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Long non-coding RNA UCA1 correlates with elevated disease severity, Th17 cell proportion, inflammatory cytokines, and worse prognosis in acute ischemic stroke patients

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Abstract

Background: This study aimed to explore the association of long non-coding RNA urothelial carcinoma-associated 1 (lncRNA UCA1) expression with disease severity, inflammation, and prognosis in acute ischemic stroke (AIS) patients.

Methods: The lncRNA UCA1 expression of blood $CD4^+$ T cells from 160 first-episode AIS patients and 160 non-AIS patients with high-stroke-risk factors (as controls) was detected by reverse transcription quantitative polymerase chain reaction. For AIS patients, interleukin (IL)-6, IL-17, and intracellular adhesion molecule-1 (ICAM1) were determined by enzyme-linked immunosorbent assay; Th17 cell ratio in $CD4^+$ T cells was detected by flow cytometry. Their follow-up data were recorded up to 36 months, recurrence of stroke or death. The recurrence-free survival (RFS) analysis was assessed according to the follow-up data.

Results: LncRNA UCA1 expression was higher in AIS patients compared to controls ($p < 0.001$), and it was positively correlated to national institute of health stroke scale score ($r = 0.436$, $p < 0.001$), Th17 cell ratio ($r = 0.398$, $p < 0.001$), IL-6 ($r = 0.204$, $p = 0.010$), IL-17 ($r = 0.326$, $p < 0.001$), and ICAM1 ($r = 0.276$, $p < 0.001$) in AIS patients. Regarding prognosis, lncRNA UCA1 expression was elevated in 2-year recurrence/death AIS patients compared to those patients without recurrence or death within 2 years ($p = 0.033$), also increased in 3-year recurrence/death AIS patients compared to those patients without recurrence or death within 3 years ($p = 0.008$). Furthermore, high lncRNA UCA1 expression was associated with worse accumulating RFS ($p = 0.017$) in AIS patients.

Conclusion: LncRNA UCA1 might sever as a candidate prognostic biomarker in AIS patients, suggesting its potency for AIS management.

KEYWORDS
acute ischemic stroke, disease severity, inflammation, Long non-coding RNA urothelial carcinoma-associated 1, prognosis

1 | INTRODUCTION

Acute ischemic stroke (AIS) is a cerebrovascular disease characterized by blockage of the cranial vasculature, which leads to insufficient oxygen and nutrients supply to the affected brain area.[1] Notably, it is the third most common cause of death for elderly people with prevalence rate of 36.8% for patients above 60 years in high-income countries.[2] Regarding the current treatment for AIS, it involves thrombolysis and embolectomy surgery to resolve the thrombosis and re-establish normal blood flow in the vessels.[3] However, AIS patients tend to develop restenosis and recurrence due to prolonged ischemic period and profound affected brain area, which results in poor prognosis and reduced quality of life in them.[3] Therefore, finding a potential and novel factor to monitor disease progression and predict prognosis in AIS patients is necessary.

Long non-coding RNA (lncRNA) is a type of RNA whose length over 200 nucleotides with less coding function, which has been reported to be highly involved in angiogenesis and inflammation, and some of them have been identified as key regulators in carido-cerebrovasular pathophysiology, especially in the stroke.[4-6] As a commonly studied lncRNA, lncRNA urothelial carcinoma-associated 1 (lncRNA UCA1) is initially found in bladder carcinoma and serves as a tumor enhancer in various types of cancer (such as colorectal cancer, gastric cancer, and hepatocellular carcinoma).[7-10] More interestingly, accumulating evidence suggests the crucial role of lncRNA UCA1 in various cadio-cerebovascular diseases.[11-13] For instance, lncRNA UCA1 regulates microRNA (miR)-18a expression to promote hypoxia injury and neural cell apoptosis in the cell model of cerebral ischemia.[14] Moreover, small RNA interference targeting lncRNA UCA1 decreases pro-inflammatory cytokines (such as IL-6 and ICAM1) at gene level and prevents their induction in lipopolysaccharide (LPS) treated human dermal microvascular endothelial cells, indicating the correlation of lncRNA UCA1 with inflammatory cytokines.[11] Besides, inflammation plays an important role in the pathogenesis of AIS since inflammation leads to unstable atherosclerotic plaque with higher risk of rupture. In the clinical practice, lncRNA UCA1 expression is upregulated in acute myocardial infraction (AMI) patients, which shares similar pathophysiological mechanism as AIS since both of these two diseases characterized by the blockage of blood vessels due to atherosclerosis plaque formation.[12] In addition, we performed a preliminary research in a smaller sample size and discovered that lncRNA UCA1 expression was upregulated in tested AIS patients. Based on those abovementioned evidence, we hypothesized that lncRNA UCA1 may play a critical role in AIS. However, no relevant research has been conducted yet. Therefore, we conducted this study and aimed to investigate the correlation of lncRNA UCA1 expression with disease severity, inflammation, and prognosis in AIS patients.

2 | METHODS

2.1 | Study population

This study consecutively recruited 160 first-episode AIS patients treated in our hospital between May 2015 and July 2017. AIS patients were eligible for inclusion if they met the following criteria: (i) confirmed diagnosis of AIS; (ii) admitted to our hospital within 12 hours

of stroke onset; (iii) age ≥18 years old; and (iv) no intracranial hemorrhage was found. Patients with any of following conditions were excluded from study: (i) had medical history of stroke before study recruitment; (ii) known neurological diseases (eg, glioma); (iii) history of severe infections or systemic inflammatory disease; (iv) treatment with immunosuppressive drugs with 3 months before enrollment; (v) history of hematological malignancies (eg, leukemia) or solid tumors; and (vi) female patients in pregnant. In addition, 160 non-AIS patients with high-stroke-risk factors in our hospital were recruited as study controls during the same period. The eligible criteria for controls were as follows: (i) had at least 2 risk factors of ischemic stroke listed in World Health Organization (WHO) recommendations on stroke prevention, diagnosis, and therapy (including arterial hypertension, diabetes, heart disease, obesity, transient ischemic attack occurrence, platelet hyper-aggregability, smoking, elevated blood lipid level, and cerebral infection) [15]; (ii) had age and gender matched with enrolled AIS patients; (iii) had no history of neurological diseases (eg, stroke or glioma) or severe cardiovascular and cerebrovascular diseases (eg, coronary heart disease or acute coronary syndrome); (iv) had no history of severe infections or systemic inflammatory disease; (v) had no treatment with immunosuppressive drugs with 3 months before enrollment; (vi) had no history of hematological malignancies (eg, leukemia) or solid tumors; and (vii) non-pregnant woman. This study was approved by Institutional Review Board of our Hospital. The written informed consents were collected from the participants or their family members.

2.2 | AIS patients' characteristics and blood sample collection

After recruitment, basic characteristics of AIS patients were documented for analysis, including demographic information and underlying disease. National Institute of Health stroke scale (NIHSS) score was evaluated on the day of admission, which was used for disease severity assessment.[16] Peripheral blood samples of AIS patients were also collected on the day of admission, which were divided into two parts: one was processed by centrifugalizing to separate serum samples for measurement of interleukin-6 (IL-6), interleukin-17 (IL-17), and intercellular cell adhesion molecule-1 (ICAM1); the other was treated with Dynabeads™ FlowComp™ Human CD4 Kit (Invitrogen) to isolate CD4$^+$ T cells from blood samples according to the manufacturer's instructions. After isolating CD4$^+$ T cells, the Th17 cell ratio in the CD4$^+$ T cells was measured immediately by flow cytometry. The total RNA in CD4$^+$ T cells was extracted using TRIzol™ Reagent (Invitrogen) and stored at −80℃ for lncRNA UCA1 detection.

2.3 | Controls' characteristics and blood sample collection

The characteristics of controls (demographic information and underlying disease) were also collected after enrollment. The blood samples of controls were collected to isolate the CD4$^+$ T cells as same as that in AIS patients. The total RNA in CD4$^+$ T cells was extracted

using TRIzol™ Reagent (Invitrogen) and stored at −80℃ for lncRNA UCA1 expression detection as well.

2.4 | LncRNA UCA1 expression detection

For the AIS patients and controls, the freeze-stored total RNA from CD4$^+$ T cells was used to detect lncRNA UCA1 expression by reverse transcription quantitative polymerase chain reaction (RT-qPCR). In brief, iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, California, USA) was used for reverse transcription to convert RNA into complementary DNA. Furthermore, the THUNDERBIRD® SYBR® qPCR Mix (Toyobo) was applied for qPCR reaction. The entire thermal cycles were conducted as follows: 95℃ for 5 minutes, followed by 40 cycles of 95℃ for 5 seconds and 61℃ for 30 seconds. The lncRNA UCA1 expression was calculated by $2^{-\Delta\Delta Ct}$ method using GADPH as internal reference. Primers were designed according to a previous study [11]: LncRNA UCA1: Forward – 5′-CATGCTTGACACTTGGTGCC-3′, Reverse–5′-GGTCGCAGGTGGATCTCTTC-3′; GAPDH: Forward-5′-TGACCACAG TCCATGCCATCAC-3′, Reverse–5′- GCCTGCTTCACCACCTTCT TGA-3′.

2.5 | Th17 cell, IL-6, IL-17, and ICAM1 measurement

Th17 cell ratio in the CD4$^+$ T cells of AIS patients was measured by flow cytometry with Human Th17 phenotyping kit (Becton Dickinson). The Cellquest software (Becton Dickinson) and FLowJo software (Becton Dickinson) were used for cell capture and data analysis, respectively. The IL-6, IL-17, and ICAM1 level in the serum of AIS patients were determined by Enzyme Linked Immunosorbent Assay (ELISA) using ICAM1 Human ELISA Kit, IL-6 human ELISA Kit, and IL-17 human ELISA Kit (Invitrogen). ELISA was performed according to the manufacturer's instructions.

2.6 | RFS evaluation

All AIS patients were followed up every 3 to 6 months through clinic visits or telephone contact, and the follow-up was continued until the completion of 36-month visit, the recurrence of stroke, or death. Recurrence-free survival (RFS) was assessed according to the follow-up data. The RFS was defined as the duration from hospitalization to stroke recurrence or death. The patients who lost follow-up were censored at the last visit date.

2.7 | Statistical analysis

Statistical analysis and graphs construction were performed using SPSS 24.0 software (IBM) and GraphPad Prism 7.01 software (GraphPad Software Inc.). Comparison between two groups was determined by Student's t test, Wilcoxon rank sum test, or chi-square test. Correlation analysis was determined by Spearman's rank correlation test. Receiver-operating characteristic (ROC) curve and the area under the curve (AUC) with 95% confidence interval (CI) were used to assess the ability of lncRNA UCA1 in predicting recurrence/death risk. RFS was displayed using Kaplan-Meier curve, and the comparison of RFS between two groups was analyzed by log-rank test. p Value <0.05 was considered statistically significant.

3 | RESULTS

3.1 | Study flow

A total number of 207 AIS patients admitted in our hospital were invited (Figure 1). However, 47 AIS patients were excluded (including 31 patients who disobeyed the inclusion criteria or who met the exclusion criteria, and 16 patients who refused to participate in this study). The remaining 160 first-episode AIS patients were recruited. For AIS patients, their lncRNA UCA1 expression and Th17 cell ratio in CD4$^+$ T cells, IL-6, IL-17, and ICAM1 level in serum were detected. Furthermore, all 160 AIS patients were followed up to stroke recurrence, death or 36 months. Then, 24 patients who lost follow-up were censored at the last visit date for RFS analysis. Eventually, all 160 AIS patients were included in the final analysis. Meanwhile, 246 non-AIS patients complicated with high-stroke-risk factors were screened. However, 74 patients were excluded (including 54 patients who disobeyed the inclusion criteria or who met the exclusion criteria and 22 patients who refused to participate in this study). The remaining 160 non-AIS patients who complicated with at least 2 high-stroke-risk factors were recruited as controls. For controls, their lncRNA UCA1 expressions were detected.

3.2 | Patients' clinical characteristics

The mean age in controls and AIS patients was 62.3 ± 9.4 years and 63.1 ± 10.5 years, respectively (Table 1). Meanwhile, there were 37 (23.1%) females and 123 (76.9%) males in controls, then 42 (26.3%) females and 118 (73.7%) males in AIS patients. Interestingly, higher percentage of patients with hypertension (p = 0.015) and hyperuricemia (p = 0.026) was found in AIS patients compared to controls. However, no difference of age (p = 0.481), gender (p = 0.517), BMI (p = 0.876), current smoke (p = 0.145), hyperlipidemia (p = 0.179), diabetes mellitus (p = 0.147), or CKD (p = 0.140) was observed between these two groups. The detailed clinical characteristics was shown in Table 1.

3.3 | LncRNA UCA1 expression in AIS patients and controls

Interestingly, lncRNA UCA1 expression was increased in AIS patients compared to controls (2.955 [2.184–3.760] vs. 1.000 [0.750–1.327], p < 0.001) (Figure 2).
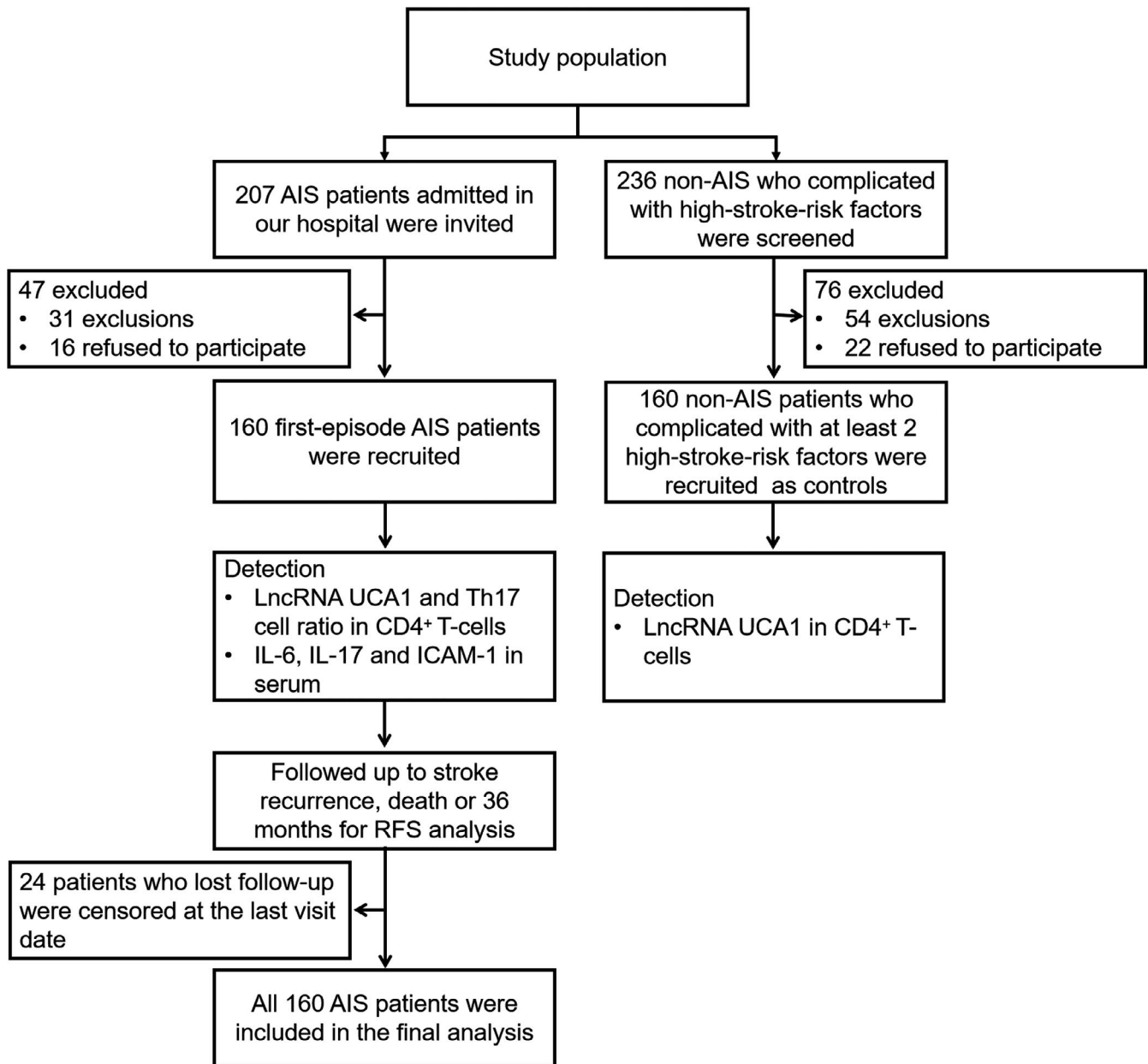
FIGURE 1 Study flow. AIS, acute ischemic stroke; ICAM1, intracellular adhesion molecule-1; IL-17, interleukin-17; IL-6, interleukin-6; lncRNA UCA1, long non-coding RNA urothelial carcinoma-associated 1; RFS, recurrence-free survival

3.4 | Correlation of lncRNA UCA1 expression with disease severity and inflammation in AIS patients

As to disease severity, lncRNA UCA1 expression was positively correlated to NIHSS score ($r = 0.436$, $p < 0.001$) (Figure 3). Regarding inflammation, lncRNA UCA1 expression was positively related to Th17 cell proportion ($r = 0.398$, $p < 0.001$) (Figure 4), IL-6 ($r = 0.204$, $p = 0.010$) (Figure 5A), IL-17 ($r = 0.326$, $p < 0.001$) (Figure 5B), and ICAM1 ($r = 0.276$, $p < 0.001$) (Figure 5C).

3.5 | Correlation of lncRNA UCA1 expression with prognosis in AIS patients

No difference of lncRNA UCA1 expression was found between 1-year recurrence/death AIS patients and those patients without recurrence or death within 1 year ($p = 0.127$) (Figure 6A). Consequently, lncRNA UCA1 failed to predict 1-year recurrence/death risk in AIS patients with AUC of 0.617 (95% CI: 0.478–0.755) (Figure 6B). Meanwhile, lncRNA UCA1 expression was elevated in 2-year recurrence/death AIS patients compared to those patients without recurrence or

TABLE 1 Comparison of clinical characteristics

Items	Controls (N = 160)	AIS patients (N = 160)	p Value
Age (years), mean ± SD	62.3 ± 9.4	63.1 ± 10.5	0.481
Sex, No. (%)			
Female	37 (23.1)	42 (26.3)	0.517
Male	123 (76.9)	118 (73.7)	
BMI (kg/m^2), mean ± SD	24.2 ± 2.9	24.2 ± 2.3	0.786
Current smoke, No. (%)			
No	93 (58.1)	80 (50.0)	0.145
Yes	67 (41.9)	80 (50.0)	
Hypertension, No. (%)			
No	34 (21.3)	18 (11.3)	0.015
Yes	126 (78.7)	142 (88.7)	
Hyperlipidemia, No. (%)			
No	89 (55.6)	77 (48.1)	0.179
Yes	71 (44.4)	83 (51.9)	
Hyperuricemia, No. (%)			
No	114 (71.3)	95 (59.4)	0.026
Yes	46 (28.7)	65 (40.6)	
Diabetes mellitus, No. (%)			
No	128 (80.0)	117 (73.1)	0.147
Yes	32 (20.0)	43 (26.9)	
CKD, No. (%)			
No	143 (89.4)	134 (83.7)	0.140
Yes	17 (10.6)	26 (16.3)	

Abbreviations: AIS, acute ischemic stroke; BMI, body mass index; CKD, chronic kidney disease; SD, standard deviation.

FIGURE 2 LncRNA UCA1 expression. AIS, acute ischemic stroke; lncRNA UCA1, long non-coding RNA urothelial carcinoma-associated 1

FIGURE 3 Correlation of lncRNA UCA1 expression with NIHSS score in AIS patients. AIS, acute ischemic stroke; LncRNA UCA1, long non-coding RNA urothelial carcinoma-associated 1; NIHSS, National Institute of Health stroke scale

death within two years (p = 0.033) (Figure 6C). Furthermore, lncRNA UCA1 showed a predictive value for 2-year recurrence/death risk in AIS patients with AUC of 0.630 (95% CI: 0.513–0.748) (Figure 6D). Besides, lncRNA UCA1 expression was higher in 3-year recurrence/death AIS patients compared to those patients without recurrence or death within 3 years (p = 0.008) (Figure 6E). Meanwhile, it showed a predictive value for 3-year recurrence/death risk in AIS patients with AUC of 0.649 (95% CI: 0.541–0.756) (Figure 6F). More importantly, high lncRNA UCA1 expression was related to worse accumulating RFS (p = 0.017) (Figure 7).

4 | DISCUSSION

In the present study, we discovered that (a) lncRNA UCA1 expression was increased in AIS patients compared to controls, (b) lncRNA UCA1 expression was positively correlated to NIHSS score and inflammation (including Th17 cell proportion, IL-6, IL-17, and ICAM1) in AIS patients, and (c) increased lncRNA UCA1 expression was related to poor prognosis in AIS patients.
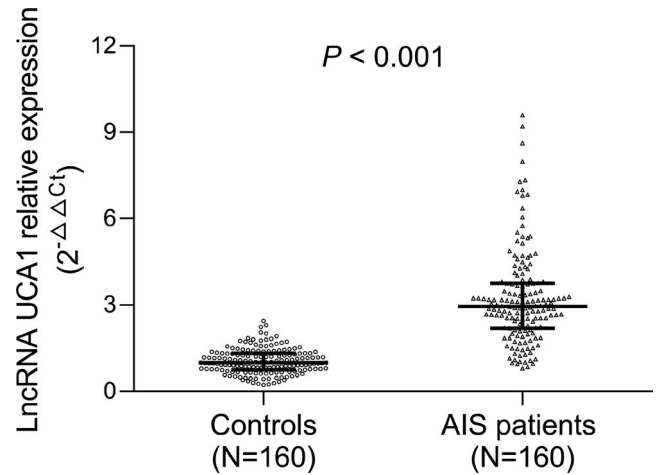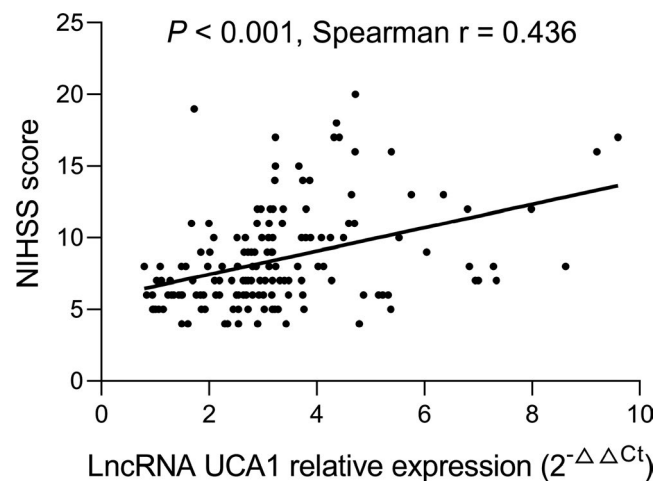
Accumulating evidence suggests that lncRNA UCA1 may play an important role in multiple neurological diseases. For example, elevated lncRNA UCA1 expression is found in both mice brain tissue and cell model of PD.[17] In addition, lncRNA UCA1 expression also has been reported to be reduced in epilepsy mice brain tissue.[18] Apart from the dysregulation of lncRNA UCA1 in various neurological diseases, several studies also explore the mechanism of lncRNA UCA1 underlying neurological diseases. For example, abnormal expression of lncRNA UCA1 promotes miR-18a expression leading to hypoxia injury and neuronal apoptosis in cell model of cerebral ischemia.[14] Moreover, lncRNA UCA1 promotes PI3 K/AKT signaling pathway to enhance oxidative stress and inflammation in mice model of PD.[13] Furthermore, lncRNA UCA1 promotes apoptosis-related pathway (such as caspase-3 activity) and enhances neuronal apoptosis in cell model of PD.[17] Taken together, lncRNA UCA1 is obviously involved in the pathophysiology of neurological diseases.

More interestingly, lncRNA UCA1 has been identified as a potential factor in cardio-cerebrovascular diseases. For instance, lncRNA UCA1 expression is increased in AMI patients compared to control subjects.[12] Another study demonstrates that lncRNA UCA1 expression varies in AMI patients which is reduced initially, then increased in Day 3 after AMI occurrence.[19] Besides, lncRNA UCA1 regulates pro-inflammatory cytokines production (such as IL-6 and ICAM1) in LPS treated endothelial cells.[11] Moreover, lncRNA UCA1 expression is elevated, which also promotes neuron injury via miR-18a/SOX6 axis in PC12 cellular cerebral ischemia model.[14] Since lncRNA UCA1 is under heavy investigation in both cardio-cerebrovascular and neurological diseases, also previous studies suggest that lncRNA UCA1 participates in endothelial cell inflammation process and cerebral ischemia related injury, thus we hypothesized that lncRNA UCA1 might also play a crucial role in AIS patients. However, no clinical research regarding the role of lncRNA UCA1 in AIS patients has been performed. In the present study, we discovered that lncRNA UCA1 expression was elevated n AIS patients compared to controls. The possible reasons were

as follows: (a) Abnormal lncRNA UCA1 expression might regulate miR-18a expression, thereby leaded to hypoxia injury and neuronal apoptosis in cerebral ischemia.[14] Thus, abnormal lncRNA UCA1 expression was found in AIS patients. (b) lncRNA UCA1 might regulate pro-inflammatory cytokines expression to enhance local inflammatory response.[11] Meanwhile, accumulating evidence suggested that local inflammatory response assisted in the pathogenesis of ischemic events.[5,6] Therefore, elevated lncRNA UCA1 expression was correlated with higher AIS risk.

The correlation of lncRNA UCA1 with inflammation in cardio-cerebrovascular disease has been explored. For instance, an interesting study finds that lncRNA UCA1 regulates IL-6 and ICAM1 in LPS treated endothelial cells.[11] In our study, we found that lncRNA UCA1 was positively correlated NIHSS score and inflammation (including Th17 cell ratio, IL-6, IL-17, and ICAM1) in AIS patients. The possible reasons were as follows: (a) lncRNA UCA1 might promote several inflammation signaling pathway (such as PI3 K/AKT signaling pathways) to enhance the production of pro-inflammatory cytokines in AIS patients,[13] (b) lncRNA UCA1 might be related to the production of several pro-inflammatory cytokines (above mentioned), thereby correlated with worse inflammation and subsequently leaded to disease severity in AIS patients. (c) lncRNA UCA1 might enhance the activity of apoptotic protein (such as caspase-3), thereby leaded to neural cell apoptosis and eventually resulted in more severe diseases in AIS patients.[17] Taken together, elevated lncRNA UCA1 expression was related to increased inflammation and more severe disease in AIS patients.

Limited study reports the prognostic power of lncRNA UCA1 in cardio-cerebrovascular diseases. In order to fill this vacancy, we conducted this study and found that lncRNA UCA1 showed a predictive value for 2-year and 3-year recurrence/death risk; also, its high level was related to worse accumulating RFS in AIS patients. The possible reasons were as follows: (a) lncRNA UCA1 was positively related to disease severity as discussed earlier. Hence, more severe AIS disease (such as patients with multiple vascular bed affected) was related to increased risk of recurrence ischemic stroke events, thereby related to poor prognosis.[20] (b) lncRNA UCA1 might promote caspase-3 activity in neurons, thereby leaded to neuronal apoptosis and eventually resulted in poor prognosis in AIS
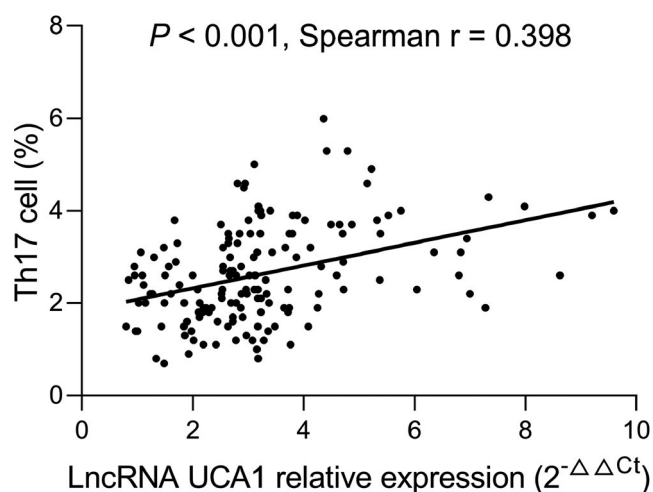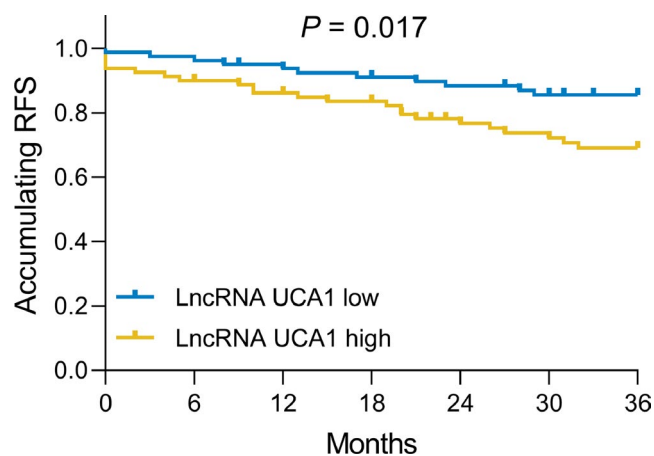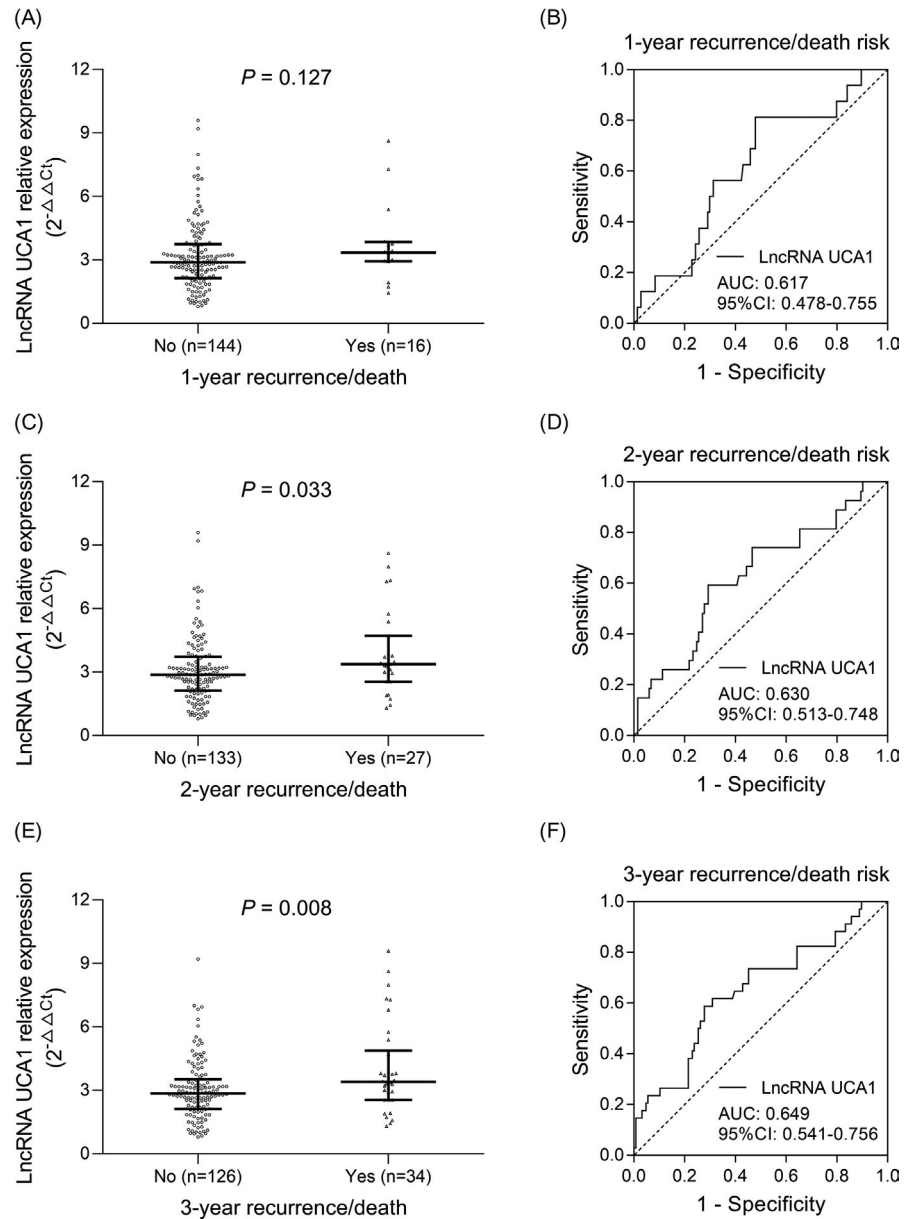

FIGURE 4 Correlation of lncRNA UCA1 expression with Th17 cell proportion in AIS patients. AIS, acute ischemic stroke; LncRNA UCA1, long non-coding RNA urothelial carcinoma-associated 1; Th17, T helper 17
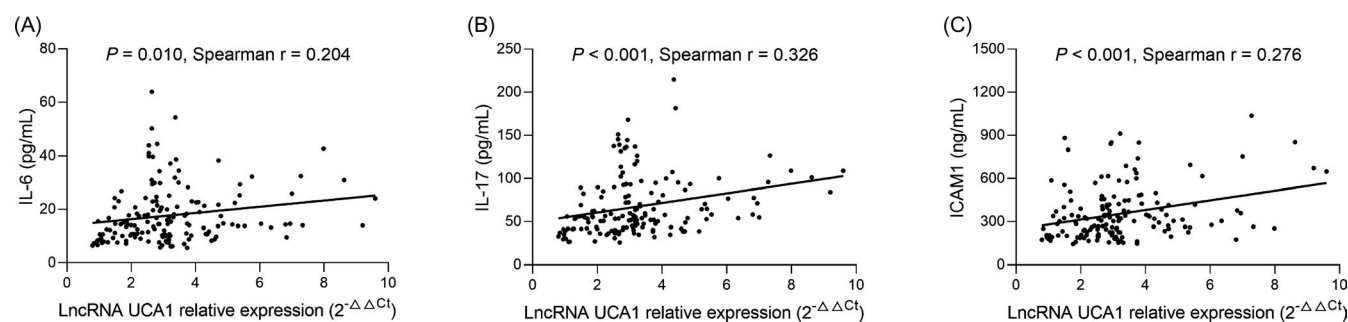
FIGURE 5 Correlation of lncRNA UCA1 expression with inflammatory cytokines in AIS patients. Correlation of lncRNA UCA1 expression with IL-6 (A), IL-17 (B) and ICAM1 (C) in AIS patients. AIS, acute ischemic stroke; ICAM1, intracellular adhesion molecule-1; IL-17, interleukin-17; IL-6, interleukin-6; lncRNA UCA1, long non-coding RNA urothelial carcinoma-associated 1

FIGURE 6 Correlation of lncRNA UCA1 expression with recurrence/death risk in AIS patients. (A) Difference of lncRNA UCA1 expression between AIS patients with or without 1-year recurrence/death. (B) The predictive value of lncRNA UCA1 expression for 1-year recurrent/death risk in AIS by ROC curve. (C) Difference of lncRNA UCA1 expression between AIS patient with or without 2-year recurrence/death. (D) The predictive value of lncRNA UCA1 expression for 2-year recurrence/death risk in AIS by ROC curve. (E) Difference of lncRNA UCA1 expression between AIS patients with or without 3-year recurrence/death. (F) The predictive value of lncRNA UCA1 expression for 3-year recurrence/death risk in AIS by ROC curve. AIS, acute ischemic stroke; AUC, area under curve; CI, confidence interval; LncRNA UCA1, long non-coding RNA urothelial carcinoma-associated 1; ROC, receiver-operating characteristic

FIGURE 7 Correlation of lncRNA UCA1 expression with accumulating RFS in AIS patients. LncRNA UCA1, long non-coding RNA urothelial carcinoma-associated 1; RFS, recurrence-free survival

patients.[17] Therefore, increased lncRNA UCA1 was related to poor prognosis in AIS patients.

There were some limitations in this study. Firstly, this study only recruited non-AIS with high-stroke-risk factors as positive controls; however, the age and gender-matched health as negative controls was not set in our study; thus, further study was necessary to comprehensively analyze lncRNA UCA1 expression. Meanwhile, the inflammatory cytokines (such as IL-6, IL-17, and ICAM-1) were not detected in controls; thus, further study was necessary. Moreover, the sample size in our study was relatively small, which leaded to low statistical power; thus, further study with larger sample size to validate this was needed. Although lncRNA UCA1 regulates miR-18a to induce hypoxia injury in the cell model of cerebral ischemia according to a previous study, the correlation of lncRNA UCA1 with miR-18a was not explored in our study for it was not the main objective; thus, further study could investigate this aspect. Meanwhile, further study with larger sample size to validate whether lncRNA UCA1 was

an independent risk factor for AIS recurrence would be conducted in the following studies. Finally, the underlying mechanism in the effect of lncRNA UCA1 on Th17 cell ratio and inflammatory cytokines (including IL-6, IL-7, and ICAM1) was not explored; thus, further study could investigate this aspect.

In conclusion, lncRNA UCA1 is correlated with elevated disease severity, Th17 cell proportion, inflammatory cytokines, and worse prognosis in AIS patients.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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