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Changes in Serum LncRNA MEG3/miR-181b and UCH-L1 Levels in Patients with Moderate and Severe Intracerebral Hemorrhage

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ABSTRACT

AIM: To study on the association of lnc-MEG3/miR-181b and UCH-L1 in the progression of cerebral hemorrhage. Further facilitation the development of novel strategies that use their potential as a therapeutic targets.

MATERIAL and METHODS: In this study, we studied the lncRNA MEG3 and miR-181b expression in 30 patients with ICH who were admitted to the General Hospital of Xinjiang Military Command from January 2021 to May 2021 by quantitative polymerase chain reaction. Serum levels of UCH-L1 were detected by enzyme-linked immune sorbent assay, and disease severity was evaluated using the Glasgow Coma Scale. We also recorded ICH-related deaths in hospital.

RESULTS: We found that lnc-MEG3 and UCH-L1 levels increased and miR-181b levels decreased in the serum of patients with ICH. lnc-MEG3, miR-181b, and UCH-L1 levels were also associated with the severity of the condition.

CONCLUSION: Our data indicated that lnc-MEG3, miR-181b, and UCH-L1 are likely involved in the pathophysiology of ICH, and could form the basis of future studies on potential targets for the treatment of traumatic CNS injuries. UCH-L1 could also find application in ICH management. Further studies on the plasma lncRNA-MEG3, miR-181b, and UCH-L1 levels in patients with ICH could yield novel biological targets for the prediction and treatment of ICH.

KEYWORDS: Intracerebral hemorrhage, Long non-coding RNA, Ubiquitin C-terminal hydrolase L1, miR-181b

INTRODUCTION

Spontaneous intracerebral hemorrhage (ICH) is a subtype of stroke, and is a neurosurgical disease that is difficult to treat. The mortality of patients with ICH within 30 days of onset is 30%–50%, and the incidence of disability in surviving patients is as high as 75% (3). The lifetime treatment cost of patients with ICH exceeds 123,500 United States dollars (US$), and the average daily hospitalization cost is US$1,396 (5). This high treatment cost adds to the heavy social burden, which has led to extensive research aimed at alleviating this burden. The prognosis in patients with ICH is closely related to the degree of brain damage. The assessment tools currently used to determine the severity of brain injury such as the Glasgow Coma Scale (GCS) and pupil response are mostly subjective. More objective laboratory indicators are needed to help doctors assess and monitor changes in the condition of patients with ICH. Laboratory indicators based on minimally invasive blood specimens have become a research hot spot in the evaluation of brain function in recent years. A novel objective assessment strategy would be of great value in determining the severity of brain injury, prognostic evaluation, and development of strategies to treat ICH.

Long non-coding RNA (lncRNA) are approximately 200-nucleotide RNA transcripts that lack an open reading frame (2,5). Long non-coding RNA maternally expressed gene 3 (lnc-MEG3) is located on human chromosome 14q32.3. lnc-MEG3 is involved in the regulation of neuron apoptosis, neurite outgrowth, and inflammation, and participates in neuronal

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apoptosis (8). Further, lnc-MEG3 indirectly regulates proteins by "sponging" members of the miRNA-181 family; miR-181b plays an important role in the regulation of neural ischemic injuries by targeting ubiquitin carboxyl-terminal hydrolase isozyme L1(UCH-L1), heat shock protein A5, and cylindromatosis tumor suppressor (7,10-12). UCH-L1 is a deubiquitinating enzyme (DUB), and can identify and remove unwanted, misfolded, oxidized, or aggregated proteins. UCH-L1 mainly occurs in the neuronal cell body cytoplasm and is over expressed in central nervous injury or disease (1).

In this study, we hypothesized that changes in lncMEG3 and miRNA-181b expression occur during ICH injury, and that these changes could have neuroprotective effects. We also assessed the possible use of UCH-L1 as a therapeutic target in neurotrauma.

MATERIAL and METHODS

Thirty patients with ICH with moderate (GCS11–9, n=19) or severe (GCS ≤ 8, n=11) disturbance of consciousness under the same surgical way, and thirty age and gender matched controls were consecutively recruited from The General Hospital of Xinjiang Military Region from January 2021 to May 2021 for this study. Diagnosis of ICH was made by a neurosurgeon and confirmed by head computed tomography (CT). The site of the bleed was the left basal ganglia, and the diagnosis was made according to the Chinese Guidelines for the diagnosis and treatment of ICH issued by the Neurology Society of Chinese Medical Association in 2019. Based on survival status at hospital discharge, patients with ICH were classified into survivors (n=22) and non-survivors (n=8).

Patients were excluded if they had craniocerebral surgery, tumors, trauma, severe liver or kidney insufficiency, severe diabetes, or mental or neurodegenerative diseases. The control group was age and gender matched to the patient group, and the individuals in the control group had no central nervous system (CNS) diseases (confirmed by head CT/MRI).

All patients and their families provided informed consent and the investigation was approved by the Ethics Committee of Xinjiang Military General Hospital.

Collection and Testing of Venous Blood

Fasting blood was collected and studied after the diagnosis of ICH at several intervals: within 12 hours (T1), one (T2), two (T3), three (T4), four (T5), seven (T6), ten (T7), 12 days (T8), 15 days (T9), and 20 days (T10) after disease onset. The fasting blood was collected from healthy controls in the early morning. Serum was collected after natural coagulation by centrifugation at 1500–2000×*g* for 10 min. The serum samples were stored at –80°C for future use.

ELISA

Serum UCH-L1 levels in each group were detected using enzyme-linked immune sorbent assay (ELISA) kits purchased from Wuhan Elabscience Biotechnology Co.Ltd.

Quantitative Reverse Transcription PCR (qRT-PCR)

To measure MEG3 expression, 25–8ng/μl of total serum RNA was obtained using the TRIzol reagent (Invitrogen), and cDNA was generated using PrimeScript™ RT Master Mix (TaKaRa, Dalian, China). Quantitative reverse transcription PCR (qRT-PCR) was performed using a SYBR® Premix Ex Taq™ II kit (TaKaRa, Dalian, China) with a LightCycler480 Real-Time PCR System (Cabas Z480, Shanghai, China). Amplification conditions were 95°C, 30 s for start template denaturation, 95°C, 5 s for PCR cycle denaturation, 60°C, and 30 s for annealing and extension, for 40 cycles. To study miR-181-5p expression, miRNA was extracted from the serum by using a miRcute miRNA isolation kit purchased from Tiangen Biotechnology Co. Reverse transcription and real-time PCR were performed using PrimeScript™ Reverse Transcription Supermix and QuantiNova SYBR Green PCR Kits, respectively (both from TIANGEN, Beijing, China). Amplification conditions were 95°C, 15 min for start template denaturation, 94°C, 20 s for PCR cycle denaturation, 60°C, and 34 s for annealing and extension, for 40 cycles. Relative Lnc-MEG3 and miR-181-5p expression was determined using the $2^{-\Delta\Delta Ct}$ method, with GAPDH and cel mi RNA-39 (TIANGEN, CR100-01) as the control and external reference, respectively. The primers used are listed in Table I.

Statistical Analysis

Data were analyzed using the Statistical Product and Service Solutions (SPSS) 22.0 software. Graph were created using Graphpad Prism 6.0. Normal continuous variables were reported as mean ± standard deviation (SD). Categorical variables were reported as number (percentage). Skewness continuous variables were reported as median and interquartile range (IQR). Student's *t*-test, Wilcoxon rank-sum test, and chi-squared test were used to compare differences between the two groups. Correlation analysis was performed using the Spearman rank test. Variables to evaluate how well the indicators discriminated patient outcome were calculated by receiver operating characteristic (ROC) curve analysis. $p<0.05$ was considered statistically significance.

RESULTS

Clinical Features of the Study Cohort

The difference in occurrence of hypertension was statistically significant between the patients and control groups. No significant difference in age, gender, smoking, diabetes mellitus, hyperlipidemia, and drinking were observed between the ICH and control groups was observed. Detailed patient information is shown in Table II.

LncRNA Expression in the Plasma of Patients with ICH

Relative lnc-MEG3 expression was higher (0.03 [0.012–0.147]) in patients with ICH than in controls (0.009 [0.0002–0.023] p=0.004; Figure 1A). Fasting blood was studied after the diagnosis of ICH within 12 hours (T1).

Plasma miRNA Expression in Patients with ICH

Serum miR-181b levels were abnormally low in patients with ICH (0.6 [0.426–0.9] $\times 10^{-4}$) compared with that in controls (1.3 [0.8–1.9] $\times 10^{-4}$; $p<0.0001$; Figure 1B). Fasting blood was studied after the diagnosis of ICH within 12 hours (T1).

Pearson Correlation of lnc-MEG3 and miR-181b Expression

Pearson correlation of lnc-MEG3 and miR-181b levels in the ICH group showed a negative correlation between lnc-MEG3 and miR-181b (p=0.023, r=–0.414; Figure 2A). Fasting blood was studied after the diagnosis of ICH within 12 hours (T1).

Pearson Correlation of lnc-MEG3, miR-181b, and lnc-MEG3/miR-181b Axis with GCS Score in the ICH Group

Relative lnc-MEG3 relative expression (p<0.037, r=––0.383) and lnc-MEG3/miR-181b axis (p<0.045, r=–0.369) were negatively associated with GCS score in the ICH group (Figure 3A, 3C). Relative miR-181b expression (p<0.045, r=0.369) was positively associated with GCS score in the ICH group (Figure 3B). Fasting blood was studied after the diagnosis of ICH within 12 hours (T1).

Assessment of lnc-MEG3, miR-181b, and lnc-MEG3/miR-181b Axis for Differentiating Between Moderate and Severe ICH

Lower lnc-MEG3 expression (0.022 [0.004–0.066]) was observed in patients with moderate disturbance of consciousness (GCS 11–9) than in those with severe disturbance of consciousness (GCS≤8; 0.101 [0.022–0.272]); lnc-MEG3/miR-181b axis expression exhibited a similar trend. Relative expression of miR-181b was higher (0.682 [0.541–0.888]) in patients with moderate disturbance of consciousness (GCS

Table I: qRT-PCR Primer Sequences

RNA	Primer sequences
lncMEG3	forward 5′ CTCCCCTTCTAGCGCTCACG 3′ reverse: 5′-CTAGCCGCCGTCTATACTACCGGCT-3′
GAPDH	forward 5′-TC CCAGCTTAGGTTCATCAGG-3′ reverse 5′-ATGAAGGGGTCGTTGAT GGC-3′
miR-181-5p	forward: 5′ AACAUUCAUUGCUGUCGGUGGGU 3’

Table II: Clinical Features of the Study Population

Variable	ICH patients (n=30)	Controls (n=30)	Z/t□	p
Age (year, x±s)	50.1 ± 1.71	40.8 ± 1.25	-0.2	0.766
Males, (n,%)	22 (72.5)	18 (60.0)	-1.138	0.255
Hypertension, (n,%)	26 (87.5)	8 (25.7)	-5.385	<0.001
Diabetes, (n,%)	8 (27.5)	3 (11.4)	-1.724	0.085
Dyslipidemia, (n,%)	11 (35)	5 (17.1)	-1.733	0.083
Smoking, (n,%)	17 (55)	11 (37.1)	-1.536	0.125
Drinking, (n,%)	14 (47.5)	12 (40)	-0.648	0.517

Data are shown as mean ± SD or number (percentage). p<0.05 (Student's t-test or chi-squared test).

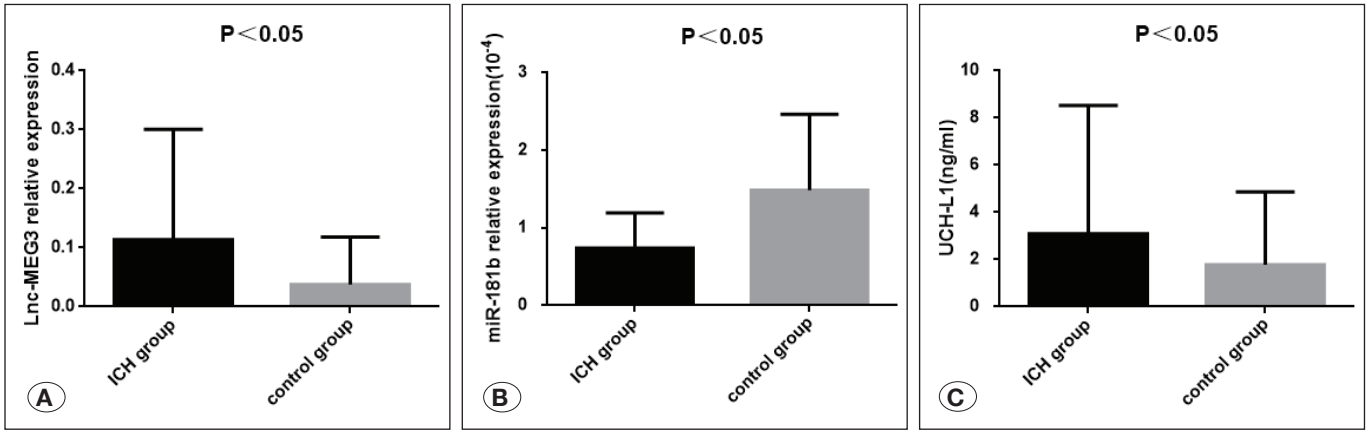

Figure 1: Relative expression of lnc-MEG3 **(A)**, miR-181b **(B)**, and initial levels of UCH-L1 **(C)** in patients with ICH and controls. Horizontal lines indicate median and IQR (Wilcoxon rank-sum test).

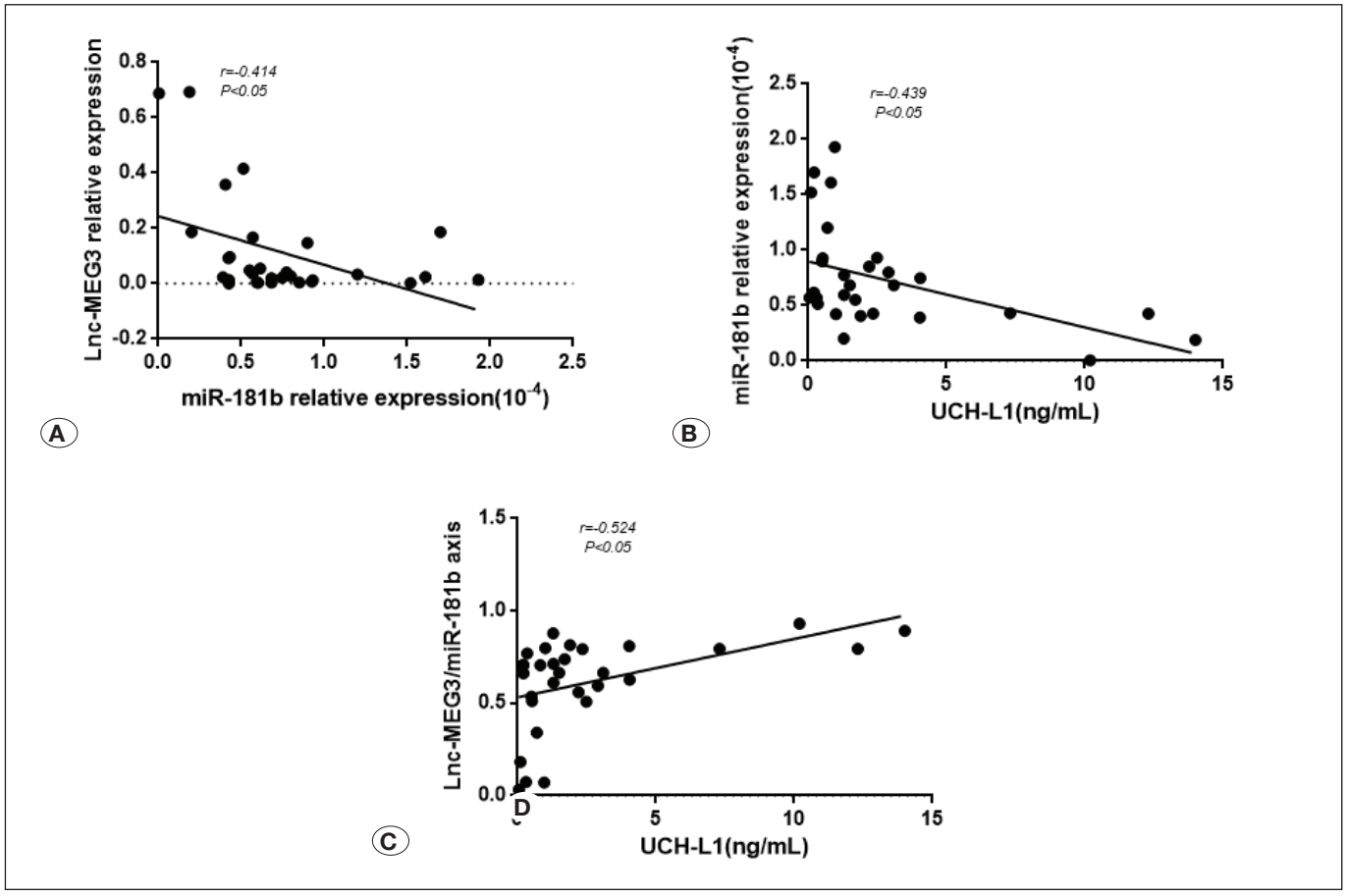

Figure 2: Correlation of relative lnc-MEG3 and miR-181b expression **(A)**, correlation of miR-181b **(B)** and lnc-MEG3/miR-181b axis **(C)** with UCH-1 protein expression in patients with ICH. Data ware evaluated by Pearson's rank correlation test. p<0.05 was considered significant.

11–9) than in those with severe disturbance of consciousness (GCS≤8; 0.488 [0.295–0.680]). However, these differences were not statistically significant (p<0.044, z=–2.017; p<0.033, z=–2.135; p<0.039, z=2.066, respectively; Figures 4A, 4C, 4B). Fasting blood was studied after the diagnosis of ICH within 12 hours (T1).

Plasma UCH-L1 Levels in Patients with ICH

Serum UCH-L1 levels were significantly higher in the ICH group (1.4 [0.5–4.1]) than in the control group (0.52 [0.06–1.85]; p<0.001; Figure 1C). Fasting blood was studied after the diagnosis of ICH within 12 hours (T1). By plotting UCH-L1 levels at different times (T1, T2, T3, T4, T5, T6, T7, T8, T9, T10).we found increases in UCH-L1 levels in the first 2 to 4 days of rupture onset in the ICH group (Figure 5).

Correlation Between miR-181b, lnc-MEG3/miR-181b Axis, and UCH-1 Protein Expression in Patients with ICH

miR-181b expression was negatively correlated with UCH-L1 levels (p=0.015, r=–0.439; Figure 3B). Further, there was a positive correlation between lnc-MEG3/miR-181b axis and UCH-L1 (p=0.003, r=0.524) levels in the ICH group (Figure 2C). Fasting blood was studied after the diagnosis of ICH within 12 hours (T1).

Initial Serum UCH-L1 Levels in Moderate and Severe ICH

Serum UCH-L1 levels were negatively correlated with GCS score in the ICH group (p=0.032, r=–0.392; Figure 4D), and higher UCH-L1 levels (2.49 [1.8–4.1]) were observed in patients with severe disturbance of consciousness than in patients with moderate disturbance of consciousness (0.82 [0.33–1.85]; p=0.025). Moreover when the cut-off value of serum UCH-L1 proteins to differentiate between severe disturbance and moderate disturbance of consciousness was 1.6 ng/ml, UCH-L1 reached a specificity of 81.8% and sensitivity of 73.7%. ROC area under the curve was 0.799 (95% CI, 0.567–0.940; p<0.05; Figure 7). Fasting blood was studied after the diagnosis of ICH within 12 hours (T1).

Initial Levels of UCH-L1 in Non-Survivors and Survivors at Hospital Discharge

By plotting UCH-L1 levels at different times (T1, T2, T3, T4, T5, T6, T7, T8, T9, T10), we found UCH-L1 levels were significantly higher in patients that did not survive than in patients who were discharged (p<0.001) at the time of hospital discharge. The survivors demonstrated steady increases in UCH-L1 levels with median levels from Day 2 to Day 4. In contrast, non-survivors showed significant elevations with peak from Day 2 to Day 4 (Figure 6).

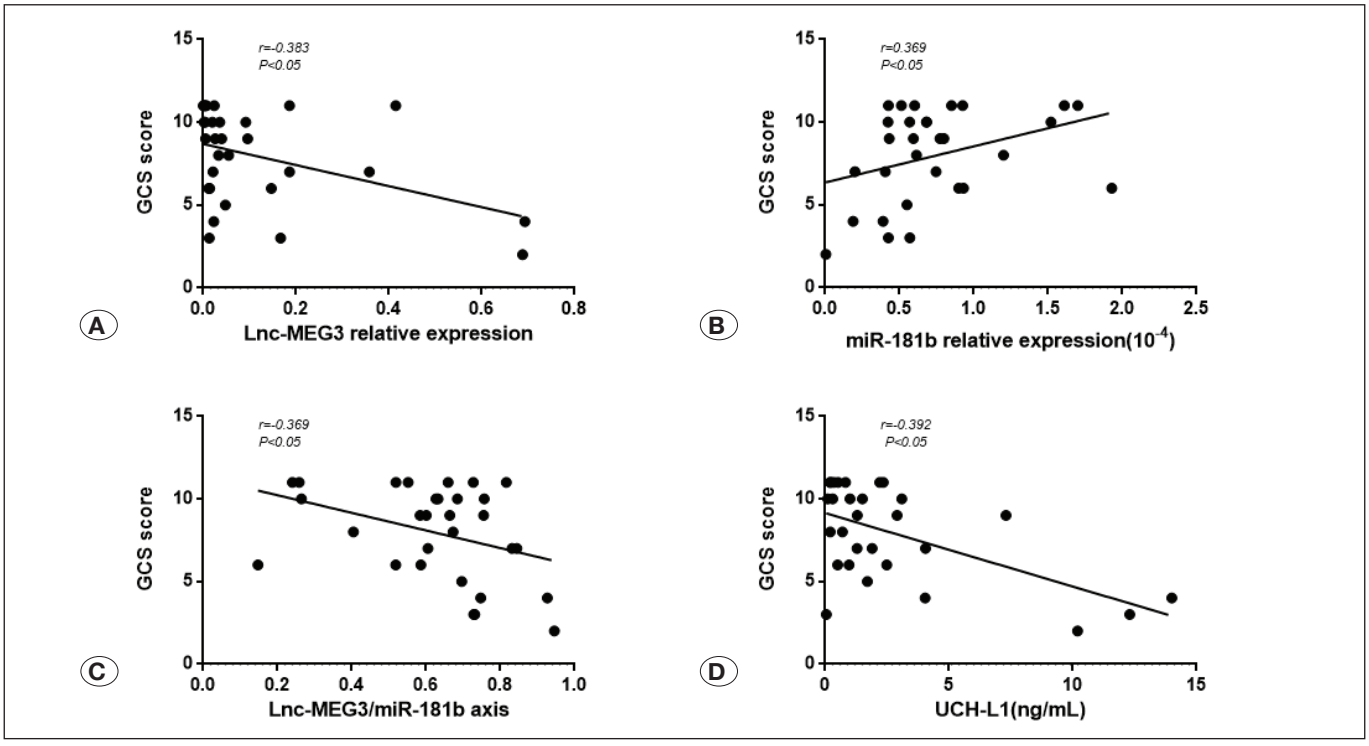

Figure 3: Associations between relative lnc-MEG3 expression **(A)**, miR-181b expression **(B)**, lnc-MEG3/miR-181b axis **(C)**, initial levels of UCH-L1 **(D)**, and GCS score in patients with ICH. Data were evaluated by Pearson's rank correlation test. p<0.05 was considered significant.

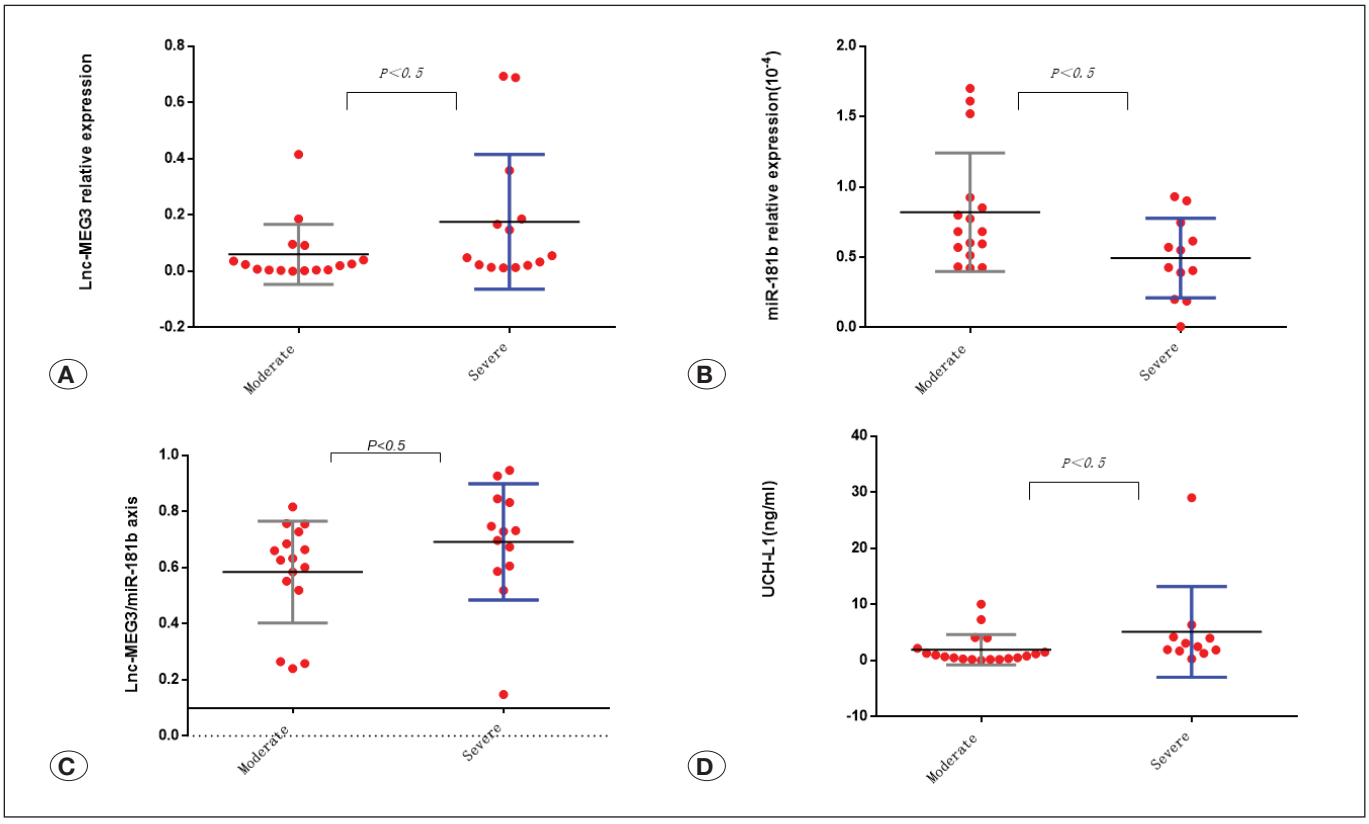

Figure 4: Relative lnc-MEG3 expression **(A)**, miR-181b expression **(B)**, lnc-MEG3/miR-181b axis **(C)**, and initial levels of UCH-L1 **(D)** in moderate and severe ICH. Horizontal lines indicate median and IQR (Wilcoxon rank sum test).

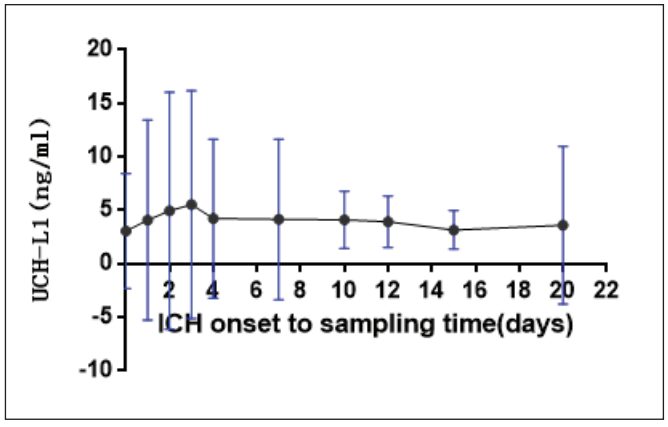

Figure 5: Temporal profile of UCH-L1 in the ICH group.

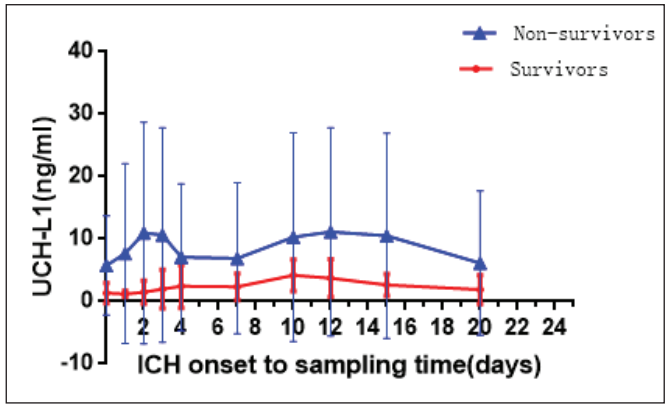

Figure 6: Temporal profile of UCH-L1 in survivors and non-survivors at the time of hospital discharge.

The optimal cut off value of serum UCH-L1 to predict death in patients with ICH was 1.6 ng/ml (Table III). Fasting blood was studied after the diagnosis of ICH within 12 hours (T1).

DISCUSSION

Although previous studies have shown differential Lnc-MEG3 expression in CNS disease, the clinical relevance of Lnc-MEG3 in ICH remained unclear. lnc-MEG3 acts as a ceRNA for miR-181b (4), binds to the miRNA via common miRNA response elements (MREs), and facilitates the translation of the target mRNA.

miR-181b downregulation has neuroprotective effects in mouse brain tissue (13). To determine whether miR-181b upregulation in ICH has favorable or adverse effects, the effect of its downstream target UCH-L1 was studied. UCH-L1 levels determined from minimally invasive samples could potentially be used as a proposed biomarker of ICH (14). We studied the association between LncMEG3/miR-181b/UCH-L1 and severity of intracerebral hemorrhage. We found that Lnc-MEG3 and UCH-L1 levels were elevated, and miR-181b was downregulated in patients with ICH. Further lnc-MEG3 expression was correlated with miR-181b expression. Further, lnc-MEG3/miR-181b axis and miR-181b was correlated with UCH-L1. Lnc-MEG3, UCH-L1 and lnc-MEG3/miR-21 axis

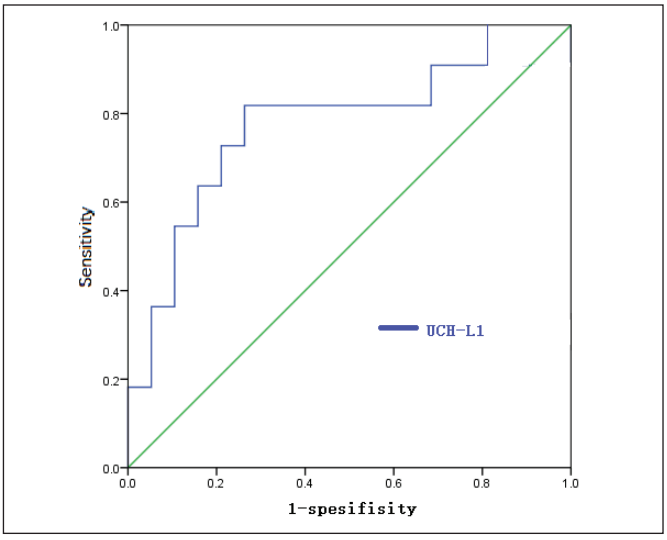

Figure 7: ROC Curve for distinguishing between moderate ICH and severe ICH.

Table III: UCH-L1 Level in the Prediction of Survival Until Hospital Discharge

Cutoff Point	Sensitivity (%)	Specificity (%)
1.4-1.49 (ng/mL)	81.8	68.4
1.5-1.59 (ng/mL)	81.8	68.4
1.6-1.69 (ng/mL)	81.8	73.7
1.7-1.79 (ng/mL)	72.7	73.7
1.8-1.89 (ng/mL)	63.6	73.7

were negatively correlated with GCS score in patients with ICH; however, miR-181b and UCH-L1 was positively correlated with GCS score. Moreover, lnc-MEG, miR-181b, UCH-L1, and the lnc-MEG3/miR-21 axis could be used to distinguish between severe and moderate ICH. Serum UCH-L1 has a numerically superior predictive value for death before hospital discharge, and UCH-L1 had a significant association with neurological impairment in ICH.

UCH-L1 functions primarily as a ubiquitin processing enzyme and has limited function as a hydrolase of ubiquitinated proteins that participates in the addition or removal of ubiquitin during protein metabolism. The activation of the ubiquitin-proteasome system (UPS) is central to the regulation of the common pathophysiological pathway after brain injury because it controls protein activity and production. UCH-L1 expressed by brain tissue in the blood can be used as an objective indicator to determine the normal physiological function or pathological conditions and study ICH pathology. We found that UCH-L1 could be used to differentiate between moderate and severe disturbance of consciousness with a sensitivity of 81.8% and specificity of 73.7%. The temporal profile of UCH-L1 was compared in survivors at hospital discharge and non-survivors. Higher levels of UCH-1 were

observed in the non-survivors than in the survivors. Further, the cut-off value of UCH-L1 to predict mortality was 1.6 ng/ml, which was consistent with the cut-off value of serum UCH-L1 protein to differentiate between severe disturbance and moderate disturbance of consciousness, indicating that the concentration of UCH-L1 in the serum are strongly correlated with clinical outcome and neurological impairment in ICH brain injury. The temporal profile of UCH-L1 showed that there were initial elevations in the first 2 to 4 days of rupture onset in non-survivors, which suggests that UCH-L1 could be used as an indicator in combination with clinical signs, neurological function scores, and imaging examinations for better diagnosis. A significant advantage of serum biomarkers over imaging is that they can be continuously monitored to understand patient condition. The significance of our research is that neurological symptoms in ICH patients are very unstable in the first few days after onset. Continuous monitoring and aggressive treatment, such as early intensive antihypertensive therapy, can reduce the likelihood of death and severe disability, and facilitate functional recovery in patients who survive. Almost all patients with ICH with less bleeding survive if they receive good medical treatment (9), suggesting that good clinical management can effectively reduce the mortality and disability associated with ICH.

Further studies on the use of non-protein coding RNA as potential therapeutic target for ICH are required (6). MiR-181b acts as an upstream regulator of mUCH-L1. We therefore expected that miR-181b downregulation would result in upregulation of mUCH-L1. In our study, miR-181b downregulation protected against brain injury. miR-181b overexpression, which is typically associated with neuroprotection, could therefore exacerbate ICH.

lnc-MEG3 can cause to cell replication, senescence, or apoptosis by blocking the cell cycle via the p53 pathway. Based on our results, we speculated that lnc-MEG3 is involved in the pathophysiology of ICH by interacting with miR-181b. Downregulation of lnc-MEG3 could enhance neurobehavioral outcomes and protect against brain damage.

ICH accounts for 10% to 15% of all strokes worldwide, and a definitive treatment is currently unavailable. Our findings strongly suggest that Lnc-MEG3 and miR-181b could be potential targets for the treatment of ICH; changes in their expression following brain injury could be used to predict brain injury severity and neurological outcome. The mechanism by which lncRNA-miRNA-mRNA pairings may interfere with neuronal survival is unclear, and requires further study. A limitation of this study was that the number of cases was relatively small because of a small number of hospital admissions and low specimen collection time. The conclusions obtained in this study therefore need to be verified in larger multicenter studies.

CONCLUSION

In conclusion, a better understanding of the role of lnc-MEG3 and miR-181b in the progression of cerebral hemorrhage, and related clinical information on ICH will facilitate the development of novel strategies that use their potential as a therapeutic targets. Serum UCH-L1 level could be used as an objective indicator to evaluate normal physiological function or pathological status in the central nervous system. Further, UCH-L1 levels, together with clinical signs, neurological function score, and imaging data could be applied as an objective basis to judge the severity of brain injury, evaluate death, and study the pathology of ICH.

AUTHORSHIP CONTRIBUTION

Study conception and design: HW

Data collection: HW

Analysis and interpretation of results: HW

Draft manuscript preparation: HW

Critical revision of the article: HW

Other (study supervision, fundings, materials, etc...): HW, LW, QS

All authors (HW, LW, QS) reviewed the results and approved the final version of the manuscript.

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