Increased perihematomal neuron autophagy and plasma thrombin–antithrombin levels in patients with intracerebral hemorrhage

An observational study

Chenghan Wu, MD, Xiaohua Yan, MD, Yuansheng Liao, MD, Lianming Liao, MD, PhD, Shengyue Huang, MD, Quanling Zuo, MD, Linying Zhou, MD, PhD, Lili Gao, MD, Yinzhong Wang, MD, PhD, Jushan Lin, MD, Shiju Li, MD, Kaiyu Wang, MD, Xuming Ge, MD, Haolong Song, MD, PhD, Ruiling Yang, MD, Feng Lu, MD, PhD

Abstract
Animal studies have demonstrated that autophagy was involved in neuronal damage after intracerebral hemorrhage (ICH). Several studies showed thrombin–antithrombin (TAT) plasma levels were elevated in patients with ICH. In this study, we aimed to evaluate if autophagy occurred in patients with ICH; and the relationship between the severity of brain injury and plasma TAT levels.

A novel tissue harvesting device was used during hematoma removal surgery to collect loose fragments of tissue surrounding the affected brain area in 27 ICH patients with hematoma volumes of >30 mL in the basal ganglia. Control tissues were obtained from patients who underwent surgery for arteriovenous malformation (n = 25). Transmission electron microscopy (TEM) and immunohistochemistry for autophagy-related proteins were used to evaluate the ultrastructural and morphologic cellular characteristics; and the extent of autophagy in the recovered tissue specimens. Stroke severity was assessed by using the Glasgow Coma Scale (GCS) and the National Institutes of Health Stroke Scale (NIHSS). An enzyme-linked immunosorbent assay (ELISA) was used to measure plasma TAT levels.

Transmission electron microscopy showed autophagosomes and autolysosomes exist in neurons surrounding the hematoma, but not in the control tissues. The number of cells containing autophagic vacuoles correlated with the severity of brain injury. Immunohistochemistry showed strong LC3, beclin 1, and cathepsin D staining in ICH tissue specimens. Plasma TAT levels correlated positively with autophagic cells and ICH severity (P < .01).

Autophagy was induced in perihematomal neurons after ICH. Autophagy and plasma TAT levels correlated positively with severity of brain injury. These results suggest that autophagy and increased plasma TAT levels may contribute to the secondary damage in ICH patients.

Abbreviations: ANOVA = analysis of variance, AVM = arteriovenous malformation, CT = computed tomography, ELISA = enzyme-linked immunosorbent assay, GCS = Glasgow Coma Scale, ICH = intracerebral hemorrhage, MRI = magnetic resonance imaging, NIHSS = National Institutes of Health Stroke Scale, TAT = thrombin–antithrombin, TEM = transmission electron microscopy, TF = tissue factor.

Keywords: autophagy, beclin 1, cathepsin D, intracerebral hemorrhage, LC3, thrombin
1. Introduction

Intracerebral hemorrhage (ICH), which is defined as spontaneous, nontraumatic bleeding into the brain parenchyma, accounts for 10% to 15% of stroke patients globally.\[^{[1]}\] It is a devastating subtype of stroke with no effective therapy and reliable prognostic markers. ICH patients have the highest risk of mortality and long-term physical and neurological dysfunction among all stroke patients.\[^{[2–4]}\] Risk factors for ICH include genetic variants of apolipoprotein E, ethnic differences, and lifestyle factors such as smoking and alcohol intake.\[^{[5]}\] Several predictors of poor clinical outcomes have been identified, including initial hematoma volume, first-day expansion, location and extent of brain edema, age, and neurological status on admission.\[^{[6–8]}\] Delayed secondary brain injury may be caused by decreased local blood flow,\[^{[9–12]}\] inflammation,\[^{[13–16]}\] peripheral edema,\[^{[17,18]}\] degradation products of hemoglobin,\[^{[19,20]}\] matrix metalloproteinase, and thrombin.\[^{[21–23]}\] Neuronal apoptosis is common after ICH.\[^{[13,24]}\] Although much effort has been made to understand the pathogenesis of ICH, no effective medical or surgical therapy has been firmly established.\[^{[25]}\] Supportive care is still the mainstay management for patients with ICH. The development of new therapeutic approaches for ICH is urgently needed and should be based on the understanding of the molecular and cellular mechanisms that underlie both early and delayed brain damage after ICH.

Autophagy is a cellular degradation process in which cellular proteins and organelles are sequestered in double membrane vesicles known as autophagosomes, delivered to lysosomes, and digested by lysosomal hydrolase.\[^{[26]}\] Autophagy protects cells against stress such as ischemia and hypoxia by clearing aged intracellular organelles and misfolded proteins. Accumulating evidence indicates that autophagy contributes to cerebral ischemic stroke and is involved in ischemic brain injury.\[^{[27–39]}\] However, the relationship between autophagy and cerebral ischemia is unclear. One report suggested that autophagy protected neurons from death,\[^{[40]}\] but another study indicated a destructive role of autophagy.\[^{[41]}\] Recent studies have shown that macroautophagy was activated during cerebral ischemia and that the autophagy induction pathway could serve as a new therapeutic target for stroke.\[^{[42]}\] In addition, autophagy was observed in astrocytes after ICH\[^{[42–45]}\] and ICH-induced autophagy was found to exacerbate neurological deficits in rats.\[^{[44]}\] Although autophagy has been widely observed in neurological diseases,\[^{[46,47]}\] no studies have demonstrated autophagy in patients with ICH.

Thrombin production may reflect secondary hemostatic activation due to inflammation and endothelial injury. Our previous studies have found that the plasma concentration of thrombin–antithrombin (TAT) complexes in ICH patients is positively correlated with ICH severity.\[^{[21]}\] Animal studies also showed that thrombin could activate autophagy in the brain and was involved in ICH-induced autophagy.\[^{[48]}\] However, clinical evidence is lacking regarding the relation between autophagy and ICH. In the current study, we aimed to investigate if autophagy occurs after ICH and its relationship with plasma thrombin levels in ICH patients. We also determined the expression of autophagic vacuoles (AVs; including autophagosomes and autolysosomes) and the expression of autophagy-related proteins including microtubule-associated protein 1 light chain (LC3), beclin 1, and cathepsin D, and further evaluated the association of autophagy with the severity of brain injury and plasma TAT levels.

2. Patients and methods

2.1. Patients

This study included 27 ICH patients who were admitted for hematoma evacuation between January, 2010 and December, 2015. All surgery was performed at the Department of Neurosurgery of the Second Affiliated Clinical College of Fujian University of Traditional Chinese Medicine and Fujian Provincial Hospital. Patients were included if they met the following criteria: ICH was diagnosed based on medical history and computed tomography (CT) scan; a space-occupying ICH in the basal ganglia (>30 mL) that mandated craniotomy for removal of intracranial bleeding; patients had no cancer, trauma, and cerebellum and brain stem bleeding. Patients were excluded from the study if they had serious liver, kidney, hematopoietic, endocrine, or connective tissue diseases, mental illness, or infection. The control cohort included 25 non-ICH patients with deep intracranial arteriovenous malformation (AVM).

The study was approved by the ethic committee of the Second Affiliated Clinical College of Fujian University of Traditional Chinese Medicine, and all procedures were performed in accordance with the current guidelines. This research was conducted in accordance with the Helsinki Declaration. Written informed consent was obtained from all subjects or their family members.

2.2. Patient evaluation

Each patient was examined by brain CT and magnetic resonance imaging (MRI) for ICH assessment. Magnetic resonance angiography and digital subtraction angiography were used for AVM evaluation. Cranial CT images were analyzed by investigators blinded to the patient’s identity. ICH volume was measured using the formula $V = \frac{A \times B \times C}{2} \times 0.5$, where $A$ and $B$ represent the largest perpendicular diameters through the hyperdensity area of the CT scan, and $C$ represents the thickness (ie, the number of 5-mm slices showing evidence of hemorrhage).\[^{[49]}\] The Glasgow Coma Scale (GCS) and National Institutes of Health Stroke Scale (NIHSS) scores were evaluated in each patient. ICH patients were divided according to their GCS scores into mild (GCS 9–11), moderate (GCS 6–8), and severe (GCS 3–5) groups.

In AVM patients, brain tissue fragments and venous plasma were collected during the surgical removal of AVMs and served as controls.

2.3. Congo red staining

To exclude a possible effect of amyloidosis on autophagy, Congo red staining was used to identify suspected amyloid deposits. Tissue sections were deparaffinized and hydrated in distilled water and then stained in Congo red solution for 30 minutes. After being rinsed in distilled water, the sections were differentiated rapidly in alkaline alcohol solution. After being rinsed in tap water for 5 minutes, tissue sections were counterstained in Gill hematoxylin for 30 seconds. After dehydration in 95% and 100% alcohol followed by clearing in xylene, the sections were mounted and observed under a microscope.

2.4. Collection of brain tissue fragments

During evacuation of intracranial hematomas and surgical resection of deep AVMs, brain tissue fragments were collected
using a device with a suction apparatus attached to a filter and a flushing system for continuous flushing of cold normal saline. During craniotomy, the suction apparatus was used to collect the cerebrospinal fluid, blood, and blood clots. A small amount of soft brain tissue fragments present in the cerebrospinal fluid and blood was aspirated onto the filter and flushed with cold normal saline. White brain tissue fragments (about 1mm in diameter) were identified and collected. The locations of the collected brain tissues were recorded by surgeons. Tissues from the surrounding hematoma were collected in the ICH group, whereas tissues from the lesions on the lateral side near the basal ganglion region were collected in the AVM control group. The brain tissue fragment recovery device has been patented in China (patent no. 201110073171.3); another patent is pending in the PCT (patent application no. PCT/CN 2011/079833). Autophagy was detected according to the guidelines.\(^\text{[10]}\)

2.5. Transmission electron microscopy

Brain tissues were fixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide. After fixation, brain tissues were dehydrated in alcohol-acetone, and embedded in epoxy embedding medium. Ultrathin sections were prepared and stained with 1% uranyl acetate and lead citrate. Transmission electron microscopy (TEM) was performed using an FEI/Philips EM 208 microscope (Philips, Eindhoven, Netherlands) at a voltage of 80kV. Images were randomly chosen in a clockwise direction and analyzed. For each subject’s brain tissue, 3 embedded blocks were prepared, and 3 sections were prepared from each block. In each section, 10 neurons were randomly selected and the numbers of autophagosomes and autolysosomes were recorded. The average numbers of autophagosomes and autolysosomes in 30 neurons was calculated.

2.6. Immunohistochemistry

Brain tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. The tissues were sectioned at 1mm\(^3\) thickness, dewaxed, and alcohol-rehydrated. Endogenous peroxidase activity was quenched with 5% hydrogen peroxide. Nonspecific background staining was blocked with 3% goat serum. The sections were then immunostained with monoclonal antibodies against LC3, beclin 1, and cathepsin D (1:100; Abcam). The sections were washed and incubated with a secondary antibodies against LC3, beclin 1, and cathepsin D (1:100; Abcam). The sections were incubated with a secondary antibody against LC3, beclin 1, and cathepsin D (1:100; Abcam). The sections were incubated with a secondary antibody against LC3, beclin 1, and cathepsin D (1:100; Abcam). The sections were incubated with a secondary antibody against LC3, beclin 1, and cathepsin D (1:100; Abcam).

2.7. Enzyme-linked immunosorbent assay

Venous blood (1.8 mL) was collected in Eppendorf tubes containing natrium citricum, and centrifuged at 300g for 10 minutes. Plasma samples were stored at -70°C for further processing. TAT complex concentrations in plasma samples were determined at Shanghai Institute of Immunology, China, using commercially available Assay Max Human TAT Complex enzyme-linked immunosorbent assay (ELISA) kit (ASSAYPRO, St. Charles, MO) as previously described.\(^\text{[11]}\)

3. Results

3.1. Demographic and baseline characteristics of the study population

The demographic and baseline characteristics of the patients are shown in Table 1. A total of 27 ICH patients and 25 age and sex-matched AVM patients were included. All ICH patients had

| Variables | ICH (n = 27) | AVM (n = 25) |
|-----------|-------------|-------------|
| Age, y    | Mean ± SD   | Mean ± SD   |
|           | 59.63 ± 7.45| 50.24 ± 9.12|
| Range     | 45–76       | 39–65       |
| Male sex, n | 16          | 14          |
| GCS scores, n | 9–11        | 12          |
|           | 6–8         | 9           |
|           | 3–5         | 6           |
| NIHSS score, n | 20–23      | 12          |
|           | 24–26       | 9           |
|           | 27–30       | 6           |
| Intracerebral hemorrhage volume, mL | Mean ± SD | 49.57 ± 10.94 |
|           | Range       | 32–78       |
| Mild ICH | 39.62 ± 5.38|
| Moderate ICH | 51.0 ± 5.2 |
| Severe ICH | 66.29 ± 6.06|
| Hypertension history, n | 23 | 7 |
| Blood pressure, mm Hg | Mean ± SD | 180.1 ± 16.77 |
| Systolic | Range       | 162–260     |
|          | 115.21 ± 13.65|
| Diastolic | Mean ± SD   | 97.13 ± 10.79 |
|           | Range       | 80–160      |
|          | 72.31 ± 10.23|
| Diabetes history, n | 6 | 4 |
| Current smoking, n | 15 | 5 |
| Current drinking, n | 16 | 8 |
| Undergoing antiplatelet therapy, n | 6 | N/A |
| Different pupil sizes, n | 17 | N/A |
| Drug abuse, n | 0 | 0 |

AVM = arteriovenous malformation, GCS = Glasgow Coma Scale, NIHSS = National Institutes of Health Stroke Scale.
cranial CT scans within 10 hours of hemorrhage onset. The median duration from ICH onset to surgery was 14.8 ± 4.7 hours (range 4.0–24.0 hours). All ICH patients had a GCS score of 11 or less. Twelve patients had mild ICH, 9 had moderate ICH, and 6 had severe ICH. Neither ICH patients nor AVM patients had amyloidosis by Congo red staining. Severe ICH patients had the highest ICH volume (66.29 ± 6.06 mL), followed by moderate ICH patients (51.0 ± 5.2 mL) and mild ICH patients (39.62 ± 5.38 mL) (P < .01 for all).

3.2. Association between autophagy and brain injury in patients with ICH

We investigated autophagy by examining brain tissues surrounding the hematoma area in both ICH patients and AVM patients. TEM revealed the presence of autophagosomes and autolysosomes in the brain cells of ICH patients and fewer organelles and mitochondrial cristae (scale bar = 300 nm; Fig. 1A). Extensive cytoplasmic vacuolization was also present. More severe ultrastructural damages, such as injured myelinated axons and nuclear membrane invaginations, were seen in patients with higher NIHSS and lower GCS scores. In contrast, the control brain cells contained few vacuoles and had intact nuclei, mitochondria, synapses, endoplasmic reticula, and myelinated axons (scale bar = 300 nm; Fig. 1B). The mean numbers of both autophagosomes and autolysosomes were significantly higher in patients with severe ICH versus those with moderate or mild ICH (1-way ANOVA, P < .05) (Fig. 1C).

3.3. ICH was associated with increased expression of autophagy-related proteins

Next, we investigated the expression of autophagy-related proteins, including LC3, beclin 1, and cathepsin D, in ICH and control brain tissues by immunohistochemistry. Notably, there were more cells that were positive for LC3, beclin 1, and cathepsin D in the ICH group compared with the control group (Fig. 2). In addition, the number of LC3, beclin 1, or cathepsin D-positive autophagic neurons, and autophagic vacuoles was significantly higher in patients with severe ICH versus those with moderate or mild ICH (1-way ANOVA, P < .05 or .01) (Table 2).

3.4. TAT levels in the plasma of patients with ICH

In a previous study, we reported increased plasma TAT levels in the postoperative ICH patients. Here, we investigated whether TAT levels were associated with ICH severity. ELISA showed that plasma TAT concentrations increased with increasing severity of ICH (1-way ANOVA, P < .05 or .01) (Fig. 3). We further investigated the relationship between TAT levels and the numbers of LC3, beclin 1, and cathepsin D-positive neurons and autophagic vacuoles in brain tissue sections. Pearson correlation analysis showed that consistent with the ELISA results, there was a positive linear correlation between plasma TAT levels and the numbers of LC3, beclin 1, and cathepsin D-positive neurons and autophagic vacuoles (P < .05 for all; Fig. 4, Table 3).

4. Discussion

In the present study, we used TEM to characterize tissue specimens from the ICH patients undergoing surgical hematoma evacuation. We demonstrated an increase in the number of AVs (autophagosomes and autolysosomes) in cells surrounding hematomas and provided strong evidence of autophagy in brain cells (including neurons as indicated by axons) of ICH patients. The number of autophagic cells positively correlated with the severity of the brain insult as reflected by GCS or NIHSS scores. To the best of our knowledge, this is the first clinical study demonstrating cerebral cell autophagy in ICH, indicating a potential role of autophagy in ICH-induced neurological injury.

Transmission electron microscopy is currently considered to be the most sensitive and accurate method for determining whether cells are undergoing autophagy. We confirmed the TEM findings by immunohistochemistry staining of the autophagy-related proteins LC3 and beclin 1, and also the lysosomal marker cathepsin D. The expression of all 3 proteins appeared to correlate with stroke severity. These results indicate that autophagy is induced by ICH at least in cells in the perihematoma region. Whether autophagy mediates ICH-induced brain injury and whether increased expression of autophagy-related proteins reflects an adaptive response to neuronal injury are unclear.

Autophagy is a tightly regulated process for the bulk removal of degraded cytoplasmic macromolecules and organelles in mammalian cells via lysosomes, and thus maintaining cell homeostasis. Neurons undergo autophagy under ischemic conditions, and autophagy is activated in the brains of rats after experimental ICH. Paradoxically, this well-tuned
process can both promote neuronal survival and cause neuronal injury or death.\(^\text{[52,53]}\) The present study demonstrated that the number of autophagic neurons in patients with ICH correlated with the severity of neuronal dysfunction and bleeding volume, suggesting that autophagy is involved in the regulation of secondary brain injury after ICH. It remains unclear how autophagy is induced after hemorrhage. We hypothesize that decreased blood supply to the brain caused by edema and thrombi activates autophagy for nutritional molecule recycling.

A recent study showed that thrombin was involved in the modulation of autophagic activation by increasing AV formation in rat brain neurons.\(^\text{[36]}\) The study also found that injection of thrombin into the rat brain enhanced the conversion of LC3-I to LC3-II, increased the levels of cathepsin D, and promoted the formation of AVs in neurons. In contrast, suppression of thrombin-induced autophagic activation by the autophagy inhibitor 3-methyladenine reduced AV formation and thrombin-induced cell death, indicating that thrombin could activate autophagy and exacerbate brain injury.\(^\text{[36]}\) Thrombin, a serine protease, is released to minimize bleeding after ICH. However, thrombin in cerebral hematoma fluid may cause secondary brain injury. An animal study reported that thrombin-mediated destruction of the blood-brain barrier (BBB) and neuronal toxicity is an important mechanism of brain edema after ICH.\(^\text{[19]}\) Thus, thrombin is responsible for secondary brain injury in both

**Table 2**

|                      | Control (n=25) | GCS (9-11) (n=12) | GCS (6-8) (n=9) | GCS (3-5) (n=6) | P       |
|----------------------|----------------|-------------------|-----------------|-----------------|---------|
| LC3\(^ -*\) neuron   | 6.50±1.48      | 14.60±5.11        | 19.14±2.85      | 21.27±3.21      | <.05    |
| Beclin 1\(^ -*\) neuron | 3.83±1.179     | 12.36±2.26        | 12.99±1.52      | 15.20±1.52      | <.05    |
| Cathepsin D\(^ -*\) neuron | 3.33±1.03     | 7.77±2.49         | 12.99±1.52      | 14.17±3.87      | <.05    |
| Autophagic vacuoles  | 3.00±0.89      | 10.00±1.65        | 17.67±4.64      | 22.67±2.16      | <.05    |

GCS = Glasgow Coma Scale.
*Statistical analysis was performed using One-way ANOVA.

**Figure 2.** Immunohistochemistry staining of LC3, beclin 1, and cathepsin D in the brains of patients with intracerebral hemorrhage (ICH) and control subjects. Brain tissue fragments were collected during surgery by a brain tissue fragment recovery device. In the control group (bottom row), fewer neurons were positive for LC3, beclin 1, and cathepsin D compared with the ICH group (top row). Magnification: 400x.

**Figure 3.** Thrombin–antithrombin (TAT) levels in the plasma of patients with intracerebral hemorrhage (ICH). The levels of TAT in the 3 ICH severity groups were significantly higher compared with that of the control group (1-way ANOVA, P < .05). TAT levels increased with increasing ICH severity (data in [C] are expressed as mean±SD; *P < .05, 1-way ANOVA). ANOVA = analysis of variance, GCS = Glasgow Coma Scale, NIHSS = National Institutes of Health Stroke Scale.
cerebral ischemia and ICH by inducing brain edema and neuronal death.\cite{16,41} Interestingly, thrombin at low concentrations protected neurons and astrocytes after brain injury, whereas high concentrations of thrombin may induce inflammatory cell invasion, BBB damage, and neuronal death.\cite{3,54–56} It was reported that thrombin may cause autophagic cell death after ICH.\cite{48}

In the present study, the levels of TAT in both the hematoma fluid and plasma were elevated in patients with ICH, and the TAT concentration in the hematoma fluid showed a correlation with stroke severity and bleeding volume. Tissue factor (TF)-bearing cells, such as smooth muscle cells, fibroblasts, and cortical cells outside the blood vessel wall, have a large amount of TFs on their surface. Under normal physiological conditions, intact vascular endothelial cells keep TFs apart from clotting factors in the blood. At the presence of cerebral vascular rupture, the vascular endothelium loses its integrity and TFs are exposed to the blood, forms TF-FVIIa complex with FVIIa, which activates FX, FIX, and subsequently FV. A small amount of thrombin is then produced on the blood vessel surface. At the same time, subendothelial collagen is exposed and platelets adhere to the endothelium. Platelets are activated when a sufficient amount of thrombin is produced on the cells with TFs on their surface. FVa, FVIIa, and FIXa are activated on the surface of the platelets, and Xa is further activated to bind to the previously activated FVa to promote thrombin generation. Thus the concentration of thrombin in the hematoma fluid is sharply increased, which has a destructive effect on the brain cells and the BBB. Due to the destruction of the BBB, thrombin is released into the blood circulation system and forms TAT complex.\cite{57–59} In addition, several studies have reported elevated blood TAT levels in patients with head injury on admission.\cite{60–63} Kushimoto et al\cite{63} showed that, in isolated blunt head trauma, TAT III complex concentrations in blood samples within 3 hours of injury were significantly higher in patients whose outcomes were characterized as severe disability, vegetative state, or death than in patients whose outcomes were characterized as good recovery or moderate disability. Thus, head trauma can activate coagulation, which, in turn, may exacerbate brain injury as shown in animal experiments.\cite{16,41} It requires further investigation whether increased TAT levels in patients with ICH can induce autophagy.

This study has several limitations. First, the number of patients was relatively small because of the difficulty of obtaining brain tissue specimens. Second, the study design did not permit a conclusion of possible causal relationship between autophagy and brain injury. Enhanced autophagy may reflect more serious injury, and other forms of cell death may also be present such as ferroptosis or apoptosis. Because we did not use markers specific for these types of cell death, we cannot rule out the possibility that ICH promotes cell death in general rather than autophagy specifically.

5. Conclusions

In summary, the present study demonstrated autophagy in neurons in the perihematomal area in the brains of patients with ICH and that thrombin may contribute to the activation of autophagy in these neurons. The possibility of autophagy-targeting pharmacological intervention in ICH deserves further study.

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Author contributions

Conceptualization: Feng Lu.

Data curation: Chenghan Wu, Xiaohua Yan, Shengyue Huang, Hailong Song.
Formal analysis: Chenghan Wu, Xiaohua Yan, Lianming Liao, Lining Zhou, Yinzhou Wang, Shijiu Li, Xiuming Ge, Ruiling Yang.

Funding acquisition: Chenghan Wu.

Investigation: Shengyue Huang, Yinzhou Wang, Xiuming Ge, Hailong Song, Ruiling Yang, Feng Lu.

Methodology: Lianming Liao, Quanrting Zuo, Hailong Song, Feng Lu.

Resources: Xiaohua Yan, Yuanzheng Liao, Quanrting Zuo, Lining Zhou, Lili Gao, Shijiu Li, Kaiyu Wang.

Software: Yuanzheng Liao, Lili Gao, Jushan Lin, Kaiyu Wang.

Supervision: Yuansheng Liao, Jushan Lin.

Validation: Jushan Lin.

Writing – original draft: Chenghan Wu, Hailong Song.

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