Autophagy biomarkers in CSF correlates with infarct size, clinical severity and neurological outcome in AIS patients

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Abstract

Background: Autophagy is demonstrated to be involved in acute ischemic stroke (AIS), which, however, is confined to cells and/or animals levels. The aim of this study was to determine two autophagy biomarkers, Beclin1 and LC3B, in cerebrospinal fluid (CSF) and serum of patients with AIS, and to evaluate a possible correlation between levels of Beclin1 and LC3B and severity of neurological deficit and clinical outcome of stroke patients.

Methods: Levels of Beclin1 and LC3B were quantified by ELISA in CSF and serum collected from 37 AIS patients and 21 controls. The clinical severity at stroke onset was determined by the National Institute of Health Stroke Scale (NIHSS) and the neurological outcome was determined by the Modified Rankin Scale (mRs) and the improvement in NIHSS between stroke onset and 3 months later. Associations between autophagy biomarkers and infarct volume, NIHSS and mRs were assessed using Pearson analysis.

Results: The levels of Beclin1 and LC3B were increased both in CSF and serum of AIS patients relative to controls. In CSF, they were positively correlated with infarct volume and NIHSS scores, and negatively correlated with mRs scores, but no significant association was observed in serum. Moreover, AIS patients with higher levels of Beclin1 and LC3B in CSF had significantly higher improvement in NIHSS.

Conclusion: CSF and serum levels of autophagy biomarkers are altered in AIS patients. CSF levels of autophagy biomarkers are associated with infarct volume, clinical severity of and neurological outcome.

Keywords: Acute ischemic stroke, Autophagy, CSF, Outcome

Background

Acute ischemic stroke (AIS) is a major cause of death and disablement all over the world [1]. Despite its high prevalence and increasing burden, there are no effective neuroprotective agents in clinical use [2, 3]. The tissue plasminogen activator (tPA) is the only approved drug for AIS [4]. However, the intravenous thrombolysis is limited due to the strict therapeutic window and high risk of hemorrhagic complications [5]. Thus, exploration of effective therapeutic strategy becomes a major challenge [6]. AIS can initiate a series of biochemical reactions that directly or indirectly injury cellular structures [7, 8]. Many substances are released into the cerebrospinal fluid (CSF) and blood during brain damage.

Recent studies have revealed that autophagy may play an important role in ischemic stroke [9, 10]. Many investigations indicate that stroke induces activation of autophagy [11]. Autophagy is a critical cellular process responsible for the degradation and recycling of cellular components via the lysosomal pathway [12, 13]. This process is important for healthy cells to efficiently remove and recycle cellular constituents and maintain metabolic homeostasis [14]. Despite its essential role in cellular physiology, alterations in the process also operate as a pathological mechanism in many diseases, including brain damage [15, 16]. LC3B is a ubiquitin-like protein encoded by the mammalian homologue of autophagy.
associated with infarct volume, clinical severity and neurological outcome.

We found that Beclin1 and LC3B in CSF were correlated with the severity of autophagy in AIS patients, with Beclin1 and LC3B levels greatly increased both in intracellular components [25–27]. By contrast, some other studies report the protective role of autophagy in ischemic injury [28–31]. Moreover, in vivo evidence from patients involving autophagy is still lacking and thus the role of autophagy in patients with ischemic stroke needs more investigation [32–34].

The present study was designed to investigate the role of autophagy in AIS patients, with Beclin1 and LC3B used as markers of autophagy. Our study showed that levels of Beclin1 and LC3B greatly increased both in CSF and serum of patients with AIS. More interestingly, we found that Beclin1 and LC3B in CSF were correlated with infarct volume, clinical severity and neurological outcome.

**Subjects and methods**

**Patients**

This study was performed in the Sun Yat-Sen Memorial Hospital of Sun Yat-Sen University from Mar. 2013 through Dec. 2014. To accurately evaluate the effect of autophagy on acute ischemia injury, the patients who met the following criteria were included: (1) the first ever stroke, without history of cerebrovascular disease; (2) within 24 h after onset; (3) age ≥18 years; (4) evidence of previous infarct lesion by brain computed tomography (CT) or magnetic resonance (MR); (5) willing to sign an informed consent document. The exclusion criteria were as follows: (1) concomitant systemic disease such as cardiovascular disease; (2) concomitant malignant disease; (3) history of diabetes; (4) history of surgery or trauma recently; (5) with autoimmune disease or treated with hormone or immuneinhibitors; (6) with neurological degenerative disease, such as Alzheimer’s disease, Parkinson’s disease, motor neuron disease, and multiple system atrophy; (7) receiving thrombolysis treatment. Forty-two AIS patients were included in the experimental group, and 5 patients were excluded due to the loss to follow-up when we assessed the mRS and NIHSS scores 3 months later after the stroke.

Detailed neurological examination and brain magnetic resonance imaging (MRI) 3.0T with diffusion weighted images (DWI) were performed in all AIS patients. Acute cerebral infarction was defined as an area of high signal intensity on the DWI. The edge of each infarct lesion was draw by the manual approach and the total infarct volume of each patient was calculated automatically by software Volume Viewer 2 (GE, AW Suite 2.0, 6.5.1.z) as previously described [35, 36]. To evaluate the severity of neurological deficit after AIS, a neurological deficit grading system National Institute of Health Stroke Scale (NIHSS) [37] was performed on each AIS patient within 24 h of stroke onset. The evaluation was performed immediately after blood and CSF samples were obtained and 3 months later it was repeated. The clinical outcome of AIS was determined by the Modified Rankins Scale (mRS) assessed 3 months after stroke and the improvement in NIHSS (ΔNIHSS) as previously described [38]. The ΔNIHSS was defined as the improvement in NIHSS = NIHSS24h − NIHSS3 months [38]. The MRI studies, NIHSS and mRS were evaluated by two neurologists who were blinded to the study.

The control subjects were selected from 21 age- and gender-matched patients with no central nervous system (CNS) disease, including psychoneurosis (n = 15), benign paroxysmal positional vertigo (n = 3), trigeminal neuralgia (n = 1), hypokalemic periodic paralysis (n = 1) and progressive muscular dystrophy (n = 1).

This study was approved by the Ethical Committee of the Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University and conducted in accordance with the principles of the Declaration of Helsinki. All the participants were informed about the study and agreed to participate by signing an informed consent form.

**CSF and serum sample collection**

CSF and peripheral blood samples of patients with AIS were collected within 10–24 h of the onset of neurological symptoms. CSF samples were taken during lumbar puncture between the L3/4 or L4/5 intervertebral space using a 25-gauge needle and held immediately on ice. Peripheral blood samples were collected by venepuncture in heparinized tubes and stored on ice. The CSF samples were centrifuged at 800 rpm for 5 min at +4 °C, and the blood samples were centrifuged at 3000 rpm for 15 min at +4 °C. All samples were stored at −80 °C until they were analyzed.

**Analysis of CSF and serum**

Concentrations of two autophagy biomarkers, Beclin1 and LC3B, in CSF and blood were measured by commercial enzyme-linked immuno sorbent assay (ELISA) kits following the manufacturer’s instructions (SunLong Biotech Co., LTD, China). Briefly, for Beclin1 and LC3B evaluation CSF and serum samples were diluted 5 times. The optical density (OD) was measured by Microplate reader (Teng instrument co., LTD, USA) at a wavelength...
of 450 nm. A standard curve linear regression equation was calculated according to standards’ concentrations. Then, the concentrations of Beclin1 and LC3B in CSF and serum were calculated by the regression equation.

**Statistical analysis**

All statistical analyses were performed by SPSS Statistics, version 19.0 software (SPSS, Inc, Armonk, NY). All graphs were generated by GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA). All data were expressed as mean ± standard deviation (SD). Differences in characteristics between the AIS patients and controls were evaluated using the Student’s t-test for continuous variables or Chi square test for dichotomous variables. The Mann–Whitney U test was used to compare the autophagy biomarkers between the AIS patients and controls. Differences in good outcome group and poor outcome group assessed by the improvement in NIHSS were evaluated using Student’s t-test. Pearson’s correlation test was used to assess the linear dependence between autophagy biomarkers and infarct volume, NIHSS and mRS. P <0.05 were considered to be statistically significant.

**Results**

**Demographics**

Patients were enrolled between Mar. 2013 and Dec. 2014. From the 42 AIS patients who were primarily included, 5 patients were excluded due to the loss to follow-up. Thus the final analysis was conducted on 37 AIS patients and 21 control subjects. Their demographic clinical characteristics are presented in Table 1. There were no significant differences in age, gender, blood pressure, cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglyceride, and serum glucose between patients with AIS and controls.

**Beclin1 and LC3B concentrations**

In the observed population, samples were collected within 10–24 h of stroke onset. Firstly, we analyzed the change of levels of Beclin1 and LC3B both in CSF and serum of AIS patients. Concentrations of Beclin1 and LC3B in CSF and serum of AIS patients were significantly higher than that of control subjects (Fig. 1).

**Association between Beclin1 and LC3B and infarct volume and NIHSS**

We also investigated the association between autophagy and infarct volume and clinical severity of neurological deficit assessed by NIHSS in AIS patients. The mean infarct volume for all AIS patients was 8.82 ± 6.63 cm³. The mean NIHSS score for all AIS patients was 6.41 ± 3.578. In CSF, concentrations of Beclin1 and LC3B were positively correlated with infarct volume (Fig. 2a, c) and NIHSS (Fig. 3a, c). In serum, no significant association was observed between Beclin1 and LC3B levels and infarct volume (Fig. 2b, d), as well as the serum Beclin1 level and NIHSS (Fig. 3b). Although concentration of LC3B in serum was positively correlated with NIHSS (Fig. 3d), the correlation coefficient (r = 0.348) is low.

**Association between Beclin1 and LC3B and clinical outcome**

Then, all the AIS cases were followed up for 3 months to investigate the role of autophagy in the outcome of AIS. The primary outcome was evaluated by mRS. In CSF, levels of Beclin1 and LC3B were negatively correlated with mRS (Fig. 4a, c), but no significant association was observed in serum (Fig. 4b, d). The improvement in NIHSS (ΔNIHSS = NIHSS 24 h – NIHSS 3 months) was selected as the second outcome measures. ΔNIHSS ≥3 was considered to be good outcome and ΔNIHSS <3 was considered to be poor outcome. As a result, 19 cases of AIS patients gained good outcome and 18 cases gained poor outcome. We found that levels of Beclin1 and LC3B in CSF were significantly higher in good outcome patients than in poor outcome patients (Fig. 5a, c). However, there was no significant difference between levels of Beclin1 and LC3B in serum and improvement in NIHSS (Fig. 5b, d).

**Discussion**

It was well demonstrated that autophagy participates in ischemic stroke [39–41], while the present study firstly reported the involvement of autophagy in patients with ischemic stroke [39–41].

**Table 1 Demographics in AIS patients and controls (mean ± SD)**

| Variables | AIS (n = 37) | Con (n = 21) | p value |
|-----------|-------------|-------------|---------|
| Gender    |             |             |         |
| Male      | 17          | 10          | 0.559   |
| Female    | 20          | 11          |         |
| Age       | 59.38 ± 14.88 | 51.90 ± 15.80 | 0.078   |
| SBP (mmHg)| 130.19 ± 21.77 | 126.76 ± 20.46 | 0.558   |
| DBP (mmHg)| 81.27 ± 11.55 | 79.76 ± 9.98  | 0.618   |
| Chol (mmol/l)| 5.12 ± 1.31  | 5.22 ± 1.19  | 0.777   |
| HDL (mmol/l)| 3.13 ± 0.86  | 3.23 ± 0.75  | 0.652   |
| LDL (mmol/l)| 1.10 ± 0.30  | 1.14 ± 0.30  | 0.682   |
| TG (mmol/l)| 1.88 ± 1.54  | 1.92 ± 1.48  | 0.92    |
| GLU (mmol/l)| 4.93 ± 0.98  | 4.88 ± 0.73  | 0.852   |

AIS acute ischemic stroke, Con control, SBP systolic pressure, DBP diastolic pressure, Chol cholesterol, LDL-C low-density lipoprotein cholesterol, HDL-C high-density lipoprotein cholesterol, TG triglyceride, GLU glucose
AIS. This study found that Beclin1 and LC3B were obviously increased in both CSF and serum of patients with AIS, and that levels of Beclin1 and LC3B in CSF were positively correlated with infarct volume and severity of neurological deficit. Moreover, levels of Beclin1 and LC3B in CSF were associated with good outcome of AIS patients.

In CNS, autophagy can be activated by cerebral ischemia/hypoxia, nutrient deprivation, oxidative stress, energy crisis and neurotoxins [42–44]. The initial role of autophagy activation is to provide proper cellular response for nutrients limitation [45, 46]. Autophagy is activated for lack of essential nutrients after ischemia [47]. Transition from basal level into induced autophagy could be regulated by multiple pathways in neurons during cerebral ischemic stroke [48]. Autophagy is activated not only by PI3 K-Akt-mTORC1 pathway [49], but also by AMP-activated protein kinase (AMPK) via activation...
of ULK1 or by inhibition of mammalian target of rapamycin complex (mTORC) [50]. Furthermore, increased AMP/ATP ratio and/or [Ca^{2+}] activates AMPK kinase through activation of Ca2+/calmodulin-dependent protein kinase kinase (CaMKK) and LKB1 kinases, therefore inducing autophagy [51]. However, autophagy can be inhibited by the binding of Bcl-2 to Beclin1 via disrupting the association of Beclin1 with PI3K, hVps34 and p150 [52]. Consistent with observations, our study showed that autophagy was activated in serum and CSF of AIS patients with indicators increased, suggesting critical roles of autophagy in ischemic process.

In this study, levels of Beclin1 and LC3B in CSF were positively correlated with infarct volume and severity of neurological deficit, whereas this relation was not observed in serum. This is likely to be due to: First, the autophagy biomarkers in serum can not accurately reflect their levels in brain, and they may be affected by other organs of bodies when stroke takes place. Second, the sample size in this study was small.

More importantly, increased concentrations of Beclin1 and LC3B in CSF were found to be associated with good outcome, suggesting autophagy plays a protective role in AIS. Accumulating evidence suggests that autophagy enhanced functional recovery after stroke. It was repeatedly demonstrated that autophagy played a neuroprotective role in ischemic stroke. Urbanek et al. reported that rapamycin can effectively prevent neuro damage by induction of protective autophagy [53]. Autophagy inhibitor 3-MA can alleviate the neurological symptoms after ischemic stroke [16]. Also, Gao et al. demonstrated that ER stress-induced autophagy contributes to neuroprotective effect in cerebral ischemic preconditioning [54]. The protective role of autophagy in AIS was possibly attributed to elimination of damaged mitochondria and block of downstream apoptosis [41, 55]. However, it was also showed that autophagy could contribute to worse outcome [56, 57], and excessive activation of autophagy leads to neuronal death in ischemic stroke [58, 59]. In the neuronal system, moderate autophagy is thought to be neuroprotective [60], while excess or inadequate autophagy may promote neuronal cell death [61, 62]. In our study, the severity of neurologic deficit of most subjects was mild to moderate, so the moderate activation of autophagy exacted neuroprotective role in AIS patients.

There were some limitations in the present study. First, it is difficult to determine concentrations of Beclin1 and LC3B in CSF from normal persons as controls. Second,
because of ethical concerns, our study was restricted to CSF and serum within 10–24 h of AIS, lacking of kinetic observation of alternations of Beclin1 and LC3B. Despite the limitations of the present study, our findings provide new evidence that autophagy was involved in AIS and was associated with clinical outcome.

In summary, we observed that autophagy biomarkers in CSF and serum levels of AIS patients were increased and that levels of Beclin1 and LC3B in the CSF were associated with good clinical outcome, implicating a thorough involvement of autophagy in ischemic injury and suggesting a bright intervention target in AIS treatment.

Abbreviations
AIS: acute ischemic stroke; tPA: tissue plasminogen activator; LC3B: microtubule-associated protein B light chain 3; Atg: autophagy-related proteins; CT: computed tomography; MRI: magnetic resonance imaging; CNS: central nervous system; NIHSS: National Institute of Health Stroke Scale; mRS: modified
Rankins score; CSF: cerebrospinal fluid; ELISA: enzyme-linked immuno sorbent assay; OD: optical density; SD: standard deviation; 3MA: 3-methyladenine; SD: standard deviation; SBP: systolic pressure; DBP: diastolic pressure; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; mTORC: mammalian target of rapamycin complex; AMPK: AMP-activated protein kinase; CaMKII: Ca2+/calmodulin-dependent protein kinase kinase.

Authors’ contributions
HHL and SQW designed the study, carried out data analysis and drafted the manuscript. XPL participated in the collection of the CSF and serum samples and analysis of the data. YP and ML conceived of the study and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

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