with a tissue tearer in 600 μL lysis buffer (8 M urea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 10 mM dithiothreitol (DTT), and 40 mM Tris−HCl, pH = 8.0). The proteins were reduced with 10 mM DTT and then alkylated by 50 mM iodoacetamide (IAA) in the dark at room temperature. The treated proteins were precipitated in 80% acetone at − 20 °C overnight and redissolved in 0.8 M urea and 500 mM tetraethylammonium bicarbonate (TEAB), pH 8.5. After centrifuging at 15,000*g* at 4 °C for 30 min, an aliquot of the supernatant was taken for determination of protein concentration by the BCA assay. The proteins in TEAB were digested by trypsin at 37 °C, and the solvent was removed by Speedvac.

DiART labeling process was performed as previously described [17]. Briefly, DiART reagents were dissolved into anhydrous acetonitrile and added to trypsinized protein solution in the lysis buffer (pH 8.5). The labeling reaction was maintained for 2 h at room temperature. The reaction was quenched by adding 10 μL 5% ethanolamine dissolved in water. Next, strong cation-exchange chromatography (SCX) was performed. Then,

the mixed sample was dried in a Speedvac, and the labeled peptides were ready for LC-MS/MS.

Protein identification by LC-MS/MS was followed as previously described [26]. Briefly, a portion of the digest (5 μL) was loaded onto a C8 trap column (C8 CapTrap – Michrom Bioresources). Bound peptides were eluted from this trap column onto an analytical column packed with Magic C18 reversed phase resin (Michrom Bioresources). Peptides were separated and eluted from the analytical column with a gradient of acetonitrile. The Proxeon Easy nLC system is attached to an LTQ Orbitrap XL mass spectrometer. Following a high-resolution (30,000 res, profile) Fourier transform mass spectrometry (FTMS) scan of the eluting peptides (300–2000 *m*/*z* range), each cycle, the five most abundant peptides (reject trypsin autolysis ions) were subjected to both collision-induced dissociation (CID) peptide fragmentation (> 2500 counts, normalized collision energy (NCE) of 35%, centroid) and higher collision energy dissociation (HCD) (> 3000 counts, 7500 resolution, NCE of 45%, profile, 1st mass fixed at 100 *m*/*z*) for quantitation of the DiART sixplex-tagged peptides. Data across a total of 140 min of elution were collected. MS/MS spectra were searched against the Swiss-Prot mouse database using the Mascot 2.3.0 search engines with search type MIS. Fixed modifications were carbamidomethyl (C), DiART sixplex (K), and DiART sixplex (N-term). Variable modifications included oxidation (M). Resulting peptide hits were filtered for a maximum 5% false discovery rate. The DiART sixplex quantification method was used to calculate the reporter ratios with a peptide mass tolerance ± 0.05 Da and a fragment mass tolerance ± 0.5 Da (Supplementary Tables S1, S2, and S3).

Further details are provided in Supplemental Methods.

Recruitment of Ischemic Stroke Patients and Collection of Serum Samples

Ischemic stroke patients ($n = 70$) were recruited within 24 h of stroke onset. Control subjects ($n = 70$) were age- and comorbidity-matched. Institutional Review Board (IRB) in the Second People's Hospital of Fujian Province approved the protocol, and the subjects gave informed consent. Inclusion criteria include (1) age 18 years or older, (2) diagnosis of ischemic stroke with a measurable neurological deficit, and (3) onset of symptoms < 24 h before treatment begins. Exclusion criteria include significant head trauma or prior stroke in the previous 3 months; symptoms suggest subarachnoid hemorrhage; history of previous intracranial hemorrhage; intracranial neoplasm or aneurysm; recent intracranial or intraspinal surgery; active internal bleeding; acute bleeding diathesis; blood glucose concentration < 2.8 mmol/L; CT demonstrates multilobar infarction (hypodensity > 1/3 cerebral hemisphere); age > 80; in accordance with the guideline [27]. Neurological function after stroke was evaluated using the NIHSS score to assess stroke severity [28]. Diagnosis was confirmed by magnetic resonance imaging (MRI) and/or computed tomography (CT). Serum samples were obtained from participants and flash frozen at − 80 °C. Biochemical parameters were assayed using a Cobas® 8000 modular analyzer. All experimental groups and sample analysis were handled in a randomized, double-blind manner.

CLU and CST3 Measurement

Human CLU was measured using the enzyme-linked immunosorbent assay (ELISA) from BioVendor (Cat. No. RD194034200R) [29], and CST3 was measured using the CYS-C kit (Ningbo MedicalSystem Biotechnology Co., Ltd., Zhejiang, China) [30], according to the manufacturer's protocols. All 70 patients' serum samples were evaluated for both tests. For control group, 70 subjects' serum were evaluated for CST3, but only 53 subjects' serum were measured for CLU due to the limit number of tests could be performed using the CLU ELISA kit.

Regression Analysis

Regression analysis was performed to discover the significant relationship between concentration of CLU/CST3 with NIHSS score and time interval after stroke. Square root data normalization and Shapiro–Wilk test was conducted to check the normality of data (Supplemental Table 13). Linear regression models were fitted on the concentration of CLU (Supplemental Table 14) and CST3 (Supplemental Table 15), represented by data $\sqrt{\text{NIHSS}}$ and $\sqrt{\text{Time}}$. The regression model on CLU level can be fitted as

$$\widehat{\text{Clusterin}} = 10.7681 + 1.9034^{*}\sqrt{\text{NIHSS}} - 0.6709^{*}\sqrt{\text{Time}}$$

where the *P* of NIHSS and time interval after stroke is 0.03522 and 0.09134, respectively, and the *P* of model is 0.02837. Supplemental Table 14 showed concentration of CLU had positive correlation with NIHSS and inverse correlation with time interval after stroke. The regression plane can be viewed from Fig. 3c. Supplemental Table 15 shows the coefficients analysis of the regression model, where the concentration of CST3 can be expressed as

$$\widehat{\text{Cystatin C}} = 0.92735 + 0.02168^{*}\sqrt{\text{NIHSS}} - 0.02678^{*}\sqrt{\text{Time}}$$

Due to the non-significant *P* of coefficients and model, we can consider no direct relationship between the concentration of CST3 with NIHSS and time interval after stroke. The regression plane can be viewed from Fig. 3d. Further detail can be found at Supplemental Method.

Statistical Analysis

Unpaired t test was used to calculate *P* values for CLU and CST3 clinical data. Pearson correlation was used for linear regression

analysis of CLU or CST3 with triglyceride, total cholesterol, high-density lipoprotein (HDL), and low-density lipoprotein (LDL). Statistical analysis and graphs were made by Prism (GraphPad Software). $P < 0.05$ was considered statistically significant.

Results

Quantitative Proteomic Analysis of Protein Candidates from Ischemic Mouse Brain

BCCAo animal model has been used for ischemia/reperfusion studies because the transient lack of blood flow to major brain subregions without physical damage [24, 31, 32]. In order to identify potential biomarkers associated with cerebral ischemia, a quantitative proteomic analysis of protein candidates from sham control and ischemia mice.

Protein samples were labeled using the DiART sixplex reagents, as described previously [33] (Supplemental Fig. S1). Then, LC-MS/MS was used to characterize reporter information for a representative peptide. Using data from the proteomic analysis, we compared differentially expressed (DE) proteins between sham control and ischemia groups (with fold change in expression > 1.3 or < 1/1.3, $P < 0.05$) (Supplemental Fig. S2). We identified a total of 1838 proteins in cortex, 1809 in hippocampus, and 1718 in striatum in both control and ischemia groups. Compared to the sham group, the moderate ischemia group differentially expressed 44 proteins in cortex, 42 in hippocampus, and 26 in striatum (Fig. 1, Supplemental Fig. S3, and Supplemental Tables S4, S6, and S8). The severe ischemia group had significant changes in expression levels in cortex (91

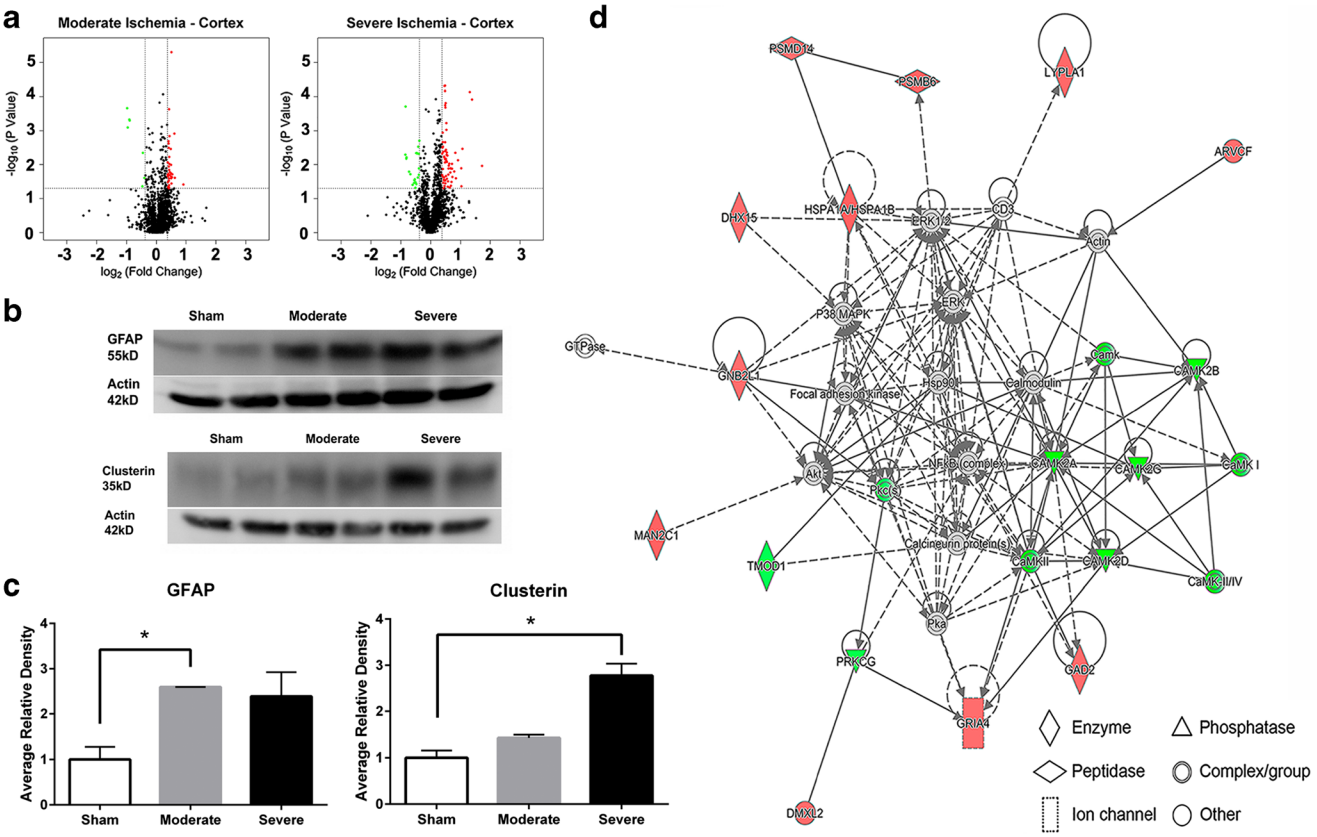

Fig. 1 Total protein changes and identification of signaling pathways in cortex in cerebral ischemia. **a** Volcano plots generated to compare all DE proteins in cortex in either moderate ischemia or severe ischemia group, compared to the sham control group. Proteins that have a fold change difference of > 1.3 or < 1/1.3 and a $P < 0.05$ were defined as significantly different. Volcano plots were generated by R statistical software. Dashed lines indicate the thresholds of $\log_2$ (fold change) and $-\log_{10}$ (P). The identified proteins are displayed as dots in red (upregulation with significant changes), green (downregulation with significant changes), or black color (no significant change). **b**, **c** Western blot validation and quantification of CLU and GFAP in the cortical tissues of sham surgery, moderate, and severe ischemia groups. GFAP expression was significantly different between sham and moderate ($P = 0.0186$). CLU expression was significantly different between sham and severe ($P = 0.0209$). Data are expressed as mean ± SEM and were analyzed by unpaired t test. **d** Post-translational modification, cell signaling, and molecular transport network in the moderate ischemia group. The identified proteins involved in the networks are displayed in red (upregulation) and green (downregulation) color. The color intensity indicates the degree of regulation. Solid lines in the network imply direct interactions between proteins and dashed lines indicate indirect interactions. Proteins in gray are part of our data set, but according to our data analysis setting (fold change > 1.3 or < 1/1.3; $P < 0.05$), they are not significantly expressed. White proteins are not part of our data set but have relationships with our proteins in the network

proteins), hippocampus (90 proteins), and striatum (85 proteins), compared with the sham group (Supplemental Tables S5, S7, and S9). Changes in expression levels of individual proteins appear to be brain region-specific, as illustrated by Venn diagram (Supplemental Fig. S5). In the cortex, hippocampus, and striatum of ischemia groups, expression levels of proteins of interest were changed as compared to sham control, including increases of heat shock 70 kDa protein 1A (HSPA1A), CLU, CST3, GFAP, vimentin (VIM), and apolipoprotein E (APOE) (Fig. 1a–c and Supplemental Figs. S3–S5). Western blot validation confirmed that cortical tissue protein expression levels of GFAP, a previously known potential biomarker of ischemic stroke, and CLU, a molecular chaperone involved in diseases related to oxidative stress, as indicated in the LC-MS/MS results (Fig. 1b, c). Principal component analysis (PCA) of expression levels of the identified proteins verified that each replicate for the individual groups strongly clustered together. These data indicate the consistency and reproducibility of our biological replicates (Fig. 2 and Table S12).

To further obtain an overview of the functions of DE proteins, machine learning-based ingenuity pathway analysis (IPA) was used to analyze top functional annotation, pathway analysis, and protein–protein interaction networks as previously described [34]. IPA identified pathways involved in inflammation and oxidative stress, including chemokine signaling, 14-3-3 mediated signaling, Nrf2 mediated antioxidant response, oxidative phosphorylation, and mitochondrial dysfunction (Supplemental Table S11). IPA also helped to identify top disease/functional networks related to post-translational modification, cell signaling, and molecular transport with involvement particularly with protein candidates CLU and CST3 (Fig. 1d, Supplemental Figs. S6 and S7). Initial proteomic identification and IPA indicated that CLU and CST3 had a relationship with ischemic injury of different brain regions. In the severe ischemia group, we observed that the expression level of cortical CLU was higher than in the moderate ischemia group (2.09 vs. 1.30, respectively). Additionally, significant fold changes in CST3 expression were found between the cortex (1.54) and striatum (1.22). (More details regarding the pilot quantitative proteomics profiling and identifications are provided in the supplemental result section.)

Evaluation of CLU and CST3 as Potential Biomarkers

The combination of signaling pathway analysis and network provides a rational and comprehensive approach to prioritizing potential candidates for validation based on existing biological knowledge [35, 36]. Using this approach, we further examined candidate proteins that had statistically significant fold changes in protein expression in pathways and networks of interest [14]. Initial proteomic identification and IPA indicated that expression of CLU and CST3 were upregulated in different regions of the ischemic mouse brain.

Based on results from the mouse ischemia model, we sought to evaluate the potential use of these two proteins as biomarkers in human patients with AIS. A preliminary testing indicated presence of both proteins in human sera. Subsequently, human serum samples were obtained for measurement of CLU and CST3 levels in age- and comorbidity-matched control subjects and in AIS patients within 24 h of stroke onset. As compared with controls, AIS patients showed a higher prevalence of hypertension, increased levels of prothrombin time, international normalized ratio (INR), LDL, and decreased level of HDL (Table 1).

Results of analysis of serum CLU indicated significantly higher levels in AIS patients as compared with controls (14.91 ± 4.03 ng/L in AIS patients vs. 12.79 ± 2.22 ng/L in control; $P = 0.0004$; Fig. 3a). Analysis of serum CST3 also indicated significantly higher levels in AIS patients as compared with controls (0.90 ± 0.19 mg/L in AIS vs. 0.84 ± 0.12 mg/L in control; $P = 0.0064$; Fig. 3b). Next, regression analysis was performed to examine the relationships of CLU / CST3 concentrations,

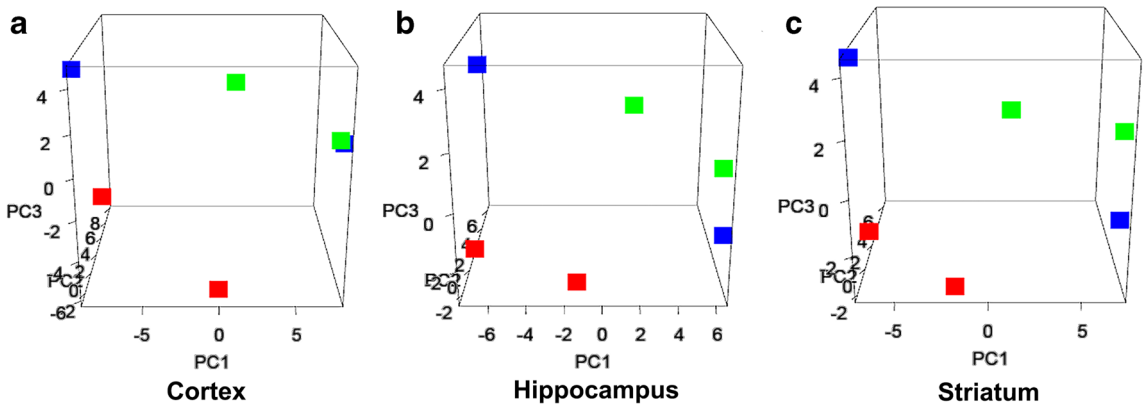

Fig. 2 Strong clustering for identified proteins using PCA analysis. PCA analysis showed that all the identified proteins profile effectively separated sham (red) from BCCAo animals (moderate: blue; severe: green) in cortex (**a**), hippocampus (**b**), and striatum (**c**) regions. For cortex, axis: PC1: PCA component 1 (72.8% variance); PC2: PCA component 2 (86.6% variance); PC3: PCA component 3 (95.7% variance). For hippocampus, axis: PC1 71.1% variance; PC2 86.8% variance; PC3 96.1% variance. For striatum, axis: PC1 75.5% variance; PC2 90.5% variance; PC3 95.8% variance (detailed analysis in Table S12)

Table 1 Clinical characteristics of cerebral ischemic stroke patients

Characteristic (mean ± SD)	Stroke	Control	*P*
No. of patients	70	70	NA
Age (years)	63 ± 11	59 ± 8	***
Male sex, no., (%)	47 (67)	35 (50)	NA
Severe hypertension, no. (> 180/120 mmHg)	8	0	NA
Heart rate (bpm)	76 ± 11	73 ± 9	*
Glucose (mmol/L)	7.6 ± 3.8	5.5 ± 1.0	****
Blood urea nitrogen (mmol/L)	5.1 ± 2.0	5.0 ± 1.1	NS
Creatinine blood test (umol/L)	66.6 ± 17.9	72.7 ± 11.0	***
Red blood cell (10^{12}/L)	7.3 ± 3.2	5.7 ± 1.2	****
Hemoglobin (g/L)	138.6 ± 20.1	145.6 ± 13.3	**
Platelet count (10^{9}/L)	228.5 ± 76.7	231.3 ± 53.7	NS
Prothrombin time (s)	12.8 ± 2.3	12.0 ± 1.0	**
International normalized ratio (INR)	1.1 ± 0.3	1.0 ± 0.2	**
Activated partial thromboplastin time (s)	32.8 ± 6.5	35.0 ± 3.5	**
Fibrinogen (g/L)	3.5 ± 1.1	3.3 ± 0.7	NS
Triglyceride (mmol/L)	1.4 ± 0.8	1.2 ± 0.6	NS
Total cholesterol (mmol/L)	4.8 ± 1.3	5.3 ± 1.1	**
High-density lipoprotein (mmol/L)	1.1 ± 0.3	1.3 ± 0.3	**
Low-density lipoprotein (mmol/L)	3.3 ± 1.2	3.1 ± 0.8	NS
NIHSS	13.8	0	****
Modified Rankin Scale (mRS)	0.07	0	*
Time interval after stroke	< 24 h (2–24)	NA	NA

INR is based on the ratio of the patient's prothrombin time and the normal mean prothrombin time

NA not applicable

NS: $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$

NIHSS scores, and time interval after stroke onset. Within 24 h, higher NIHSS score correlated with increased serum CLU levels (CLU serum level vs. NIHSS score: $P = 0.03522$, CLU serum level vs. time interval: $P = 0.09134$; multiple $R^2 = 0.10$), whereas, in the case of CST3, no direct relationship was observed with NIHSS scores ($P = 0.590$) or time interval ($P = 0.135$) (Fig. 3c, d; Supplemental Tables S13–S15).

We further implemented Pearson correlation analyses to measure the strength and direction of the linear relationship between CLU levels and concentration of biochemical parameters such as stroke risk factors that are characteristically associated with the lipid profile, including triglycerides, total cholesterol, HDL, and LDL. Results show that the CLU levels were positively correlated with total cholesterol ($r = 0.32$, $P = 0.0032$) and LDL ($r = 0.30$, $P = 0.0054$) levels in the AIS patients (Fig. 4), whereas CST3 level was not significantly correlated with any of those factors (Fig. 5).

Discussion

Using an initial quantitative proteomics study, we demonstrated the feasibility of screening and identifying DE proteins in different regions of mouse brain subjected to global cerebral ischemia. Our proteomic analysis protocol offered a more accurate quantification as compared to the semi-quantitative label-free method. This preliminary study also revealed several distinct canonical pathways and intracellular signaling networks involved in ischemic injury in mice. IPA indicated that this type of ischemic stroke is associated with inflammatory response, neuronal cell death, and movement disorder pathways. In addition, our results identified a number of proteins involved in a variety of cellular functions, including CLU (cytoprotective chaperone), CST3 (proteinase inhibitor), GFAP and VIM (cytoskeletal markers), and HSPA1A (stress/inflammatory protein), could serve as potential biomarkers of ischemic stroke. Our data are consistent with previous findings suggesting that ischemic insult is associated with an overall down-regulation of beneficial neurotrophic molecules and an upregulation of inflammatory signaling proteins and cytoskeletal components [14, 37].

Biomarkers are important tools for disease diagnosis and for determining severity and prognosis of the disease. Peripheral blood biomarkers are particularly desirable because they are readily available and can be monitored and analyzed in clinical pathology laboratories [38, 39]. To date, potential

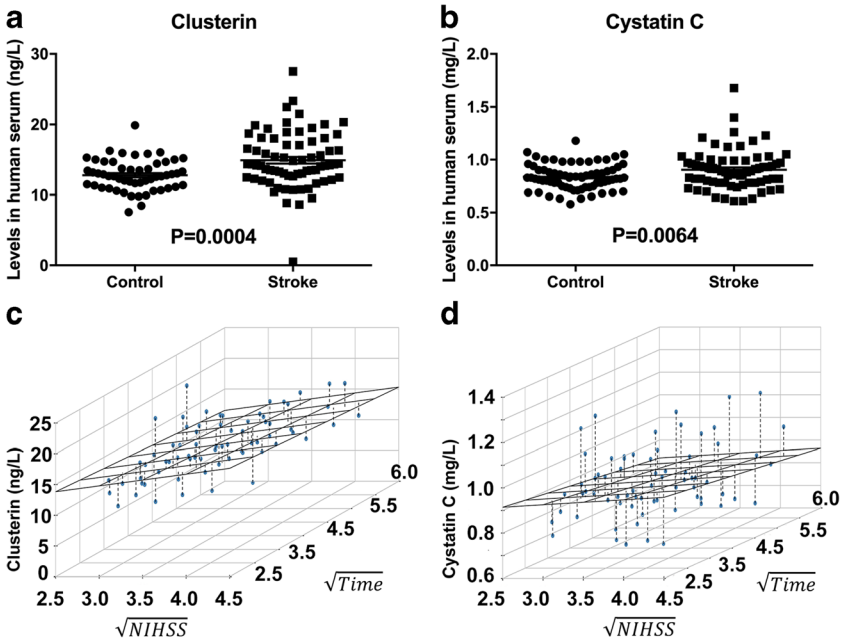

Fig. 3 Quantification of CLU and CST3 levels in human serum, comparing controls with stroke patients and their correlation with NIHSS and time. **a**) CLU levels were significantly elevated in stroke samples ($P = 0.0004$; $n = 70$ for patients and $n = 53$ for control subjects) as compared to control. **b** CST3 levels were significantly increased in stroke serum samples ($P = 0.0064$; $n = 70$ for both patients and control subjects) as compared to control. Data were analyzed by unpaired t test. **c**, **d** Fitted regression model between CLU or CST3 and NIHSS and time interval after stroke onset were visualized as blue points. The plane represented the predicted CLU or CST3 levels given NIHSS and time interval after stroke. The dashed line represented the residuals between the real and predicted CLU or CST3 levels. CLU levels were significantly associated with NIHSS score. No direct relationship was detected for CST3

biomarkers for stroke include GFAP, S100 calcium binding protein B, and matrix metalloproteinase-9 [2]. In this study, our results suggest CLU and CST3 as potential biomarkers. Besides significantly higher serum levels of CLU and CST3 in serum of AIS patients, changes in CLU levels were positively correlated with NIHSS score. Since NIHSS is used as a scale for determining stroke severity [28], it is reasonable that CLU levels may also serve as a quantitative serum surrogate to reflect stroke severity. Additional validation is required to evaluate the selectivity and specificity of CLU as a biomarker.

CLU (apolipoprotein J) is a secreted stress-induced cytoprotective chaperone that aids in the folding of other secreted proteins [40]. This disulfide-linked heterodimeric protein is associated with apoptosis and the clearance of cellular debris. Our results indicated that the immunoreactivities of both CLU and GFAP were higher in ischemic mouse brains than in sham controls. GFAP is considered as a known biomarker of ischemic stroke [2]. Prior studies indicate that sustained expression of CLU in astrocytes serves as a neuroprotective role after ischemia [41]. Interestingly, CLU is associated with the burden of fibrillary Aβ in the brain during Alzheimer's disease (AD) [18, 42]. A recent cohort study suggested that higher plasma CLU levels were associated with a lower risk of dementia (hazard ratio = 0.75 [0.60–0.95]) [18]. Corroborated with such evidence, potential mechanisms of CLU were further explored. Previous studies suggest ability for CLU levels to inhibit cell death by preventing protein aggregation, proteotoxic stress, cytochrome C release, and activation of Bax and caspase [43, 44]. CLU overexpression was shown to exert a neuroprotective role in vivo after permanent focal cerebral ischemia in mice [45]. Moreover, CLU was shown to contribute to caspase-3-independent brain injury following neonatal hypoxia–ischemia [46]. Other studies also found a significant increase in CLU in a rat model of oxygen-induced retinopathy, and in the cerebrospinal fluid (CSF) of multiple sclerosis patients [47, 48]. Our study is first to identify the association of CLU with mouse cerebral ischemia using proteomics tool, and subsequently validating with AIS patients. Also, our data suggested the positive correlation between CLU and major stroke risk factors associated with the lipid profiles, such as total cholesterol and LDL.

CST3 is a secretory protein that inactivates members of the cathepsin family of cysteine proteases and thus plays a role in protein catabolism, antigen presentation, hormone processing, and cleavage of membrane and extracellular matrix proteins during tissue remodeling [49, 50]. Serum CST3 has been used as a biomarker for renal function because of its superiority to creatinine as a proxy for glomerular filtration rate [21]. Notably, CST3 is also highly abundant in the central nervous system [51]. In serum and CSF, CST3 has a strong association with ischemic/hemorrhagic stroke and asymptomatic carotid atherosclerosis, suggesting that it may help stratify patient risk [22, 52, 53]. Other proteomic studies have shown significantly elevated levels of CST3 in CSF of AD patients and in

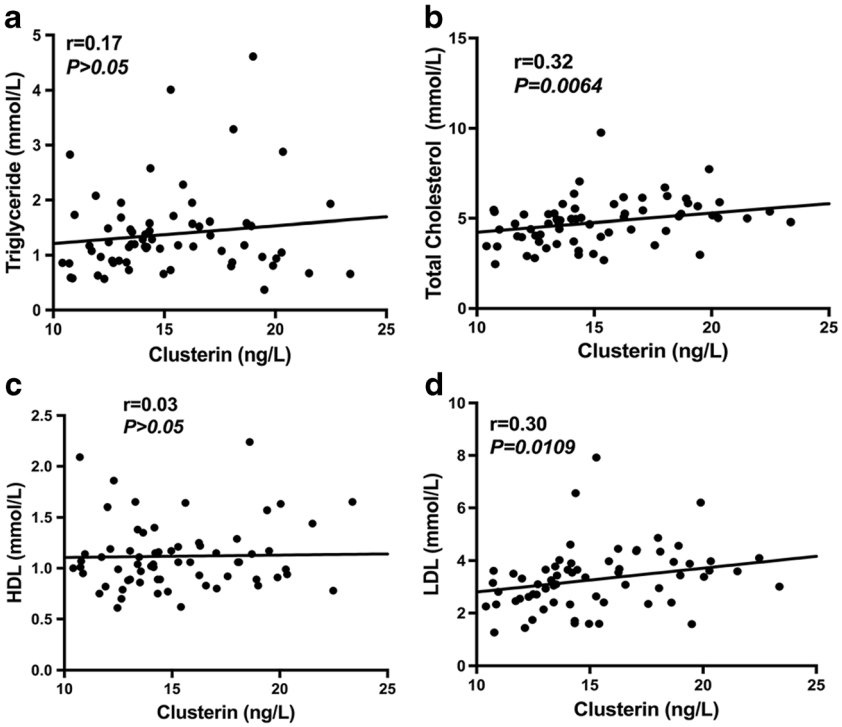

Fig. 4 Pearson correlation of CLU with triglyceride, total cholesterol, HDL, and LDL levels. The CLU level against triglyceride (**a**), total cholesterol (**b**), HDL (**c**), and LDL (**d**) in stroke patients. Pearson correlations were performed for statistical analysis. CLU levels were positively correlated with total cholesterol ($P = 0.0032$) and LDL ($P = 0.0054$) levels. Pearson r were indicated individually in each figure panels

postmortem dorsolateral prefrontal cortical brain tissue from patients with major depression compared to controls [54, 55]. A recent report also suggested that CST3 might serve as an endogenous neuroprotectant by preserving the lysosomal membrane integrity [23]. Our data also agree with a previous study showing an increase in plasma CST3 in patients with stroke as a strong predictor for the risk of vascular events [22]. Consistent with other research reports, we have identified the association of CST3 with AIS, but without significant correlation between CST3 levels and NIHSS score or time interval after ischemic stroke or lipid profiles.

The global ischemia animal model by BCCAo in mice is commonly used to make ischemic injury within brain subregions such as cortex, hippocampus, and striatum, due to the transient lack of blood flow. Though this model resembles cerebral ischemic damage in patients with cardiac arrest, the

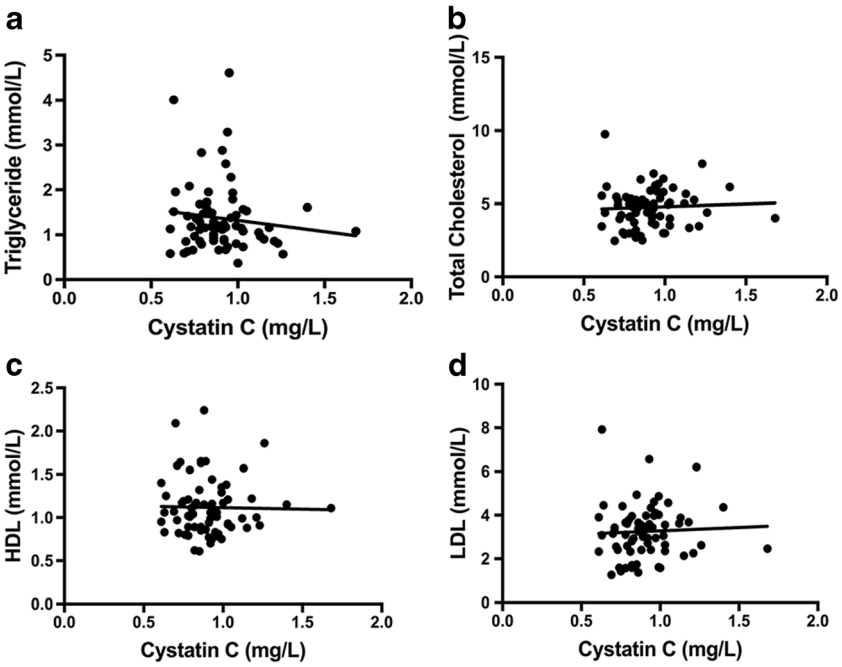

Fig. 5 Pearson correlation of CST3 with triglyceride, total cholesterol, HDL, and LDL levels. The CLU level against triglyceride (**a**), total cholesterol (**b**), HDL (**c**), and LDL (**d**) in stroke patients. Pearson correlations were performed for statistical analysis. CLU levels were not statistically correlated with any major stroke risk factors. Pearson r were indicated individually in each figure panels

brain injury after BCCAo in fact mimics the cellular events, e.g., delayed neuron cell death in the cortex and hippocampal area and glial activation, which are also found in focal ischemic stroke. Study with BCCAo animal model allows better correlation of proteomic data with the brain subregion-defined ischemic injury. In this study, analysis of the brain proteome from the global cerebral ischemia mouse model provided valuable information and helped to identify potential marker surrogates to assist clinical biomarker discovery. We chose this more suitable model over the focal ischemia model for study of molecular mechanisms by the proteomic study in order to avoid dissection of damaged tissue, e.g., infarct core and penumbral regions.

With regard to the clinical evaluations of these two candidates, there are potential limitations that can be improved in future studies. First, our sample size is relatively small as compared to large cohorts. Certain overlapping of the data is shown that could be due to heterogeneity of the ischemic patients, for instances, NIHSS and severe hypertension were found in eight AIS patients. Perhaps, to be more clinical relevant, one solution is to further increase the case numbers by classifying them into subtypes of AIS and stratifying other risk factors including hypertension, blood cholesterol/HDL levels, etc. for ongoing investigations. Additionally, as to detect and validate both CLU and CST3 levels in ischemic stroke patients, there are differences in baseline characteristics between stroke and control subjects. This could potentially affect the candidates' measurements. Future study of continuously recruiting more patients by classifying into subtypes of AIS and stratifying other risk factors with multicenter randomized trials is necessary to validate our findings and to minimize the effects of baseline differences. Secondly, serum samples were collected at single time point for each patient. Future study may consider collecting serum at multiple time points after AIS in order to gage extent of recovery. Finally, despite of positive correlation between CLU levels and NIHSS scores, follow-up evaluations may provide information regarding the extent of recovery.

In summary, from the proteomic study with mouse cerebral ischemia, two marker proteins (CLU and CST3) were identified, and subsequently testing with serum samples of AIS patients provided information that this biomarker may be relevant for diagnosing the severity of stroke in these patients. This pilot study thus contributed to progress toward preclinical screening by using animal models and translation of results from bench to bedside. Consequently, the increase in CLU levels in serum within 24 h after stroke could be an additional biomarker to aid clinical assessment of stroke and its severity.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Statement of Human Rights/Statement on the Welfare of Animals Ethical approval for human rights: "All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards."

Ethical Approval for the Welfare of Animals "All procedures performed in studies involving animals were in accordance with the ethical standards of the University of Missouri approved protocols for the Care and Use of Laboratory Animals at which the studies were conducted."

Informed consent "Informed consent was obtained from all individual participants included in the study."

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