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RESEARCH ARTICLE

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Long noncoding RNA HULC in acute ischemic stroke: Association with disease risk, severity, and recurrence-free survival and relation with IL-6, ICAM1, miR-9, and miR-195

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Abstract

Background: This study aimed to evaluate the clinical role of long noncoding RNA (lncRNA) HULC in acute ischemic stroke (AIS).

Methods: LncRNA HULC in plasma samples from 215 first episode AIS patients and 215 age/gender-matched non-AIS controls was detected by reverse transcriptional-quantitative polymerase chain reaction (RT-qPCR). Then, in AIS patients, interleukin-6 and intercellular adhesion molecule 1 (ICAM1), as well as microRNA (miR) target of lncRNA HUCL (miR-9 and miR-195), were detected by enzyme-linked immunosorbent assay and RT-qPCR, respectively. Disease severity was assessed by National Institution of Health stroke scale (NIHSS) score. AIS recurrence or death was recorded, and recurrence-free survival (RFS) was calculated.

Results: LncRNA HULC was increased in AIS patients compared to non-AIS controls ($P < .001$), and receiver operating characteristic curve showed that it was correlated with increased AIS risk (area under curve: 0.876, 95% confidence interval: 0.843-0.908). Meanwhile, lncRNA HULC was positively correlated with NIHSS score ($P < .001$, $r = .456$), interleukin-6 ($P < .001$, $r = .275$) and ICAM1 ($P < .001$, $r = .383$), whereas negatively correlated with miR-9 ($P < .001$, $r = -.438$) but not miR-195 ($P = .205$, $r = -.087$) in AIS patients. Additionally, miR-9 was negatively correlated with NIHSS score ($P < .001$, $r = -.335$), interleukin-6 ($P = .001$, $r = -.231$), and ICAM1 ($P < .001$, $r = -.280$), while miR-195 was only negatively associated with NIHSS score ($P = .041$, $r = -.139$) in AIS patients. Moreover, lncRNA HULC high expression predicted worse RFS ($P = .013$) in AIS patients.

Conclusion: LncRNA HULC is correlated with higher AIS risk, increased disease severity and worse prognosis in AIS patients. Meanwhile, it associates with higher IL-6, elevated ICAM1, and lower miR-9 AIS patients.

KEYWORDS

AIS, ICAM1, IL-6, LncRNA HULC, recurrence-free survival

1 | INTRODUCTION

Acute ischemic stroke (AIS) is one of the leading causes of disability and mortality in China, which induces tremendous economic and humanistic burden.[1,2] According to the latest estimate from the Global Burden of Diseases 2016, the age-standardized prevalence of AIS has increased by 36.6% during the past three decades in China, and AIS would still be a major public health issue in the future.[3] Moreover, the narrow treatment window of AIS restricts the application of effective treatment and partly leads to worse prognosis in AIS patients.[4] Therefore, it might be critical to search for novel biomarkers for prediction of AIS risk and surveillance of AIS severity as well as prognosis of AIS patients.

Long noncoding RNA (lncRNA) highly up-regulated in liver cancer (HULC), a lncRNA of 16 kb located on chromosome 6q24.3, is initially found to be overexpressed in hepatocellular carcinoma.[5] Recently, it is suggested that lncRNA HULC could regulate inflammation in vascular endothelial cells, thus participating in the occurrence and progression of AIS.[6] Meanwhile, previous studies reveal that lncRNA HULC promotes interleukin (IL)-6 and intercellular adhesion molecule 1 (ICAM1) expressions in human dermal microvascular endothelial cells, and both IL-6 and ICAM1 are two critical indicators for AIS risk.[7-9] Moreover, two microRNA (miR) targets of lncRNA HULC, namely miR-9 and miR-195,[6,10] play important roles in the pathogenesis and progression of AIS.[11,12] Additionally, in our pilot study, we found that lncRNA HULC was elevated in AIS patients compared to non-AIS controls. Based on the abovementioned information, we hypothesized that lncRNA HULC might be a potential biomarker for AIS; however, no relevant study had been performed.

In the present study, we consecutively enrolled 215 first episode AIS patients and 215 age/gender-matched non-AIS controls with high stroke risk. The aim of this study was to evaluate the correlation of lncRNA HULC with AIS risk, disease severity, and prognosis, and to investigate the relation of lncRNA HULC with IL-6, ICAM1 and its two miRNA targets in AIS patients.

2 | METHODS

2.1 | Participants

This study consecutively recruited 215 first episode AIS patients from our hospital between January 2015 and December 2016. All patients were aged above 18 years and confirmed as first episode AIS by cranial computed tomography (CT) scan, magnetic resonance imaging (MRI), and/or diffusion-weighted imaging within 24 hours following the onset of symptoms. The patients who suffered from cerebral hemorrhagic infarction, epilepsy, severe infections, hematological malignancies, solid tumors, or treated with immunosuppressive drugs in the past 3 months were excluded. In addition, pregnant or lactating women were also excluded from this study. During the same period, 215 subjects whose age and gender were matched with AIS patients were enrolled as non-AIS controls. The inclusion criteria for non-AIS controls were subjects who complicated with at least two of the following risk factors: hypertension, heart disease, tobacco use, hyperlipidemia, diabetes mellitus, overweight or obesity, family history of stroke, and so on[13]; age and gender were matched with enrolled AIS patients; and had no history of AIS. The exclusion criteria for non-AIS controls were as follows: had a history of stroke, hematological malignancies, solid tumors, severe infections, and pregnant or lactating women. This study was approved by the Ethics Committee of our hospital. All participants or their family members signed the informed consents before enrollment.

2.2 | Data and sample collection

After enrollment, the demographic characteristics and AIS risk factors were recorded. And the severity of AIS was assessed using the National Institute of Health stroke scale (NIHSS). The peripheral blood samples of AIS patients were collected on the day of admission, and then, the blood samples were centrifugated at 2000 g for 20 minutes (4°C) to separate plasma samples. The peripheral blood samples of non-AIS controls were collected on the enrollment, and the plasma samples were isolated from the blood samples using the same method as described above.

2.3 | LncRNA HULC detection

For the AIS patient and non-AIS patients, the expression of lncRNA HULC in plasma samples was detected by reverse transcriptional-quantitative polymerase chain reaction (RT-qPCR). Briefly, total RNA was extracted by miRNeasy Serum/Plasma Advanced Kit (Qiagen), reverse transcription was performed with an iScript™ cDNA Synthesis Kit (Bio-Rad) under the manufacturer's instruction, and qPCR was conducted by THUNDERBIRD® SYBR® qPCR Mix (Toyobo) according to the manufacturer's guidance. The expression of lncRNA HULC was calculated and normalized to the endogenous reference GAPDH by the $2^{-\Delta\Delta Ct}$ formula. The primers were listed in Table S1.

2.4 | IL-6, ICAM1, miR-9, and miR-195 detections

For the AIS patients, the levels of IL-6 and ICAM-1 in plasma samples were detected by enzyme-linked immunosorbent assay (ELISA) and the expressions of miR-9 and miR-195 in plasma samples were detected by RT-qPCR. All procedures of ELISA were conducted referring to the handbooks of Human ICAM-1 (Soluble) ELISA Kit (Thermo Fisher Scientific) and Human IL-6 ELISA Kit (Thermo Fisher Scientific). All procedures in RT-qPCR were carried out referring to detection of lncRNA HULC, while U6 was used for internal reference. The primers were listed in Table S1.

2.5 | Treatment and follow-up

After admission, all AIS patients received appropriate treatments, which were decided by their physicians based on clinical status. Regular follow-up was performed by telephone or clinical visit until 36 months, stroke recurrence, or death. Recurrence-free survival (RFS) was defined as the duration from admission to stroke recurrence or death. A total of 30 AIS patients lost follow-up, and they were censored on the date of the last visit in the finally analysis. In addition, for the patients who did not know whether they have relapsed or died at last follow-up, they were censored on the date of the last visit as well.

2.6 | Statistical analysis

Statistical analyses were performed using SPSS software version 24.0 (IBM). The figures were plotted using GraphPad Prism software version 7.01 (GraphPad Software). Comparisons between two groups were determined by Student's t test, chi-square test, or Wilcoxon rank-sum test. Correlations between two variables were analyzed by Spearman's rank correlation test. The performance of variables in discriminating AIS patients from non-AIS controls was assessed using receiver operating characteristic (ROC) curve, the area under the curve (AUC) with 95% confidence interval (CI), sensitivity, specificity, false-positive rate (FPR), and false-negative rate (FNR) at best cut-off point (defined as the point which the sum of false positives and false negatives is less than any other point). RFS was illuminated by the Kaplan-Meier curve, and the difference of RFS between two groups was determined by the log-rank test. *P* value <.05 was considered statistically significant.

3 | RESULTS

3.1 | Characteristics of all participants

The mean age was 62.7 ± 11.0 years in AIS patients and was 61.7 ± 9.3 years in non-AIS controls. There were 59 (37.4%) females and 156 (72.6%) males in AIS patients, and 50 (23.3%) females as well as 165 (76.7%) males in non-AIS controls. Further analysis showed that no difference was found in age, gender distribution, or body mass index between AIS patients and non-AIS controls (all $P > .05$). Regarding AIS risk factors, the numbers of participants with hypertension (185 (86.0%) vs 161 (74.9%)) ($P = .004$), diabetes mellitus (63 (29.3%) vs 45 (20.9%)) ($P = .045$), or CKD (38 (17.7%) vs 19 (8.8%)) ($P = .007$) were increased in AIS patients compared to non-AIS controls; whereas the numbers of participants who were current smokers, or with hyperlipidemia or hyperuricemia were similar between AIS patients and non-AIS controls (all $P > .05$). Additionally, the mean NIHSS score was 8.2 ± 3.5 in AIS patients. The detailed demographic and clinical characteristics of the participants were shown in Table 1.

3.2 | LncRNA HULC relative expression in AIS patients and non-AIS controls

LncRNA HULC was increased in AIS patients compared to non-AIS controls ($P < .001$) (Figure 1A). Meanwhile, the ROC curve showed that LncRNA HULC had good potential in discriminating AIS patients from non-AIS controls with AUC of 0.876 (95% CI: 0.843-0.908). Besides, lncRNA HULC relative expression was 1.508 at the best cut-off point; and the sensitivity, specificity, FPR, and FNR at the best cut-off point were 80.9%, 82.8%, 17.2%, and 19.1%, respectively (Figure 1B).

3.3 | Association of lncRNA HULC with NIHSS score in AIS patients

The correlation between lncRNA HULC and NIHSS score in AIS patients was investigated subsequently, and data showed that lncRNA HULC was positively correlated with NIHSS score in AIS patients ($P < .001$, $r = .456$) (Figure 2).

3.4 | Association of lncRNA HULC with IL-6 and ICAM1 in AIS patients

The correlation analysis in LncRNA HULC with pro-inflammatory cytokine IL-6 and cell adhesion molecule ICAM1 was performed. As shown in Figure 3, lncRNA HULC was positively correlated with IL-6 ($P < .001$, $r = .275$) (Figure 3A) and ICAM1 ($P < .001$, $r = .383$) (Figure 3B) in AIS patients.

3.5 | Association of lncRNA HULC with miR-9 and miR-195 in AIS patients

The correlation between lncRNA HULC and two of its target miRNAs were investigated. Data showed that lncRNA HULC was negatively correlated with miR-9 ($P < .001$, $r = -.438$) (Figure 4A); however, no significant correlation was observed between lncRNA HULC and miR-195 ($P = .205$, $r = -.087$) (Figure 4B) in AIS patients. Furthermore, miR-9 was negatively correlated with NIHSS score ($P < .001$, $r = -.335$) (Figure 5A), IL-6 ($P = .001$, $r = -.231$) (Figure 5B), and ICAM1 ($P < .001$, $r = -.280$) (Figure 5C) in AIS patients. As to miR-195, it was negatively correlated with NIHSS score ($P = .041$, $r = -.139$) (Figure 5D); however, no significant correlation was found in miR-195 with IL-6 ($P = .285$, $r = -.073$) (Figure 5E) or ICAM1 ($P = .103$, $r = -.111$) (Figure 5F) in AIS patients.

3.6 | Association of LncRNA HULC with RFS in AIS patients

Based on the median value of LncRNA HULC relative expression in AIS patients, they were further divided into LncRNA HULC high

TABLE 1 Comparison of characteristics between AIS patients and non-AIS controls

Items	Non-AIS controls (N = 215)	AIS patients (N = 215)	*P* value
Age (years), mean ± SD	61.7 ± 9.3	62.7 ± 11.0	.325
Gender, No. (%)			
Female	50 (23.3)	59 (37.4)	.318
Male	165 (76.7)	156 (72.6)	
BMI (kg/m^2), mean ± SD	24.2 ± 2.8	24.3 ± 2.3	.724
Current smoke, No. (%)			
No	125 (58.1)	113 (52.6)	.244
Yes	90 (41.9)	102 (47.4)	
Hypertension, No. (%)			
No	54 (25.1)	30 (14.0)	.004
Yes	161 (74.9)	185 (86.0)	
Hyperlipidemia, No. (%)			
No	118 (54.9)	109 (50.7)	.385
Yes	97 (45.1)	106 (49.3)	
Hyperuricemia, No. (%)			
No	151 (70.2)	137 (63.7)	.151
Yes	64 (29.8)	78 (36.3)	
Diabetes mellitus, No. (%)			
No	170 (79.1)	152 (70.7)	.045
Yes	45 (20.9)	63 (29.3)	
CKD, No. (%)			
No	196 (91.2)	177 (82.3)	.007
Yes	19 (8.8)	38 (17.7)	
NIHSS score, mean ± SD	-	8.2 ± 3.5	-

Note: Comparisons were determined by Student's *t* test or chi-square test.

Abbreviations: AIS, acute ischemic stroke; BMI, body mass index; CKD, chronic kidney disease; NIHSS, National Institute of Health stroke scale; SD, standard deviation.

expressed AIS patients and LncRNA HULC low expressed AIS patients. Data showed that RFS was shorter in LncRNA HULC high expressed AIS patients compared to LncRNA HULC low expressed AIS patients ($P = .013$) (Figure 6).

4 | DISCUSSION

LncRNA HULC is a vital regulator in the pathogenesis and progression of AIS. For example, it is reported that the overexpression of lncRNA HULC could regulate tumor necrosis factor (TNF)-α-induced apoptosis in vascular endothelial cells, thus participating in the vascular endothelial dysfunction.[6] Meanwhile, inflammation-promoted human dermal microvascular endothelial cells, the knockdown of lncRNA HULC decreases the mRNA level of IL-6 and ICAM1[7] (which are two vital pro-inflammatory mediators that contribute to the progression of AIS[14,15]). Moreover, lncRNA HULC might interact with miRNAs to regulate the progression of AIS. For instance, it is reported that lncRNA HULC is able to regulate DNA methyltransferase to modulate miR-9 expression,[6] and the latter one could target nucleotide oligomerization domain-like receptor pyrin domain-containing protein 1 (NLRP1) to ameliorate injury of neural cells after ischemic stroke in rats.[11] Additionally, lncRNA HULC is able to target miR-195,[10] which not only reduces inflammation and apoptosis by suppressing the c-Jun N-terminal kinase (JNK) pathway and the nuclear factor-κB (NF-κB) pathway in injured neural cells, but also promotes regeneration of neural stem cell by increasing cell proliferation and migration.[12] Therefore, lncRNA HULC plays a vital role in the initiation and progression of AIS. Based on this information, we hypothesized that lncRNA HULC might be a potential predictive biomarker for AIS risk. In the present study, we observed that lncRNA HULC was up-regulated in AIS patients compared to non-AIS controls. Additionally, lncRNA HULC was correlated with enhanced AIS risk. Possible explanations for our data might be that: (a) as mentioned above, the up-regulation of lncRNA HULC might increase IL-6 and ICAM1 level to promote inflammation, and to exacerbate the TNF-α-induced apoptosis in

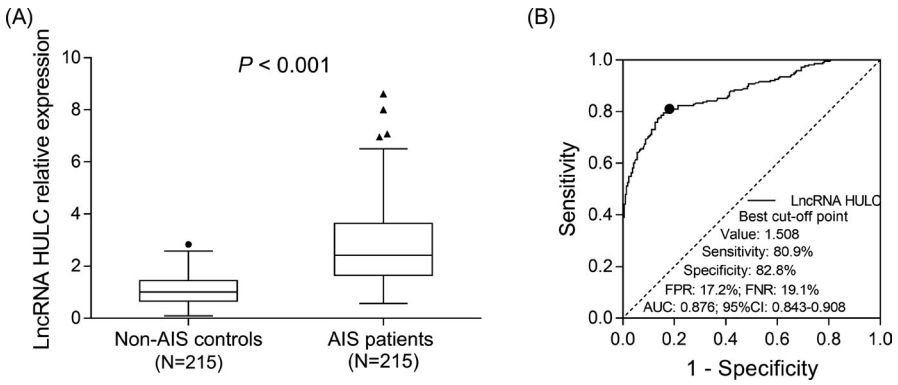

FIGURE 1 LncRNA HULC relative expression in AIS patients and non-AIS controls, and its correlation with AIS risk. A, Comparison of lncRNA HULC relative expression between AIS patients and non-AIS controls; B, Correlation of lncRNA HULC with AIS risk. AIS, acute ischemic stroke; AUC, area under curve; CI, confidence interval; FNR, false-negative rate; FPR, false-positive rate; HULC, highly up-regulated in liver cancer; LncRNA, long noncoding RNA; ROC, receiver operating characteristic

vascular endothelial cells, which contributed to the pathogenesis of AIS[14,15]; and (b) lncRNA HULC might act as a competing endogenous RNA to suppress several miRNAs that possessed anti-inflammatory function and suppressed the initiation of AIS (such as miR-9 and miR-195[10,12]). Therefore, increased lncRNA HULC was correlated with higher AIS risk.

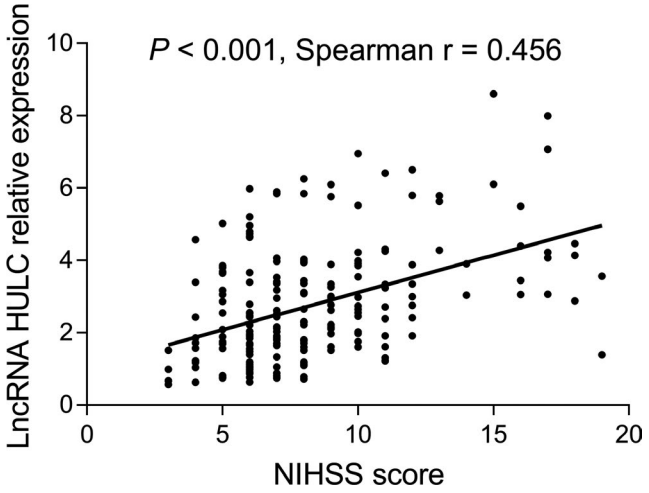

FIGURE 2 Correlation analysis between lncRNA HULC relative expression and NIHSS score in AIS patients. AIS, acute ischemic stroke; HULC, highly up-regulated in liver cancer; LncRNA, long noncoding RNA; NIHSS, National Institute of Health stroke scale

It is suggested that several lncRNAs might be indicators of disease severity in AIS[16-18]; however, no previous study had been conducted to investigate the correlation between lncRNA HULC and disease severity of AIS to the best of our knowledge. In this study, a positive correlation was observed between lncRNA HULC relative expression and NIHSS score in AIS patients, which could be explained by that: (a) the high expression of lncRNA HULC might promote several pro-inflammatory cytokines such as IL-6 (mentioned above) to up-regulate inflammation, which exacerbated the injury of neural cell and increased severity of AIS[19]; (b) increased lncRNA HULC might promote cell adhesion molecules (such as ICAM1[7]) to enhance neutrophil infiltration, which resulted in higher levels of vascular dysfunction and thrombosis,[20] thus increasing the severity of AIS; and (c) the up-regulation of lncRNA HULC might suppress its target miRNAs (such as miR-9 and miR-195[6,10]) to repress the regeneration of neural cells, thus increasing brain injury after stroke. Therefore, lncRNA HULC was positively associated with NIHSS score in AIS patients.

Additionally, we investigated the correlation of lncRNA HULC with several regulators that are highly involved in the progression of AIS (including IL-6, ICAM1, miR-9, and miR-195). Data showed that positive correlations were observed in lncRNA HULC with IL-6 and

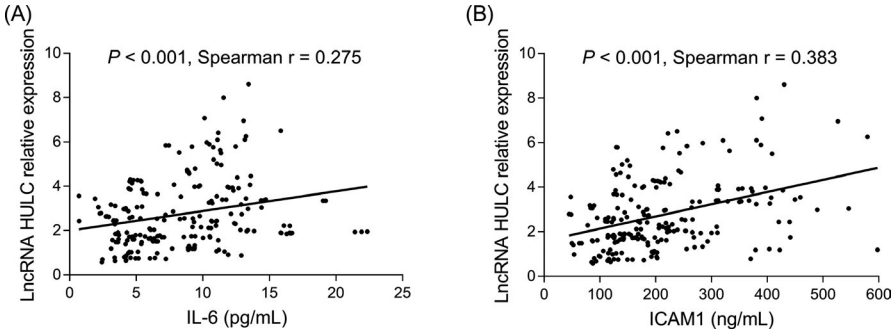

FIGURE 3 Correlation analysis of lncRNA HULC relative expression with IL-6 and ICAM1 in AIS patients. A, Correlation analysis between lncRNA HULC relative expression and IL-6; B, Correlation analysis between lncRNA HULC relative expression and ICAM1. AIS, acute ischemic stroke; HULC, highly up-regulated in liver cancer; ICAM1, intercellular adhesion molecule 1; IL-6, interleukin-6; LncRNA, long noncoding RNA.

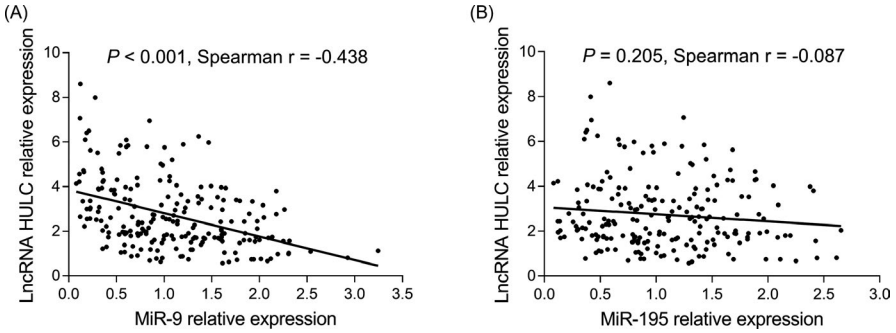

FIGURE 4 Correlation analysis of lncRNA HULC relative expression with miR-9 and miR-195 in AIS patients. A, Correlation analysis between lncRNA HULC relative expression and miR-9; B, Correlation analysis between lncRNA HULC relative expression and miR-195. AIS, acute ischemic stroke; HULC, highly up-regulated in liver cancer; LncRNA, long noncoding RNA; miR, microRNA

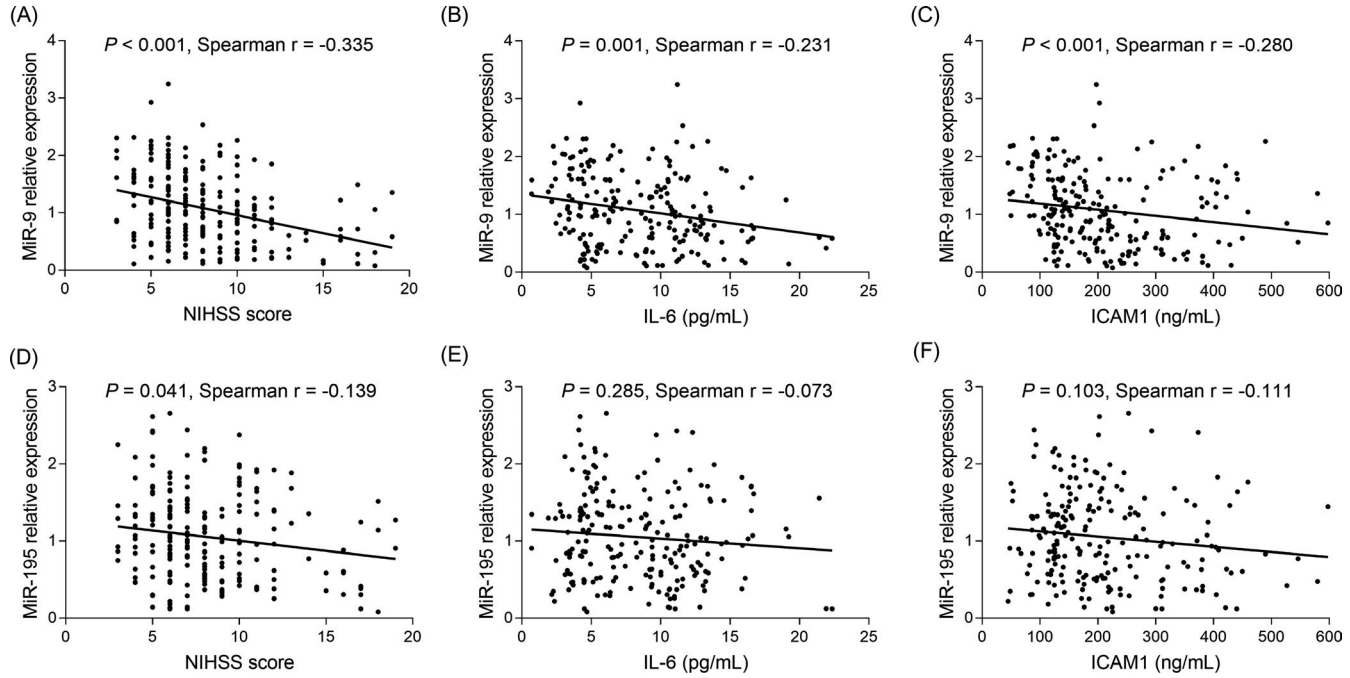

FIGURE 5 Correlation analysis of miR-9 and miR-195 with NIHSS score, IL-6, and ICAM1 in AIS patients. A, Correlation analysis between miR-9 and NIHSS score; B, Correlation analysis between miR-9 and IL-6; C, Correlation analysis between miR-9 and ICAM1; D, Correlation analysis between miR-195 and NIHSS score; E, Correlation analysis between miR-195 and IL-6; F, Correlation analysis between miR-195 and ICAM1. ICAM1, intercellular molecule 1; IL-6, interleukin-6; miR, microRNA; NIHSS, National Institute of Health stroke scale

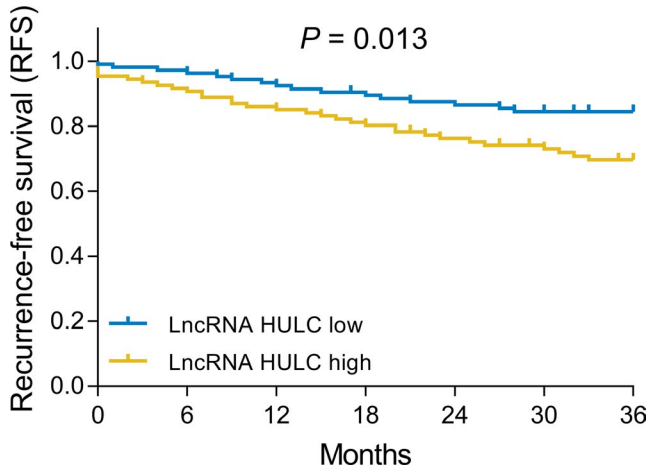

FIGURE 6 Comparison of RFS between lncRNA HULC high expressed AIS patients and lncRNA HULC low expressed AIS patients. HULC, highly up-regulated in liver cancer; LncRNA, long noncoding RNA; RFS, recurrence-free survival

ICAM1, and negative correlations were found in lncRNA HULC with miR-9 and miR-195 (although the correlation between lncRNA HULC and miR-195 was not statistically significant), which supported the viewpoint that lncRNA HULC might interact with pro-inflammatory cytokines (such as IL-6[7]), cell adhesion molecules (such as ICAM1[7]) as well as its target miRNAs (such as miR-9 and miR-195[6,10]) to regulate AIS. Moreover, negative correlations were observed in miR-9 with disease severity and pro-inflammatory mediators IL-6 as well as ICAM1; meanwhile, similar tendencies were also observed in miR-195, but less significant. Possible explanations might be that (a) miR-9 might suppress the nuclear factor-κB pathway to repress inflammation and to alleviate neural injury[21,22]; meanwhile, it might inhibit apoptosis and inflammation in vascular endothelial cells by targeting CXC chemokine receptor-4.[23] Therefore, miR-9 was negatively correlated with disease severity, IL-6, and ICAM1 in AIS patients; and (b) miR-195 might reduce neural injury through activating the JNK pathway, and thus, it was negatively correlated with disease severity in AIS patients; however, it might not affect inflammation or stability of vascular endothelial cells; therefore, no correlation was found in miR-195 with IL-6 or ICAM1 in AIS patients.

In the present study, we further investigated the prognostic value of lncRNA HULC in AIS patients. Data showed that lncRNA HULC was negatively correlated with RFS in AIS patients, which could be explained by that lncRNA HULC was correlated with enhanced disease severity (mentioned above), which indirectly resulted in worse RFS in AIS patients; meanwhile, higher lncRNA HULC might influence inflammation, vascular dysfunction, and thrombosis level, as well as injury and regeneration of neural cells,[7,12,20] thus directly worsening the RFS of AIS patients.

Several limitations existed in this study and should be clarified. Firstly, the follow-up duration of this study was relatively short, and further studies with longer follow-up duration could be conducted to investigate the long-term prognostic effect of lncRNA HULC in AIS patients. Secondly, lncRNA HULC had a negative correlation with RFS in AIS patients, indicating it might modulate the recurrence of AIS; therefore, the clinical role of lncRNA HULC in recurrent AIS patients could be investigated further. Finally, the detailed molecular

mechanism of lncRNA HULC in the regulation of AIS was not investigated in this study, which could be performed further.

Collectively, lncRNA HULC is correlated with increased AIS risk, higher disease severity, and worse RFS; meanwhile, it shows positive association with IL-6 and ICAM1 while negative association with miR-9 in AIS patients.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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