RESEARCH ARTICLE

Circulating long noncoding RNA ANRIL downregulation correlates with increased risk, higher disease severity and elevated pro-inflammatory cytokines in patients with acute ischemic stroke

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Background: To investigate the correlation of plasma lncRNA ANRIL expression with stroke risk, severity and inflammatory cytokines levels in acute ischemic stroke (AIS) patients.

Methods: A total of 126 AIS patients and 125 controls were consecutively recruited in this case-control study. Blood samples from all participants were collected, and plasma was separated. Plasma lncRNA ANRIL expression was quantified by quantitative real-time polymerase chain reaction (qRT-PCR). Plasma tumor necrosis factor- α (TNF- α) and interleukin (IL)-1 β (IL-1 β), IL-6, IL-8, IL-10, and IL-17 levels were measured by enzyme-linked immunosorbent assay (ELISA). National Institutes of Health Stroke Scale (NIHSS) scores were used to assess the stroke severity in AIS patients.

Results: Plasma lncRNA ANRIL expression was lower in AIS patients than in controls ($P < 0.001$), and the receiver operating characteristic (ROC) curve showed a good prediction value of lncRNA ANRIL for AIS risk with area under curve (AUC) of 0.759 (95% CI: 0.741-0.849). In addition, lncRNA ANRIL expression was negatively correlated with NIHSS score ($r = -0.351$, $P < 0.001$). Furthermore, while lncRNA ANRIL expression was negatively associated with hs-CRP level ($r = -0.247$, $P = 0.005$), no correlation was found between lncRNA ANRIL expression and ESR level ($P = 0.619$). For inflammatory cytokines, lncRNA ANRIL expression was inversely associated with TNF- α ($r = -0.216$, $P = 0.015$) and IL-6 levels ($r = -0.326$, $P < 0.001$), while it positively correlated with IL-10 level ($r = 0.210$, $P = 0.018$).

Conclusion: Circulating lncRNA ANRIL downregulation correlates with increased stroke risk, higher disease severity and elevated inflammation in AIS patients.

KEYWORDS

acute ischemic stroke, inflammatory cytokines, lncRNA ANRIL, National Institutes of Health Stroke Scale score, stroke risk

1 | INTRODUCTION

Stroke is the leading cause of acquired neurological disability and the second leading cause of death worldwide, with incidences

ranging from 400 to 800 people per 100 000 every year worldwide.^{1,2} In China, 2.5 million new stroke cases occur each year, and there are 7.5 million stroke survivors; approximately 15%-30% of stroke survivors are left permanently disabled.³ Stroke includes

acute ischemic stroke (AIS) and hemorrhagic stroke, with the former being responsible for almost 85% of cases.² At present, the diagnosis of AIS primarily relies on laboratory examination and neuroimaging techniques, including computed tomography or magnetic resonance imaging (MRI), digital subtraction angiography (DSA), and transcranial Doppler ultrasound (TCD), among which DSA remains the gold standard for diagnosis of AIS; however, the exorbitant cost and invasiveness are not suitable for all patients.⁴ Although several techniques are available for diagnosing AIS, the early ischemic changes in the brain of AIS patients cannot usually be detected, thus the prognosis of patients remains unsatisfactory due to the delay of diagnosis.⁵ Therefore, exploring noninvasive highly sensitive biomarkers for early diagnosis and for disease monitoring of AIS is critically important for optimized care, allocation of health-care resources and improved prognosis.

Long noncoding RNAs (lncRNAs), a subclass of RNAs, are transcripts of more than 200 nucleotides in length with unapparent coding potential.⁶ In recent years, numerous lncRNAs have been well-characterized, and dysregulation of lncRNAs is related to progression of cardio-cerebrovascular diseases, including coronary artery disease, ischemic stroke and others, providing new insights into the pathophysiology of these diseases.⁷⁻⁹ lncRNA ANRIL, a newly documented antisense noncoding RNA at the INK4 locus, plays a role in the atherosclerotic processes such as thrombogenesis and vascular remodeling and/or repair and in plaque stability and inflammatory responses.¹⁰⁻¹² In the pathogenesis of AIS, atherosclerosis and inflammatory responses are identified as two of the most crucial pathogenetic factors for development and progression of cerebral arterial occlusion.^{4,13} However, the correlation of lncRNA ANRIL expression with stroke risk and severity, as well as inflammatory cytokines levels in AIS patients is still not well documented.

Therefore, the aim of our study was to investigate the correlation of circulating lncRNA ANRIL expression with stroke risk, severity and inflammatory cytokines levels in AIS patients.

2 | METHODS

2.1 | Participants

Between July 2015 and October 2017, 126 AIS patients and 125 controls from the Central Hospital of Wuhan were consecutively recruited for this case-control study. The inclusion criteria for AIS patients were as follows: (a) diagnosis of AIS according to medical history and clinical findings, including laboratory and neurological indices, computed tomography (CT) scanning or magnetic resonance imaging (MRI); (b) admission to the hospital within 24 hours after the onset of symptoms; and (c) age greater than 18 years. The exclusion criteria were as follows: (a) presence of intracranial hemorrhage; (b) current immunosuppressive therapy or thrombolytic therapy; (c) severe infections, inflammatory or autoimmune diseases; (d) history of hematological malignancies or solid tumors,

heart failure, renal or hepatic dysfunction; and (e) pregnancy or lactation. The inclusion criteria for controls were as follows: (a) presence of peripheral vascular diseases or vascular risk factors (such as hypertension, hyperlipidemia, hyperuricemia, smoking and chronic kidney disease [CKD]); (b) age and gender matched to AIS patients; and (c) no history of stroke. The exclusion criteria of controls were as follows: (a) history of solid tumors, hematological malignancies, heart failure, renal or hepatic dysfunction, severe infection, acute/chronic inflammatory diseases or autoimmune diseases; (b) history of neurological diseases; and (c) pregnancy or lactation.

2.2 | Ethics

This study was approved by the Institutional Review Board (IRB) of The Central Hospital of Wuhan. All participants or their guardians gave informed consents forms.

2.3 | Sample collection

Blood samples from AIS patients were collected within 24 hours post-onset of symptoms and before treatment; samples were collected from controls after enrolment. Samples were drawn into serum separator tubes and were centrifuged at 1600 g for 10 minutes at room temperature. The supernatants were recovered and were further centrifuged at 12 000 g for 10 minutes at 4°C to completely remove cell debris. Subsequently, plasma was obtained and stored at -80°C for further detection.

2.4 | Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the plasma of all participants (both AIS patients and controls) using the TRIzol (Invitrogen, CA, USA) test kit following the manufacturer's instructions. Purity and concentration of RNA were determined by a NanoPhotometer spectrophotometer (IMPLEN, CA, USA) and a Qubit RNA Assay Kit in a Qubit 2.0 Fluorometer (Life Technologies, CA, USA) respectively. Then, RNA was transcribed using Transcript First-strand cDNA synthesis SuperMix (TransGenBiotech, Beijing, China) according to the manufacturer's protocol. Subsequently, qRT-PCR was performed using SYBR Premix EX Taq™ II (Takara, Dalian, China) with the ABI 7500 system (Applied Biosystems, MA, USA). The reaction was incubated at 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds, 61°C for 15 seconds and 72°C for 30 seconds. The relative expression level of lncRNA ANRIL was calculated by the $2^{-\Delta\Delta Ct}$ method using U6 as the internal reference.

2.5 | Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of tumor necrosis factor- α (TNF- α) and interleukin 1 β (IL-1 β), IL-6, IL-8, IL-10 and IL-17 in plasma from AIS patients were determined using Enzyme-Linked Immunosorbent Assay (ELISA)

commercial kits (BioSource, Kansas City, USA) in accordance with the products' manuals.

2.6 | Data collection

Demographics and medical history data of all participants were collected after enrolment, including age, gender, body mass index (BMI), smoking history, hypertension, diabetes mellitus, hyperlipidemia, hyperuricemia, and CKD. C-reactive protein (CRP) and erythrocyte sedimentation rates (ESR) of AIS patients were recorded as well.

2.7 | Assessment of stroke severity

Stroke severity of AIS patients was assessed on the day of admission by physicians using the National Institutes of Health Stroke Scale (NIHSS) score, a clinical measure of neurologic deficit with a range of 0 (no deficit) to 42 (maximum possible deficit), defined as: 0-1 point, normal or near normal; 2-4 points, mild stroke; 5-15 points, moderate stroke; 16-20 points, moderate-severe stroke; and 21-42 points, severe stroke.

2.8 | Statistics

Statistical analysis was performed using SPSS 22.0 software (IBM Corp., Ltd, New York, USA), and statistical graphs were drawn using GraphPad Prism 5.01 software (GraphPad Software Inc, USA). Data were presented as the mean $\pm$ standard deviation, count (%) or median (1/4 to 3/4 quartiles). Comparison between two groups was determined by the *t* test, Chi-square test or Wilcoxon rank sum test. Receiver operating characteristic (ROC) curves were generated to assess the value of lncRNA ANRIL expression for predicting AIS risk. The correlation of lncRNA ANRIL expression with NIHSS score, hs-CRP, ESR, TNF- α , IL-1 β , IL-6, IL-8, IL-10 and IL-17 levels were evaluated by the Spearman test. *P* < 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Characteristics of AIS patients and controls

The demographic and clinical characteristics of AIS patients (N = 126) and controls (N = 125) were listed in Table 1, revealing no differences in terms of baseline characteristics between AIS patients and controls. The mean ages of AIS patients and controls were 61.9 $\pm$ 10.4 years and 61.0 $\pm$ 8.9 years, respectively (*P* = 0.881). The numbers of male and female were 89 and 37 in AIS patients and 87 and 38 in controls (*P* = 0.858). The percentages of patients with hypertension (*P* = 0.220), diabetes mellitus (*P* = 0.334), hyperlipidemia (*P* = 0.474), hyperuricemia (*P* = 0.374), and CKD (*P* = 0.292) were 91.3%, 19.8%, 43.7%, 36.5%, and 16.7% in AIS patients, and 86.4%, 15.2%, 39.2%, 31.2%, and 12.0% in controls, respectively. Other detailed characteristics of AIS patients and controls were shown in Table 1.

TABLE 1 Characteristics of participants

Items	AIS patients (N = 126)	Controls (N = 125)	P value
Age (y)	61.9 $\pm$ 10.4	61.0 $\pm$ 8.9	0.881
Gender (male/female)	89/37	87/38	0.858
BMI (kg/m ²)	24.5 $\pm$ 2.8	24.2 $\pm$ 2.6	0.314
Smoke (n/%)	63 (50.0)	64 (51.2)	0.849
Hypertension (n/%)	115 (91.3)	108 (86.4)	0.220
Diabetes mellitus (n/%)	25 (19.8)	19 (15.2)	0.334
Hyperlipidemia (n/%)	55 (43.7)	49 (39.2)	0.474
Hyperuricemia (n/%)	46 (36.5)	39 (31.2)	0.374
CKD (n/%)	21 (16.7)	15 (12.0)	0.292

Data were presented as mean $\pm$ standard deviation or count (%). Comparison was determined by *t* test or Chi-square test. *P* < 0.05 was considered statistically significant.

AIS, acute ischemic stroke; BMI, body mass index; CKD, chronic kidney disease.

3.2 | Predicting value of lncRNA ANRIL expression for the risk of AIS

As shown in Figure 1, lncRNA ANRIL expression in AIS patients (0.590 [0.358-1.123]) was lower than in controls (1.637 [0.912-2.536]) (*P* < 0.001, Figure 1A). To evaluate the value of lncRNA ANRIL expression for distinguishing AIS from controls, an ROC curve was generated and revealed that lncRNA ANRIL expression predicted AIS risk, with an area under curve (AUC) of 0.759 (95% CI: 0.741-0.849), a sensitivity of 72.2%, and a specificity of 71.2% at the best cut-off point, yielding the maximum value of sensitivity plus specificity (Figure 1B).

3.3 | Correlation of lncRNA ANRIL expression with NIHSS score

Stroke severity in AIS patients was evaluated by NIHSS score. To explore the association of lncRNA ANRIL expression with stroke severity in AIS, the Spearman test was performed. The result showed that lncRNA ANRIL expression was negatively associated with NIHSS score (*r* = -0.351, *P* < 0.001, Figure 2).

3.4 | Correlation of lncRNA ANRIL expression with hs-CRP and ESR levels

The correlation of lncRNA ANRIL expression with hs-CRP and ESR in AIS patients was displayed in Figure 3. lncRNA ANRIL expression was negatively associated with hs-CRP level (*r* = -0.247, *P* = 0.005, Figure 3A), while there was no correlation of lncRNA ANRIL expression with ESR levels (*r* = -0.045, *P* = 0.619, Figure 3B).

FIGURE 1 Predicting value of lncRNA ANRIL expression for the risk of AIS. lncRNA ANRIL expression was decreased in AIS patients compared with controls (A), and ROC curve showed that lncRNA ANRIL had a good value for predicting AIS risk (B). Comparison between two groups was determined by Wilcoxon rank sum test. ROC curve was conducted to evaluate the predicting value of lncRNA ANRIL expression for AIS risk. $P < 0.05$ was considered statistically significant. AIS, acute ischemic stroke; ROC, receiver operating characteristic

FIGURE 2 Correlation of lncRNA ANRIL expression with NIHSS score. The expression of lncRNA ANRIL was negatively associated with NIHSS score. Spearman test was used to evaluate the correlation of lncRNA ANRIL expression with NIHSS score. $P < 0.05$ was considered statistically significant. NIHSS, National Institutes of Health Stroke Scale

3.5 | Correlation of lncRNA ANRIL expression with inflammatory cytokines

As presented in Figure 4, lncRNA ANRIL expression negatively correlated with TNF- α ($r = -0.216$, $P = 0.015$, Figure 4A) and IL-6 levels ($r = -0.326$, $P < 0.001$, Figure 4C) but positively associated with IL-10 level ($r = 0.210$, $P = 0.018$, Figure 4E), while there were no correlation of lncRNA ANRIL expression with IL-1 β ($r = 0.089$, $P = 0.312$, Figure 4B), IL-8 ($r = -0.164$, $P = 0.067$, Figure 4D) or IL-17 levels ($r = -0.037$, $P = 0.683$, Figure 4F).

4 | DISCUSSION

In the present study, we observed that: (a) lncRNA ANRIL expression in AIS patients was lower than in controls, revealing good potential to predict AIS risk; (b) lncRNA ANRIL expression was negatively associated with disease severity as assessed by NIHSS score in AIS patients; and (c) lncRNA ANRIL expression negatively correlated with hs-CRP, TNF- α and IL-6 levels while it was positively associated with IL-10 levels.

FIGURE 3 Correlation of lncRNA ANRIL expression with hs-CRP and ESR levels. lncRNA ANRIL expression was negatively correlated with hs-CRP level (A), but was not correlated with ESR level (B). Spearman test was used to assess the correlation of lncRNA ANRIL expression with hs-CRP and ESR levels. $P < 0.05$ was considered statistically significant. CRP, C-reactive protein; ESR, erythrocyte sedimentation rate

FIGURE 4 Correlation of lncRNA ANRIL expression with inflammatory cytokines levels. lncRNA ANRIL expression was negatively correlated with TNF- α (A) and IL-6 levels (C), but positively associated with IL-10 level (E), while no correlation of lncRNA ANRIL expression with IL-1 β (B), IL-8 (D) or IL-17 (F) levels was discovered. Spearman test was used to assess the correlation of lncRNA ANRIL expression with inflammatory cytokines levels. $P < 0.05$ was considered statistically significant. TNF- α , tumor necrosis factor- α ; IL, Interleukin

Acute ischemic stroke, a major cerebrovascular disease, is caused by vascular occlusion that decreases cerebral blood flow to an area of brain perfused by an occluded artery.² Atherosclerosis is the main cause of AIS, and inflammatory responses are involved in endothelial dysfunction and propagation of atherosclerosis, contributing to ischemic brain injuries.^{13,14} In recent years, lncRNAs have been proposed as key regulators of posttranscriptional gene expression/function in pathological aspects of ischemic stroke.^{9,14} For example, an in vitro and in vivo study reports that lncRNA Malat1 protects cerebral microvasculature and parenchyma from cerebral ischemic injury by benefiting from the inhibition of endothelial cell (EC) death and pro-inflammatory cytokines expression.⁹ Another in vivo study shows that lncRNA SNHG1 prolongs brain microvascular endothelial cells survival by regulating HIF-1 α /VEGF signaling, thereby exerting a neuroprotective effect.¹⁴ These data indicate that dysregulated lncRNAs are involved in the development and progression of AIS.

lncRNA ANRIL, one of the most frequently studied lncRNAs located on the 9p21 locus, has been reported to mediate proliferation, apoptosis, inflammation, and angiogenesis in inflammatory and cardio-cerebrovascular diseases.^{10,15-17} One study reports that in inflammatory bowel disease (IBD) patients, lncRNA ANRIL expression was lower in inflamed intestinal mucosa than in noninflamed intestinal mucosa or normal intestinal mucosa from healthy controls.¹⁶ Another study in CAD patients shows that lncRNA inhibits pre-rRNA maturation in vascular smooth muscle cells and macrophages via binding to pescadillo homolog 1, leading to nucleolar stress and p53 activation, thereby enhancing apoptosis and restraining proliferation in vascular cells and tissues, subsequently attenuating atherosclerosis progression.¹⁷ Partially in line with these findings, our

results showed that lncRNA ANRIL was lower in AIS patients than in controls, and the ROC curve showed good prediction value of lncRNA ANRIL expression for AIS risk. These results may be explained by the protective roles of lncRNA ANRIL in atherosclerosis and inflammatory response, two of the most prominent pathological features of AIS.^{4,15-17}

National Institutes of Health Stroke Scale score was used to evaluate stroke severity of AIS patients in our study, and the results showed that lncRNA ANRIL expression was negatively associated with NIHSS score. A considerable number of studies have illustrated positive correlations of pro-inflammatory cytokines levels and negative correlations of anti-inflammatory cytokines levels with stroke severity of AIS.^{18,19} Furthermore, as a freshly discovered nuclear component of the NF- κ B pathway that is inseparable from inflammatory process, lncRNA ANRIL has been reported to mediate several cytokines (such as TNF- α , IL-6/8 or IFN- γ) in the inflammatory response of ECs by binding to Yin Yang 1 and thus modulating atherosclerosis.^{15,20} Based on the results of these studies, the negative correlation of lncRNA ANRIL with NIHSS score in our study might be explained by a mechanism in which lncRNA ANRIL suppresses the expression of pro-inflammatory cytokines and inhibits the degree of atherosclerosis; thus, its expression negatively correlates with stroke severity of AIS.¹⁸⁻²⁰

The critical role of inflammation in the development of AIS has been previously demonstrated, and markers of inflammation, including hs-CRP and IL-6 have been associated with AIS progression.^{18,21} To further elucidate the correlation of lncRNA ANRIL with inflammation, levels of hs-CRP, ESR and six common inflammatory cytokines (including pro-inflammatory and anti-inflammatory cytokines)

in plasma of AIS patients were measured in this study. We found that lncRNA ANRIL expression was negatively correlated with hs-CRP, TNF- α and IL-6 levels, but was positively associated with IL-10 levels in AIS patients. These results further suggested that lncRNA ANRIL acted as an anti-inflammatory gene to participate in AIS progression probably by regulating the expression of inflammatory cytokines.

There were some limitations to this study: (a) The sample size was relatively small, possibly causing lower statistical efficiency compared to studies with larger sample sizes; thus, further confirmation performed on larger sample sizes would be advantageous; (b) all patients enrolled in this study were from a single center, possibly leading to selection bias; thus, multicenter research is necessary in the future; and (c) this was a case-control study and the effect of lncRNA ANRIL on prognosis was not assessed; therefore, a cohort study should be conducted in the future to evaluate the role of lncRNA ANRIL in AIS prognosis.

In conclusion, circulating lncRNA ANRIL downregulation correlates with increased stroke risk, higher disease severity and elevated inflammation in AIS patients.

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