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Original Article

The significance of low plasma miR-335 level in patients with acute cerebral infarction may be associated with the loss of control of *CALM1* expression

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Abstract: Calcium overload, mediated by calmodulin (CaM), plays an important role in ischemia/reperfusion injury during acute ischemic brain damage. However, the regulation of CaM expression in cerebral ischemia is still not fully addressed. This study showed that the levels of plasma miR-335 were significantly down-regulated in 152 patients with acute cerebral infarction (0.60 ± 0.31) compared to 136 age-matched healthy controls (1.16 ± 0.30) ($P < 0.01$). Moreover, the levels negatively correlated with the National Institutes of Health Stroke Scale (NIHSS) scores in acute cerebral infarction patients ($r = -0.680$; $P < 0.01$). Transfection of miR-335 mimics and inhibitors inhibited *CALM1* mRNA and CaM protein expressions in HUVECs. This study suggests that miR-335 is an important biomarker of acute cerebral infarction, and which functions are through regulating the expression of the CALM1 gene during acute ischemic brain injury. Our findings highlighted the potential of miR-335 as a target to develop agents to treat acute cerebral infarction.

Keywords: Atherosclerotic cerebral infarction, calmodulin, NIHSS, miRNA-335

Introduction

Cerebral ischemia occurs when the blood flowing to the brain does not adequately meet metabolic demand. Restoring perfusion to ischemic brain regions may cause additional, or even more serious, brain damage termed "reperfusion injury". This ischemia/reperfusion injury plays a pivotal role in the development of brain damage in patients with acute cerebral infarction [1, 2]. Calcium overload is believed to be a crucial event during the development of ischemia/reperfusion injury [3] and plays an important role in brain damage after ischemic stroke. Calcium overload is mediated by calmodulin (CaM). A recent study in a rat model of cerebral ischemia/reperfusion injury observed increased CaM activity in the brain [4]. However, the upstream regulators of calmodulin have not been fully identified.

MicroRNAs (miRNAs) are small non-coding RNAs (ncRNA), which often act as "fine-tuning" mechanisms for gene expression by binding to the 3' untranslated regions (3'UTRs) of target mRNAs via either promoting degradation of target mRNAs or inhibiting their translation. MiRNAs have been demonstrated to play essential roles in numerous pathophysiological processes, including neurogenesis, neural stem cells differentiation, and neuronal apoptotic cell death [5-7]. miRNAs have been found to play several roles in cerebral ischemia [8, 9]. Recent studies also revealed that miRNAs also play crucial roles in ischemia/reperfusion injury. For example, calcium/calmodulin dependent protein kinase II (CaMKII) is a crucial regulator of Ca^{2+} signaling and mediates signaling pathways responsible for reperfusion injury. Cha et al study revealed that miR-145 represses CaMKIIδ protein expression and Ca^{2+} overload [10]. miR-1 is a key regulator of CaM [11]. However, no miRNA has been reported to regulate CaM expression in ischemic stroke.

miR-335 was recently reported to be down-regulated in the rat brain in a middle cerebral artery occlusion model [12]. However, the

pathogenesis of miR-335 in acute cerebral infarction is still unknown. In this study, plasma miR-335 was measured in patients with acute cerebral infarction comparing with age-matched healthy controls, and the relationship between miR-335 and *CALM1* gene expression was validated in HUVECs.

Materials and methods

Study population

This study protocol was approved by the Human Ethics Committees of the Second Affiliated Hospital, University of South China (Hengyang, China), and signed informed consent forms were obtained from all participants.

A total of 152 patients with acute cerebral infarction and 136 age-matched healthy volunteers without acute cerebral infarction were recruited for this study between June 2012 and October 2013 at the Department of Neurology, Second Affiliated Hospital of University of South China. Acute cerebral infarction was diagnosed based on a patient's medical history, lab examination, neurological deficit, magnetic resonance imaging, and magnetic resonance angiography findings. Cerebral infarction was diagnosed by following the criteria of the Fourth National Cerebrovascular Disease Congress in China and the TOAST classification [13]. The severity of stroke and neurological deficits in patients were evaluated at admission according to the National Institutes of Health Stroke Scale (NIHSS) criteria. The patients were included in the study if: 1) They were diagnosed with large artery atherosclerotic cerebral infarction, 2) The length of hospital stay was 14 days or less (≤ 14 days). Patients were excluded from the study if they had a history of liver ailment, nephrosis, hematological diseases, autoimmune diseases, pregnancies, acute infectious disease, or malignant tumors.

Sample collection

10 ml of whole blood was collected with tubes containing EDTA from patients into within 24 hrs of admission and from the age-matched healthy controls, respectively. Plasma was separated by centrifugation at 1,000 g for 10 min at room temperature. All extracted plasma samples were stored at -80°C until use.

Total RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) of miR-335

Total RNA was extracted from each plasma sample using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and purified using mirVana miRNA Isolation Kit (Ambion, USA) according to the manufacturer's instructions. The cDNA was synthesized from total RNA using the ReverTra Ace qPCR RT Kit (TOYOBO, Tokyo, Japan). The quantitative PCR (qPCR) was carried out using the SYBR Premix Ex TaqTM kit (TakaRa, Tokyo, Japan) and processed on a 7500HT analyzer (Applied Biosystems, USA). The sequence-specific reverse transcription primers for miR-335 and the internal control cel-miR-39 were 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACATTT-3' and 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC AAAGC-3', respectively. miR-335 was amplified using forward primer; 5'-CTCCAGCTGTCAAGAGCAATAACGAA-3' and reverse primer: 5'-TCCAGTGCAGGGTCCG-AGGT-3'. Cel-miR-39 was amplified using forward primer: 5'-CACTCCGTCACCGGGTGTAAATC-3' and reverse primer: 5'-TCCAGTGCAGGGTCCGAGGT-3'. The primers were synthesized by Sangon company (Shanghai, China). qPCR was performed in triplicate. The relative miR-335 level was analyzed using the $2^{-\Delta\Delta CT}$ method.

Bioinformatics analysis

The relationship between miR-335 and *CALM1 gene* was predicted with TargetScan (http://www.targetscan.org/) and Miranda (http://www.microrna.org/microrna/home.do) miRNA databases.

Cell culture and transfection

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Basel, Switzerland) and maintained as previously described [13]. miR-335-mimics, negative control for miRNA mimics, miR-335-inhibitors, and negative control for inhibitors of miRNAs were purchased from Genepharma (Shanghai, China). HUVECs were seeded on 96-well plates 24 hrs before transfection. The transfection of miRNA mimics, inhibitors, and controls were performed using Lipofectamine 2000 (Invitrogen, CA, USA) by following the manufacturer's instructions. 48 hrs later, HUVECs were har-

Table 1. Demographic and clinical characteristics of participants

Characteristic	ACI (n = 152)	Controls (n = 136)
Age (years)	63.8 ± 11.4	61.7 ± 11.8
Gender (M/F)	100/52	88/48
BMI (kg/m^2)	23.8 ± 2.0	23.4 ± 1.7
Hypertension (%)	71 (46.7%)	47 (34.5%)*
Current smoking (%)	61 (40.1%)	37 (27.2%)*
Current drinking (%)	47 (30.9%)	32 (23.5%)
LDL (mmol/L)	2.74 ± 0.85	2.43 ± 0.80*
HDL (mmol/L)	1.24 ± 0.39	1.43 ± 0.43*
Diabetes (%)	36 (23.7%)	17 (12.5%)*

*P < 0.05 for patients with ACI versus controls. ACI: acute cerebral infarction; M: male; F: female; BMI: body mass index; LDL: low-density lipoprotein; HDL: high-density lipoprotein.

vested for mRNA or protein expression analysis.

qRT-PCR of CALM1 gene

Total cellular RNA was extracted using TRIzol reagent (Invitrogen) and reverse-transcribed into cDNA using M-MLV First-Strand cDNA synthesis Kit (Invitrogen). *CALM1 (calmodulin*) mRNA was detected by quantitative PCR as described above. β-actin was amplified as an internal control. *CALM1* gene was amplified using forward primer: 5'-TGAAGTGGATGCTGATGGTAATGG-3' and reverse primer: 5'-CATAGTTGACTTGTCCGTCTCCAT-3'. Human β-actin was amplified using forward primer: 5'-CACTCTTCCAGCCTTCCTTCCT-3' and reverse primer: 5'-ACTCGTCATACTCCTGCTTGCT-3'. The melt curves for each PCR were analyzed to determine any non-specific amplification. qPCR was performed in triplicate. Relative *CALM1* expression was analyzed using the comparative cycle threshold method ($2^{-\Delta\Delta ct}$).

Western blot

HUVECs were homogenized and 50 μg protein were loaded per lane and run on 10% SDS/PAGE gels and then transferred to PVDF membranes (Millipore, MA, USA). After blocking with 5% fat-free milk in TBS buffer at 25°C for 1 hr, the membranes were incubated with rabbit anti-human CaM or β-actin antibody (1:1000 dilution, ABclonal, USA) overnight at 4°C, followed by secondary goat anti-rabbit IgG (H+L) conjugated with HRP (1:5000 dilution, ABclonal) at 25°C for 2 hrs. The blots were developed with electrochemiluminescence (ECL) reagent (GE, USA).

Statistical analysis

Quantitative data were presented as mean ± standard deviation (SD) and analyzed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Normally distributed data between two groups were analyzed using the two tail Student's *t*-test. Spearman correlation was performed between the two variables. Results were considered significant at $P < 0.05$.

Results

Clinical characteristics of patients

The demographic and clinical characteristics of the patients and the age-matched controls were presented in **Table 1**. No significant differences in median age, gender, BMI, and percentage of patients that drink were observed between the acute cerebral infarction patients and age-matched healthy controls. However, significantly higher percentage of acute cerebral infarction patients smoked and had hypertension, diabetes, and dyslipidemia compared to the controls.

Plasma miR-335 was decreased in acute cerebral infarction patients

Plasma miR-335 level in acute cerebral infarction patients was examined by using qRT-PCR. As shown in **Figure 1A**, the level was significantly lower in patients with acute cerebral infarction (0.60 ± 0.31) than in age-matched healthy controls (1.16 ± 0.30) ($P < 0.01$), and it was analyzed by dividing acute cerebral infarction patients into high NIHSS score group (score > 5, n = 82) and low NIHSS score group (score ≤ 5, n = 70) (**Figure 1B**). The levels of plasma miR-335 were significantly lower in patients with high NIHSS scores (0.48 ± 0.25) compared to patients with low NIHSS scores (0.74 ± 0.32) ($P < 0.05$).

Correlation of plasma miR-335 level with the severity of ischemic stroke

Significantly negative correlations were observed between plasma miR-335 levels and

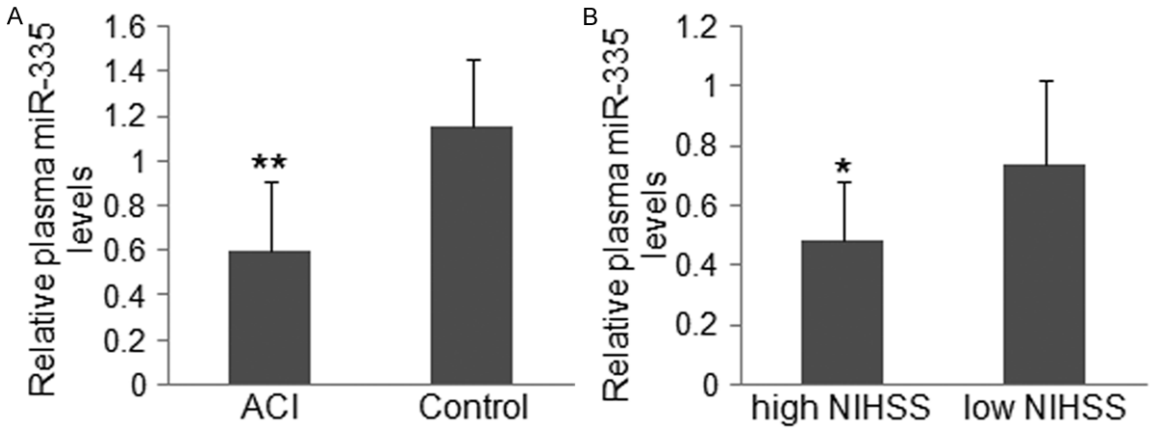

Figure 1. Plasma miR-335 levels. Plasma miR-335 was examined by qRT-PCR and presented as relative levels. A. Comparison of miR-335 levels between acute cerebral infarction (ACI) patients and age-matched healthy controls (control). $^{**}P < 0.01$. B. Comparison of miR-335 levels between ACI patients with high and low NIHSS scores. $^{*}P < 0.05$.

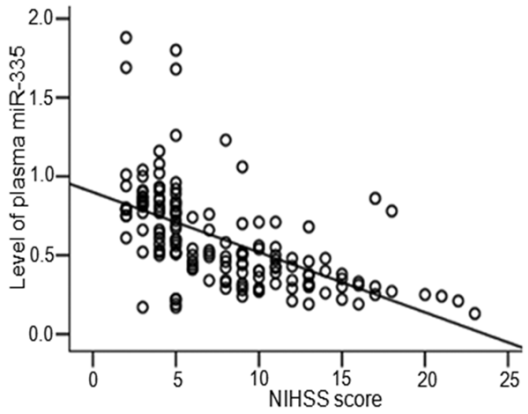

Figure 2. Correlation analysis between plasma miR-335 levels and NIHSS scores in ACI patients. The negative relation was detected (Spearman's correlation analysis, r = -0.680; P < 0.01).

NIHSS scores in acute cerebral infarction patients (r = -0.680; $P < 0.01$) (**Figure 2**), suggesting a significant association between plasma miR-335 level and the severity of stroke in acute cerebral infarction patients.

MiR-335 down-regulates CALM1 gene expression

TargetScan and Miranda databases were employed to analyze the targeting site of miR-335 in the CALM1gene, and one targeting site of miR-335 was found in the 3'UTR of *CALM*1 mRNA (**Figure 3A**).

Transfection of miR-335 mimics, rather than the blank or the negative control (NC), obviously decreased *CALM1* mRNA (**Figure 3B**) and CaM protein (**Figure 3C**, **3D**) expression in HUVECs 48 hrs after transfection (**Figure 3B**). Transfection of miR-335 inhibitors strongly increased the expression of *CALM1* mRNA and CaM protein in HUVECs (**Figure 3B-D**). These results suggested that miR-335 could negatively regulate *CALM1*/CaM expression in HUVECs.

Discussion

Ischemic stroke is the major cause of mortality and morbidity worldwide and constitutes 60%-80% of all strokes. However, the etiological factors affecting ischemic stroke remain uncertain. It is widely known that ischemic stroke is a multifactorial disease influenced by unhealthy lifestyles, hypertension, dyslipidemia, and diabetes. This study also revealed that smoking, hypertension, diabetes, low level of high-density lipoprotein, and high level of low-density lipoprotein are important risk factors of acute cerebral infarction. During the past several decades, the molecular and cellular mechanisms driving the development of acute cerebral infarction have been intensively investigated. Numerous signaling molecules, including microRNAs, have been demonstrated to play roles in ischemia/reperfusion injury. However, the key molecular markers remain unknown.

A previous study reported that miR-335 was down-regulated in the brain of rats with a transient middle cerebral artery occlusion [12]. Moreover, miRNAs were found to freely pass

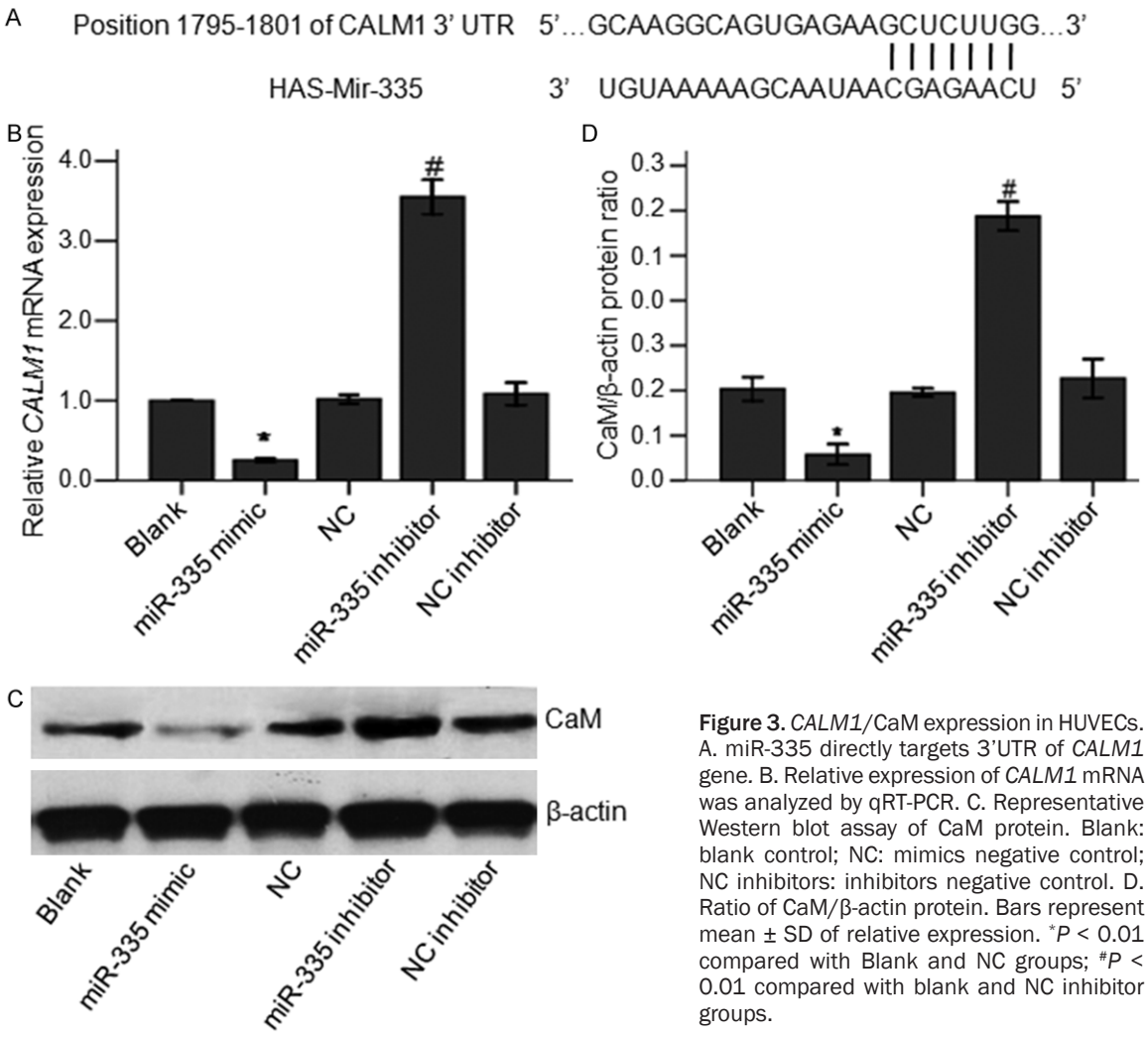

Figure 3. *CALM1*/CaM expression in HUVECs. A. miR-335 directly targets 3'UTR of *CALM1* gene. B. Relative expression of *CALM1* mRNA was analyzed by qRT-PCR. C. Representative Western blot assay of CaM protein. Blank: blank control; NC: mimics negative control; NC inhibitors: inhibitors negative control. D. Ratio of CaM/β-actin protein. Bars represent mean ± SD of relative expression. $^{*}P < 0.01$ compared with Blank and NC groups; $^{\#}P < 0.01$ compared with blank and NC inhibitor groups.

through the blood-brain barrier [14], and circulating miR-335 level was found to parallel the level of miR-335 in the ischemic hemisphere [15, 16]. In this study, we found that the plasma miR-335 level was significantly lower in acute cerebral infarction patients compared to age-matched healthy controls. Moreover, plasma miR-335 level was also significantly lower in patients with high NIHSS scores than in patients with lower NIHSS scores. Correlation analysis showed that plasma miR-335 levels negatively correlated with NIHSS scores in acute cerebral infarction patients. Thus, our study suggests that plasma miR-335 is a marker of acute cerebral infarction, and its level can reflect the severity of brain injury. However, the role of miR-335 in brain injury is unknown.

Calmodulin is a calcium-binding protein and regulates transmembrane calcium transportation, absorption, and secretion. An increase in calmodulin activity means an elevation in intracellular Ca^{2+} concentration [17]. Calcium overload has been demonstrated to induce neuron necrosis during ischemia/reperfusion injury [3]. A recent study demonstrated that calmodulin is regulated by miR-1 [11]. However, miR-1 is thought to be one of the miRNAs specifically expressed in muscles [11, 18]. Therefore, the role of plasma miR-1 in ischemia/reperfusion injury is questionable. Interestingly, in this study, we found that the calmodulin gene (*CALM1*) is a target of miR-335. Overexpression of miR-335 significantly decreased the expression of *CALM1* mRNA and CAM protein in HUVECs and this process can be blocked with miR-335 inhibitors. This suggests that miR-335 can directly regulate CaM expression in endothelial cells. Indeed, endothelial dysfunction has been widely reported to affect the occur-

rence of cerebral infarction [19]. While acute cerebral infarction occurs, ischemic damage and then hemorrhagic transformation or ischemia/reperfusion injury can further aggravate ischemic brain damage [20, 21]. Imbalance of neuronal calcium (Ca^{2+}) homeostasis is an important aggravating factor in cerebral ischemia/reperfusion injury [22]. Thus, downregulated miR-335 expression may be an important factor in brain injury of patients with acute cerebral infarction. Downregulated miR-335 may lead to an increase in calmodulin expression and subsequent calcium overload in cerebral vessel endothelium and/or neuronal tissues, which further induces neuronal necrosis during ischemia/reperfusion injury.

We acknowledge that there were several limitations to this study. We provided no direct evidence on whether miR-335 expression in the brain was actually reduced in patients with acute cerebral infarction. Also, we provided no direct evidence on whether miR-335 regulated calmodulin expression in cerebral endothelial cells and neuronal cells. These issues, however, could be evaluated in animal models of acute cerebral infarction or in human autopsy specimen.

In conclusion, this study suggests that plasma miR-335 level can serve as a potential biomarker in acute cerebral infarction patients, and miR-335 targets the *CALM1* gene and inhibits its expression in endothelial cells. Our finding implies that miR-335 could be a potential therapeutic target in acute cerebral infarction.

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Disclosure of conflict of interest

None.

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