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Upregulated lncRNA NORAD can diagnose acute cerebral ischemic stroke patients and predict poor prognosis

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Abstract

Introduction: *Acute cerebral ischemic stroke (AIS) dramatically influences patients' quality of life. lncRNA NORAD (NORAD) has been studied in cerebrovascular diseases, which are potential risk factors for AIS. The specific significance of NORAD is unclear. This study aimed to assess the role of NORAD in AIS, and to provide therapeutic value for its' treatment.*

Material and methods: *A total of 103 AIS patients and 95 healthy individuals (control) were enrolled into this study. Expression level of NORAD in the plasma of all participants was analyzed by PCR. Diagnostic potential of NORAD in AIS was evaluated by ROC analysis, while Kaplan-Meier and Cox regression analyses were conducted to assess its' prognostic value in AIS.*

Results: *A significantly increased level of NORAD was observed in AIS patients compared with healthy individuals. The upregulation of NORAD could dramatically discriminate AIS patients from healthy individuals with high sensitivity (81.60%) and specificity (88.40%). NORAD was positively correlated with patients' high-sensitivity C-reactive protein (hs CRP, r = 0.796), matrix metalloproteinase-9 (MMP9, r = 0.757), and NIHSS scores (r = 0.840), and negatively related to pc-ASPECTS scores (r = –0.607). Moreover, NORAD upregulation was associated with patients' unfavorable prognosis and served as an independent prognostic biomarker, together with NIHSS and pc-ASPECTS scores of AIS patients.*

Conclusions: *NORAD was upregulated in AIS, which can discriminate AIS patients, and was closely correlated with severe development and poor prognosis of patients.*

Key words: *acute cerebral ischemic stroke, lncRNA NORAD, diagnosis, prognosis.*

Introduction

Continuous blood flow as well as sufficient oxygen and glucose levels in the blood are essential requirements for maintaining vitality and function of the brain. Deficient blood supply caused by cerebral vascular occlusion can induce stroke [14]. Acute cerebral ischemic stroke (AIS) is a common and frequently occurring disease in neurology, which is of a high morbidity fatality and leads to neurological dysfunction. With aging of the society, the incidence and mortality of AIS are gradually rising, causing a financial burden on families. The pathogens of AIS include, but are not limited to, hypertension, arterial stenosis, atherosclerosis, hyperglycemia, and thrombosis [15]. Existing diagnosis methods for AIS, such as brain computed tomography and digital subtraction angiography, are limited to patients' disease conditions [16,22]. To obtain completed results, the diagnosis process is always complicated, and requires blood analysis combined with head imaging. Patients with acute illness cannot receive timely, rapid, and effective treatment, missing the golden opportunity for treatments.

To improve the diagnosis and therapy efficiency of AIS, an increasing number of research have been devoted to the identification of biomarkers, which

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indicate the onset and development of patients' illness. Among those studies, non-coding RNAs (ncRNAs) have been extensively investigated in AIS [3,24]. lncRNA NORAD (NORAD) is a long non-coding RNA that has been reported to play roles in various human diseases. For example, in osteosarcoma, NORAD was suggested to regulate cell proliferation and sensitivity to cisplatin [25]. It was reported that NORAD could mediate vascular endothelial cell injury and suppress the incidence and progression of atherosclerosis [1,5,10]. Additionally, NORAD was found to suppress mesangial cell proliferation, inflammation, and fibrosis under high-glucose conditions [23]. Due to the potential of atherosclerosis and hyperglycemia-induced AIS, NORAD was speculated to be correlated with the occurrence and development of AIS, which was validated in the present study.

Susceptibility weighted imaging (SWI) is a novel technology that has been widely used in the diagnosis of brain tumors, neurodegenerative disease, brain injury, and cerebrovascular disease [7,13]. In AIS, SWI could reflect the position, size, and morphology of the thrombus, therefore helping the diagnosis and therapy of AIS. In this study, we took the posterior circulation ASPECTS (pc-ASPECTS) based on SWI into the evaluation of AIS patients' disease conditions, so as to assess the potential application of SWI in AIS detection and treatment.

Table I. Statistical analysis of the basic clinical information of all subjects

Measurements	Healthy individuals (n = 95)	Patients (n = 103)
Age (years)	61.24 ±7.00	61.54 ±8.36
BMI	23.31 ±3.95	23.73 ±3.69
Gender (n)		
Male	54	57
Female	41	46
Hypertension (n)		
Yes	22	34
No	73	69
Hyperlipidemia (n)		
Yes	7	14
No	88	89
Diabetes (n)		
Yes	13	17
No	82	86
Smoker (n)		
Yes	10	15
No	85	88
hs CRP (mg/dl)	0.45 ±0.26	0.92 ±0.31***
MMP9 (µg/l)	81.34 ±10.35	187.38 ±54.00***
NIHSS	–	9.47 ±3.44
pc-ASPECT	–	6.56 ±2.72

*BMI – body mass index, hs CRP – high-sensitivity C-reactive protein, MMP9 – matrix metalloproteinase-9, *** $p < 0.01$, healthy individuals compared with patients with acute ischemic stroke. Data expressed as n or mean ± standard deviation*

Material and methods

Study subjects

One hundred and three patients primarily diagnosed with AIS by brain computed tomography/magnetic resonance imaging (CT/MRI) examination according to the World Health Organization (WHO) diagnostic criteria and treated at The First Affiliated Hospital of Bengbu Medical College during 2013-2015 were included in the AIS group in this study. Onset time of patients were within 24 hours, and the pathogenesis included internal carotid artery, middle cerebral artery, and vertebral or basilar artery occlusion. Patients diagnosed by CT/MRI with cerebral hemorrhage or with other comorbidities were excluded. Meanwhile, another ninety-five healthy individuals who underwent physical examination were enrolled in the control group. Main clinical information of the participants are summarized in Table I. Peripheral blood samples were collected from each participant and separated plasma samples stored at –80°C. AIS patients were followed up for 90 days to obtain disease development. Endpoint events were defined as any cause-induced death and modified ranking scale over 2. This study had been approved by the Ethics Committee of The First Affiliated Hospital of Bengbu Medical College, and informed consent was obtained from participants or their families.

Imaging analysis

The pc-ASPECTS was assessed based on SWI imaging analysis. According to the susceptibility results, the position of the thrombus signs was divided as proximal to M1 in the middle cerebral artery, distant to M1 in the middle cerebral artery, and distal the middle cerebral artery. pc-ASPECTS score of 10 indicated no visible posterior circulation ischemia, and score of '0' indicated visible ischemic changes in all territories.

RNA extraction and NORAD evaluation

RNA was extracted from the collected samples with QIAamp RNA blood mini kit (Qiagen). The concentration and purity of extracted RNA were evaluated with BioPhotometer analyzer. cDNA was generated using iScript cDNA synthesis kit (Bio-Rad, USA), and PCR was evaluated with SYBR qPCR mix (Toyboo) on CFX96 PCR system (Bio-Rad, USA). Thermotical cycles were conducted

as follows: 2 min at 94°C, 30 s at 94°C, 30 s at 60°C, 30 s at 60°C, 35 s at 72°C for 20 cycles; 5 min at 72°C. Relative expression of NORAD was calculated with $2^{-\Delta\Delta ct}$ method normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primers used were as follow: NORAD forward 5'-AGCGAAGTCCCGAACGACGA-3', reverse 5'-TGGGCATTTCCAACGGGCCAA-3', GAPDH forward 5'-ACCCACTCCTCCACCTTTGAC-3', reverse 5'-TGTTGCTGTAGCCAAATTCGTT-3'.

Statistical analysis

Difference between the control and AIS groups was evaluated using Student's *t*-test, followed by Duncan's post-hoc test. A correlation between NORAD and major indicators was evaluated by Pearson's correlation test. Potential of NORAD in distinct AIS patients and predicted patients' prognosis was assessed using ROC and Kaplan-Meier and Cox regression analysis, respectively. Statistical significance was marked by $p < 0.05$.

Results

Basic clinical features of study subjects

The control group was comprised of 54 males and 41 females, with an average age of 61.24 ±7.00 years, while the AIS group included 57 males and 46 females, with an average age of 61.54 ±8.36 years. Significant higher high-sensitivity C-reactive protein (hs CRP) and matrix metalloproteinase-9 (MMP9) levels were observed in the AIS group compared with the control group ($p < 0.001$). Insignificant differences were observed in other characteristics between the two groups ($p > 0.05$, Table I).

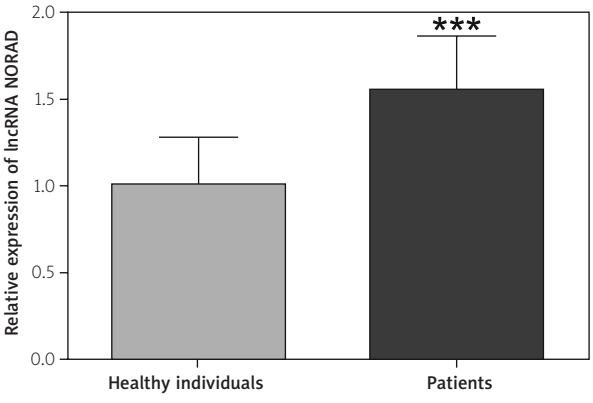

Fig. 1. Comparison of NORAD expression in acute cerebral ischemic stroke (AIS) patients and healthy individuals. A significant upregulation of NORAD was observed in AIS patients compared with healthy individuals. *** $p < 0.001$.

Expression of NORAD and its' diagnostic value in acute cerebral ischemic stroke

The expression of NORAD in AIS patients was found to be significantly higher than that of healthy individuals ($p < 0.001$, Fig. 1). Meanwhile, the results of ROC showed that NORAD can discriminate AIS patients from healthy individuals, with high sensitivity (81.6%) and specificity (88.4%) (AUC = 0.903, $p < 0.001$, Fig. 2).

Additionally, NORAD was illustrated to closely correlate with the major indicators of AIS, including hs CRP ($r = 0.796$), MMP9 ($r = 0.757$), NIHSS ($r = 0.840$), and pc-ASPECTS ($r = –0.607$) (Table II, $p < 0.001$).

Prognostic value of NORAD in acute cerebral ischemic stroke

According to the mean expression of NORAD, patients in the AIS group were divided into high expres-

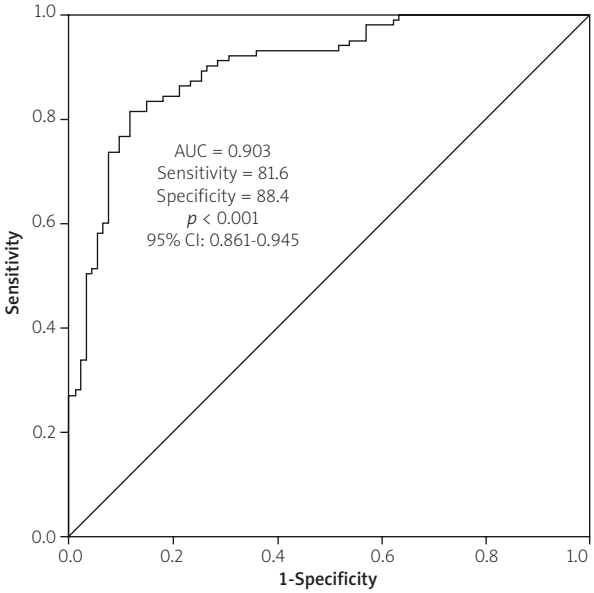

Fig. 2. ROC curve to assess the diagnostic value of NORAD. NORAD can dramatically distinguish acute cerebral ischemic stroke (AIS) patients from healthy controls, with AUC of 0.903 ($p < 0.001$).

Table II. Correlation between lncRNA NORAD and various indicators

Variables	Pearson (*r*)	*p*-value
hs CRP	0.796	< 0.001
MMP9	0.757	< 0.001
NIHSS	0.840	< 0.001
pc-ASPECT	–0.607	< 0.001

hs CRP – high-sensitivity C-reactive protein, MMP9 – matrix metalloproteinase-9

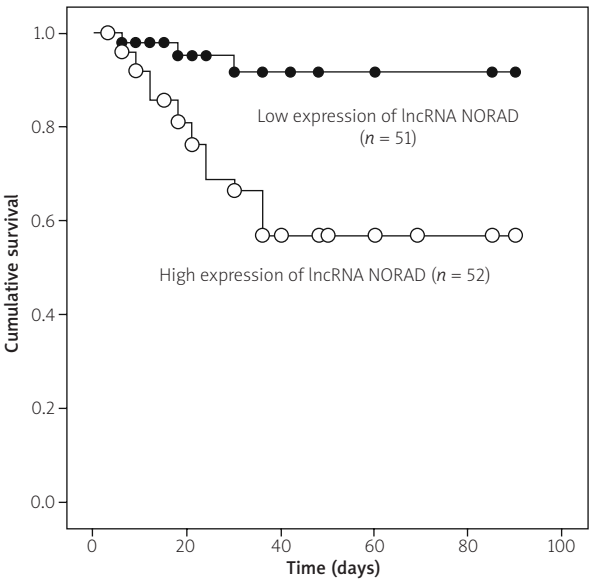

Fig. 3. Kaplan-Meier curve of acute cerebral ischemic stroke (AIS) patients based on the average expression of NORAD. High expression of NORAD was found to relate to the unfavorable prognosis of AIS patients. Log-rank, $p = 0.001$.

Table III. Multivariate Cox analysis

Name of index	HR	95% CI	*p*-value
lncRNA NORAD	7.034	1.636-10.238	0.009
Age	2.360	0.728-7.648	0.152
BMI	2.174	0.802-5.892	0.127
Gender	1.222	0.423-3.535	0.711
Hypertension	1.979	0.589-6.652	0.270
Hyperlipidemia	2.516	0.696-9.095	0.159
Diabetes	1.218	0.294-5.053	0.786
Smoker	1.468	0.308-6.993	0.630
hs CRP	2.673	0.919-7.773	0.071
MMP9	2.137	0.572-7.980	0.259
NIHSS	3.137	1.125-8.745	0.029
pc-ASPECT	0.303	0.096-0.961	0.043

BMI – body mass index, hs CRP – high-sensitivity C-reactive protein, MMP9 – matrix metalloproteinase-9

sion group and low expression group, with 52 and 51 patients, respectively. In the 90-day follow-up survey, the AIS patients with high expression of NORAD showed a worse survival rate compared with patients in low expression group, and the difference was significant (log-rank, $p = 0.001$, Fig. 3). Furthermore, multivariate Cox analysis demonstrated a significant prognostic value of NORAD, with HR value of 7.034 ($p = 0.009$) as well as NIHSS (HR = 3.137, $p = 0.029$) and pc-ASPECTS (HR = 0.303, $p = 0.043$) scores (Table III).

Discussion

lncRNAs have been demonstrated to be widely expressed in the brain, and be involved in the physiological and pathophysiological processes, such as neural development, cerebral ischemic injury, and neurodegeneration. Also, it was shown to mediate pathogenic processes, including neuroinflammation and apoptosis, oxidative stress, and excitotoxicity, which are closely correlated with cerebral ischemia and post-stroke recovery [3,24]. For example, lncRNA MIAT can promote the apoptosis and autophagy of neural cells, which further enhance the malignant development of ischemic stroke [6]. lncRNA MEG8 participated in the angiogenesis and can alleviate cerebral ischemia after ischemic stroke [20]. In this study, we evaluated the significance of NORAD in AIS early screening and progression monitoring. NORAD was previously reported to promote thyroid cancer development via negatively modulating miR-451 and miR-202-5p [8,19]. In non-small cell lung cancer (NSCLC), NORAD was found to upregulate, promoting tumor progression and regulate chemo-resistance of NSCLC cells to cisplatin [9]. Prior studies also revealed the involvement of NORAD in various human diseases, including several potential risk factors of AIS. It was reported that NORAD can attenuate the brain damage, oxidative, inflammation, and cell apoptosis induced by cerebral ischemia/reperfusion injury [28]. Hyperglycemia is one of the incentives of AIS, as it can cause insufficient cerebral blood supply, inducing abnormal metabolism of glycolipids and leading to the onset of atherosclerosis. NORAD silencing was indicated to protect against inflammation and fibrosis of mesangial cells induced by high glucose, implying its' potential function in the pathogen and development of AIS. We observed a significant upregulation of NORAD in AIS patients relative to healthy individuals, which is consistent with its' dysregulation in atherosclerosis [5,10].

The upregulated NORAD was identified as a reliable diagnostic biomarker of AIS patients, where NORAD could differentiate AIS patients from healthy control with high sensitivity and specificity. Meanwhile, we conducted SWI in the assessment of AIS patients' disease conditions, and used pc-ASPECTS scores to evaluate the severity of AIS patients. SWI could locate the thrombus in the brain of patients, such as in the internal carotid artery and M1 segment of the middle cerebral artery, according to the blood oxygen level-dependent effect and difference in susceptibility [4,12]. NORAD was observed to be negatively correlated with SWI-based pc-ASPECTS scores of AIS patients, confirming the potential diagnostic significance of SWI-

based pc-ASPECTS scores. Except for pc-ASPECT scores, NORAD was also closely associated with hs CRP, MMP9 levels, and NIHSS scores of AIS patients, which are reliable indicators of the progression and severity of AIS patients, indicating underlying involvement of NORAD in the development of AIS [11,18,21].

The prognostic value of NORAD in human diseases was reported in previous investigations. A meta-analysis reviewed the association between NORAD and clinical outcomes of various cancer patients, and found that high NORAD levels related to a positive lymph node metastasis status and unfavorable survival of patients, which concluded NORAD a valuable prognostic biomarker of human cancers [26]. Downregulation of NORAD in neuroblastoma was demonstrated to predict patients' poor prognosis and advanced tumor stage [27]. With the employment of a 90-day follow-up survey, the prognostic status of AIS patients was evaluated. AIS patients who had a relatively high expression of NORAD, showed worse outcomes compared with patients with lower NORAD levels. NORAD was identified as a favorable indicator together with NIHSS and pc-ASPECTS scores of AIS patients.

The relatively small sample size might limit the statistical power, which needs further evaluation with larger cohort. Other limitation of the present study is that there were no mechanism results explaining the function of NORAD in AIS. lncRNAs have been confirmed to display corresponding functional roles through regulating their ceRNAs [2,17]. For example, NORAD was reported to promote the progression of thyroid cancer by targeting miR-451, and regulating drug resistance and sensitivity via miR-410-3p [19,25]. Therefore, future studies should pay more attention to the underlying mechanism of NORAD's role in AIS.

According to the above findings, the upregulation of NORAD in AIS patients could serve as the biomarker for early screening and progression monitoring of AIS. Additionally, NORAD could predict the poor prognosis of AIS patients. Further mechanism research should be carried out to explore the correlated molecular involvement in AIS development.

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Disclosure

The authors report no conflict of interest.

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