

Original Article

Serum miR-155 and its target gene hypoxiainducible factor 1 α (HIF1A) are associated with acute ischemic stroke

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Abstract: Acute ischemic stroke (AIS) is one of the leading causes of death and disability all over the world. MicroRNAs (miRNAs) have been identified as a potential biomarker in diagnosis, treatment and prognosis of AIS. In this study, the expression levels of miR-23b, miR-106b, miR-130a, miR-155, and miR-425 were detected in serum of AIS patient and healthy control by using reverse transcription quantitative polymerase chain reaction (RT-qPCR) assay. We found that miR-155 was significantly up-regulated in serum of AIS patients compared with healthy control ($P < 0.05$). Using miRBase and TargetScan databases, we identified miR-155 as a possible regulator of the HIF1A (hypoxiainducible factor 1 α). Then, we validated that HIF1A is a target for miR-155 with dual luciferase reporter and Western blot assays. Furthermore, the mRNA expression levels of HIF1A was significantly down-regulated in serum of AIS patients ($P < 0.05$), and its expression was significantly negative correlated with miR-155 ($P < 0.05$). Interestingly, high miR-155 expression and low HIF1A mRNA expression, alone or in combination, were all significantly associated with high total cholesterol ($P < 0.05$), high LDL ($P < 0.05$) and low HDL ($P < 0.05$) of AIS patients. In addition, multivariate logistic regression analysis demonstrated that high miR-155 expression ($P < 0.05$), low HIF1A mRNA expression ($P < 0.05$), and combined expression of miR-155 and HIF1A mRNA ($P < 0.05$) were all significant and independent predictor for determining the presence of AIS. Our findings indicate that up-regulation of serum miR-155 and its target gene HIF1A are associated with AIS, which may be benefit for the development of miRNA-directed diagnostics and therapeutics against AIS.

Keywords: AIS, serum, miRNAs, miR-155, HIF1A

Introduction

Stroke is the second leading causes of mortality and serious long-term disability all over the world [1, 2]. The acute ischemic stroke (AIS) is the predominant type in stroke, the diagnosis of AIS relies mainly on time-consuming processes, including medical history, neuroimaging, and clinical examination [3]. Although computed tomography (CT) scans and magnetic resonance imaging (MRI) have facilitated diagnosis of AIS in the past few decades, the diagnostic and prognostic strategies are frequently limited in AIS management [1, 4]. Moreover, the only effective therapeutic approach for AIS is intravascular thrombolysis, however, less than 3% AIS only can receive treatment due to narrow time windows. Therefore, there is need in

urgent development of new, effective and sensitive diagnostic biomarkers and therapeutic strategies to the adverse situation.

MicroRNAs (miRNAs) are non-coding RNA molecules of 18-24 nucleotides in length, which bind to their target gene mRNA 3' un-translated region (UTR) via partial or complete complementarity resulting in degradation or translational inhibition of the transcript [5]. Previous studies have suggested that miRNAs are involved in various cellular physiological processes, including cell development, differentiation, growth, proliferation and apoptosis [6]. Emerging evidences have demonstrated that a number of miRNAs are abnormally expressed in many human diseases, such as cancer, inflammation, hypertension, myocardial infarction etc [6-9].

Table 1. The primer sequences for RT-qPCR

Gene	Sense (5'-3')	Antisense (5'-3')
miR-23b	TTAGGGACCGTTACT	GTGCAGGGTCCGAGG
miR-106b	ACGTGACAGTCGTGAA	GTGCAGGGTCCGAGG
miR-130a	CGGGAAAATTGTAACGT	GTGCAGGGTCCGAGG
miR-155	GATAGTGCTAATCGTAAT	GTGCAGGGTCCGAGG
miR-425	TGCCCTACTAGCACAGT	GTGCAGGGTCCGAGG
U6	GCGCGTCGTGAAGCGTTC	GTGCAGGGTCCGAGGT
HIF1A	CCCTAACTAGCCGAGGA	CACAAATCAGCACCAAGC
GAPDH	GCCTCAAGACCTTGGGCT	CAGTCCAGCCCAAGGTCT

Recent studies have found that serum miRNAs expression profiles in AIS patients and similar animal models are profoundly changed [10-12], suggesting these miRNAs may serve as potential biomarkers for diagnosis, treatment and prognosis of AIS. For example, miR-let-7e is a potential circulating biomarker of AIS [13]; miR-210 mediates vagus nerve stimulation-induced anti-apoptosis responses and antioxidant stress following cerebral ischemia/reperfusion injury in rats [14]; miR-424 protects against permanent focal cerebral ischemia injury involving suppressing microglia activation in mice [15].

Sequencing for profiling expression of miRNAs in serum of AIS patient showed that many miRNAs were aberrantly expressed, including miR-23b, miR-106b, miR-130a, miR-155, and miR-425 [16, 17]. The present study was undertaken to verify these miRNAs expression in serum of AIS patients, and evaluate the utility of these miRNAs as AIS serum biomarkers.

Materials and methods

Ethical approval

This study was approved by the ethics committee of the Fifth Affiliated Hospital of Zhengzhou University (Zhengzhou, China). Written informed consent was also obtained from all the patients and/or their family.

Clinical samples collection

A total of 100 AIS patients and 100 healthy individuals were collected from the Fifth Affiliated Hospital of Zhengzhou University (Zhengzhou, China) from January 2009 to January 2014. The diagnosis of AIS required consensus of two

certified neurologists, and confirmed from the result of brain CT or MRI imaging. The AIS patients with immunosuppressive and thrombolytic therapy, infection and lymphoma were excluded from this study. The healthy control had no history of stroke, peripheral vascular diseases or myocardial infarction.

Biochemical test

5 ml peripheral blood of each person was collected in Qiagen blood tube (Qiagen Inc., Valencia, CA, USA) before treatment. Serum samples were acquired from peripheral blood by centrifugation at 3000 g for 10 min at room temperature. Body mass index (BMI) was defined as weight divided by height squared (kg/m^2). Serum triglycerides, total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), apolipoprotein A1 (ApoA1), and apolipoprotein B (ApoB) were detected by using a Toshiba 200FR Neo Chemistry Analyzer (Toshiba Medical Systems, Tokyo, Japan).

RNA isolation and reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated by using a Qiagen blood miRNA kit (Qiagen, Chatsworth, CA, USA), according to the manufacturers protocol. RNA was predominantly extracted from circulating leukocytes, such as monocytes, granulocytes, T-cells, B-cells. Each RNA with OD 260/280 ratios between 1.8-2.1 was stored frozen at liquid nitrogen (Beyotime Biotechnology, Suzhou, China) and used for further experiments.

The complementary deoxyribonucleic acid (cDNA) was synthesized from each RNA by using a miScript II RT kit (Qiagen, Chatsworth, CA, USA). After that, cDNA template was diluted 10 times and then used for RT-qPCR reaction. RT-qPCR was conducted with a miScript SYBR[®] Green PCR kit (Qiagen, Valencia, CA, USA) on a Roche Light-Cycler 480 Real-Time PCR System (Roche Diagnostics, Indianapolis, IN, USA), according to the manufacturer's protocols. The reaction condition was presented as follows: 95°C for 10 min; and 45 cycles of 95°C for 10 sec and 60°C for 30 sec. The primer sequences of each gene were shown in **Table 1**. Small nuclear U6 snRNA (U6) and glycer-

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aldehyde-3-phosphate dehydrogenase (GAPDH) were used as internal controls. The relative expression levels of each gene were quantified by the $2^{-\Delta\Delta CT}$ method [18].

Bioinformatics analysis

The target gene of miR-155 was predicted by using miRBase (<http://www.targetscan.org/>) and TargetScan (<http://www.targetscan.org/>) algorithms.

Cell culture and transfection

The HEK-293T cells was obtained from American Type Culture Collection (ATCC, Rockville, Maryland, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, CA, USA) with 5% CO₂ at 37°C, supplemented with 10% fetal bovine serum (FBS, Invitrogen, CA, USA), 1% of 100 U/ml penicillin and 1% of 100 mg/ml streptomycin sulfates.

miR-155 mimics and mimics control were purchased from Suzhou GenePharma Co., Ltd. (Shanghai, China). Approximately 6×10^6 HEK-293T cells were seeded in 6-well plates, transfected with miR-155 mimics or mimics control by using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's protocols.

Western blot assay

Total proteins were extracted from HEK-293T cell with RIPA lysis buffer (Invitrogen, CA, USA), according to the manufacturer's protocols. Approximately 40 µg proteins were used for Western blot assay. The proteins were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were treated with 0.5% low-fat milk in Tris-buffered saline (TBS) buffer. After that, the membranes were cultured with mouse monoclonal anti-HIF1A antibody (ab199004, 1:1000, Abcam, Cambridge, UK) and mouse monoclonal anti-GAPDH antibody (ab8245, 1:5000, Abcam, Cambridge, UK) overnight at 4°C. The membranes were washed with TBS and then incubated with horseradish-peroxidase-conjugated (HRP) secondary anti-mouse antibody (ab6-789, 1:10000, Abcam, Cambridge, UK). Finally, the band of proteins was detected by an en-

hanced chemiluminescence (Pierce, Rockford, IL, USA).

Plasmid construction and dual luciferase reporter assay

The mRNA 3'-UTR of HIF1A containing putative binding sites (5'-AGCAUUA-3') of miR-155 was sub-cloned into the empty psiCHECK-2 reporter plasmid (Promega, Madison, WI, USA), to generate the wide type (WT) recombination reporter vector (psiCHECK2-HIF1A-WT). The mutant type (MUT) recombination reporter vector (psiCHECK2-HIF1A-MUT) was generated by changing the putative binding sites to 5'-GAUGCCG-3' in the complementary sites for the seed region of miR-155. All the sequences of constructed plasmids were verified by using Sanger/DNA sequencing.

For the dual luciferase reporter assay, approximately 4×10^4 HEK-293T cells were seeded in 24-well plates, co-transfected with psiCHECK2-HIF1A-WT or psiCHECK2-HIF1A-MUT reporter plasmid and miR-155 mimics or mimics control by using Lipofectamine 2000. After 24 h transfection, the Firefly and Renilla Luciferase of each group were detected on a Modulus™ Single Tube Multimode Reader (Turner Biosystems, Sunnyvale, CA, USA) by using a Dual-Luciferase Reporter Assay kit (Promega, Madison, WI, USA).

Statistical analysis

The data was analyzed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Experiment results were presented as mean ± standard deviation (SD) and performed in triplicate for three independent times. The independent-sample *t* test was used to compare clinical characteristics between AIS patients and healthy control. The ANOVA test was used to determine the statistical differences in high miR-155 group, low HIF1A mRNA group and high miR-155 + low HIF1A mRNA group. The correlation between miR-155 and HIF1A mRNA expression was analyzed using Pearson correlation analysis. Multivariate logistic regression analysis was performed to determine the association between presence of AIS and miR-155, HIF1A expression and the other clinical manifestations. All tests were two-sided, and a value of $P < 0.05$ were considered to be statistically significant.

Table 2. Clinical characteristics of AIS patients and healthy controls

Characteristics	AIS patients (n=100)	Healthy control (n=100)	P value
Age (years)	55.4 ± 6.7	56.3 ± 7.2	0.325
Gender			
Male (%)	65 (32.5%)	71 (35.5%)	0.571
Female (%)	35 (17.5%)	29 (14.5%)	
BMI ratio	22.6 ± 5.9	21.7 ± 6.4	0.236
Smoking (%)	42 (42%)	49 (49%)	0.667
Drinking (%)	32 (32%)	29 (29%)	0.532
Hypertension (%)	57 (57%)	47 (47%)	0.126
Diabetes mellitus (%)	23 (23%)	18 (18%)	0.665
Total cholesterol (mmol/L)	4.93 ± 1.32	3.59 ± 1.13	0.023*
Triglycerides (mmol/L)	1.36 ± 0.47	1.22 ± 0.36	0.479
ApoA1 (g/L)	1.36 ± 0.29	1.43 ± 0.18	0.517
ApoB (g/L)	0.75 ± 0.30	0.82 ± 0.43	0.421
HDL (mmol/L)	0.73 ± 0.44	1.34 ± 0.24	0.009*
LDL (mmol/L)	2.61 ± 0.91	1.74 ± 0.87	0.016*

AIS: acute ischemic stroke; BMI, body mass index; HDL: high-density lipoprotein; LDL: low-density lipoprotein; ApoA1: apolipoprotein A1; ApoB: apolipoprotein B. Data are presented as mean ± standard deviation, *P<0.05.

Results

Clinical characteristics of samples

The clinical characteristics of samples were shown in **Table 2**. There was no difference in age, gender, smoking, drinking, BMI, hypertension, and diabetes mellitus between AIS patients and healthy control. Among the biochemical data, AIS patients had higher levels of total cholesterol and LDL compared to healthy control, and lower level of HDL. The triglycerides, ApoA1 and ApoB have no difference between AIS patients and healthy control.

miR-155 expression increased significantly in AIS patients compared with healthy control

Five miRNAs (namely miR-23b, miR-106b, miR-130a, miR-155, and miR-425) were selected from the results of microarray-based miRNAs expression profiling analysis, which were reported to be aberrant expressed in serum of AIS patients [16, 17]. The expression levels of these miRNAs in 10 cases of AIS patients and healthy control was detected by RT-qPCR analysis. The results showed that no difference in the expressions of miR-23b, miR-106b, miR-

130a and miR-425 (**Figure 1A-D**), while the expression of miR-155 in AIS patients was significantly up-regulated compared with healthy control (**Figure 1E**, P<0.05). Then, the up-regulation of miR-155 in serum of AIS patients was further confirmed in larger study group (**Figure 1F**, P<0.05). These results prompted us to focus on miR-155 in the following study.

HIF1A was a direct target gene of miR-155

Bioinformatics analysis was used to predict the potential target gene of miR-155. MiRanda algorithms showed that there have a potential binding sites between miR-155 and HIF1A mRNA 3'UTR (**Figure 2A**). In order to confirm whether HIF1A is a target gene of miR-155, the psiCHECK2-HIF1A-WT or psiCHECK2-HIF1A-MUT reporter plasmid was

co-transfected with miR-155 mimics or mimics control into HEK-293T cell. The results showed that the relative luciferase activity was inhibited by miR-155 mimics in the psiCHECK2-HIF1A-WT reporter vector, while the inhibitory effect of miR-155 mimics was vanished in the psiCHECK2-HIF1A-MUT reporter vector (**Figure 2B**, P<0.05). In additional, as shown in **Figure 2C**, protein expression levels of HIF1A in was decreased in miR-155 mimics treated HEK-293T cell, as compared with the mimics control.

HIF1A was down-regulated and negatively correlated with miR-155 expression in serum of AIS patients

RT-qPCR analysis was also performed to detect HIF1A mRNA expression levels in 100 cases of AIS patients and healthy control. The results revealed that the mRNA expression of HIF1A were significantly down-regulated in serum of AIS patients compared with healthy control (**Figure 3A**, P<0.05). Meanwhile, the correlation analysis showed expression of HIF1A mRNA was significantly negative correlated with miR-155 in serum of AIS patients (**Figure 3B**, P<0.05).

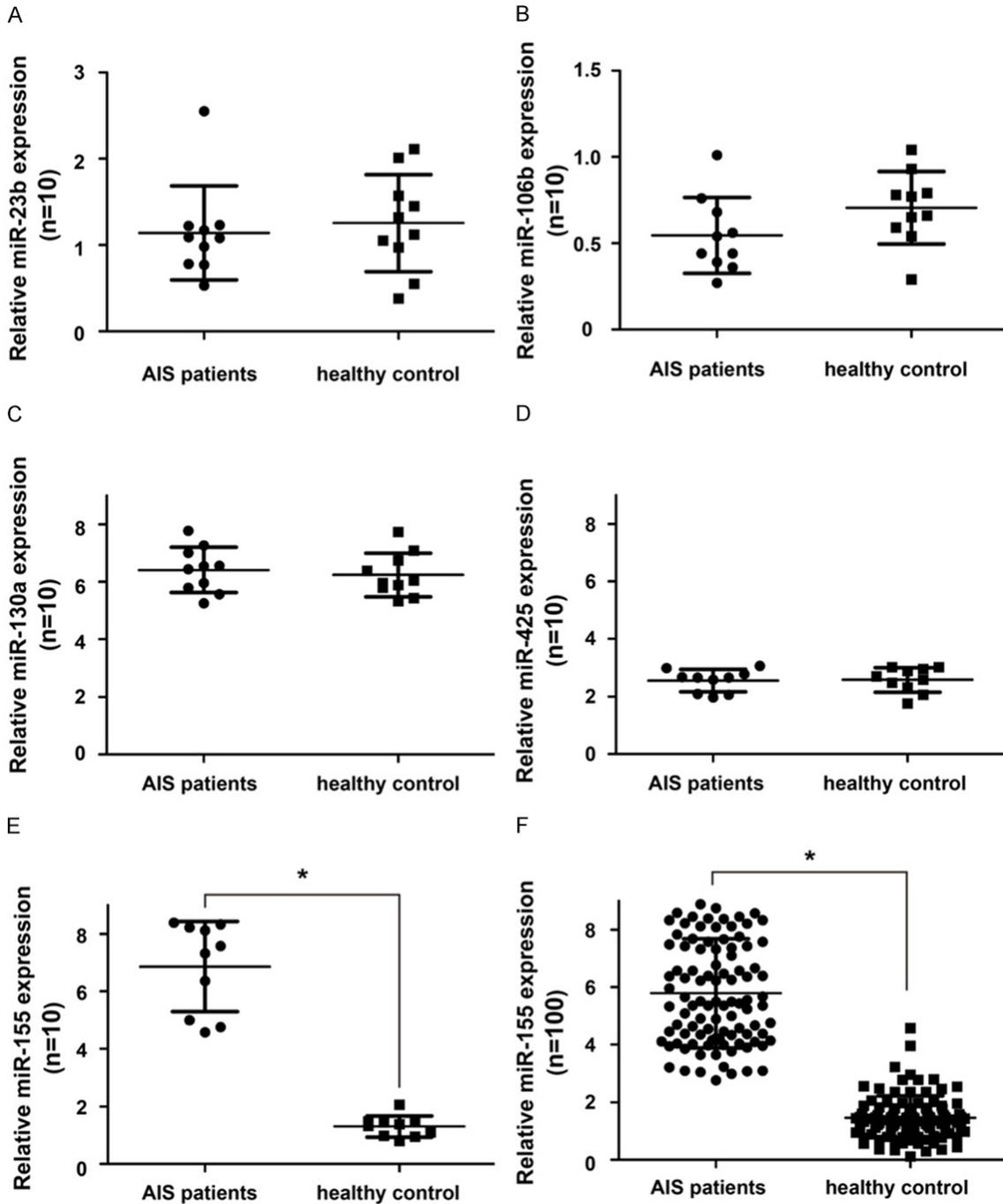


Figure 1. Expressions of miR-23b, miR-106b, miR-130a, miR-155, and miR-425 in serum of AIS patients. Analysis of the expression levels of miR-23b (A), miR-106b (B), miR-130a (C), miR-155 (D), and miR-425 (E) in serum of AIS patients and healthy control by RT-qPCR. (F) The expression of miR-155 was significantly up-regulated in serum of AIS patients compared to healthy control. U6 was used as an internal control. *P<0.05.

The aberrant expression of miR-155 and HIF1A were significantly associated with poor responses in AIS patients

Given there were no definite criteria for the expression levels of serum miR-155 and HIF1A mRNA in AIS patients, we selected the

median value of miR-155 and HIF1A mRNA as the threshold. The value of high miR-155 expression group ≥ 5.84 and low HIF1A expression group ≤ 3.52 .

As shown in **Table 3**, the data showed that high miR-155 expression and low HIF1A mRNA

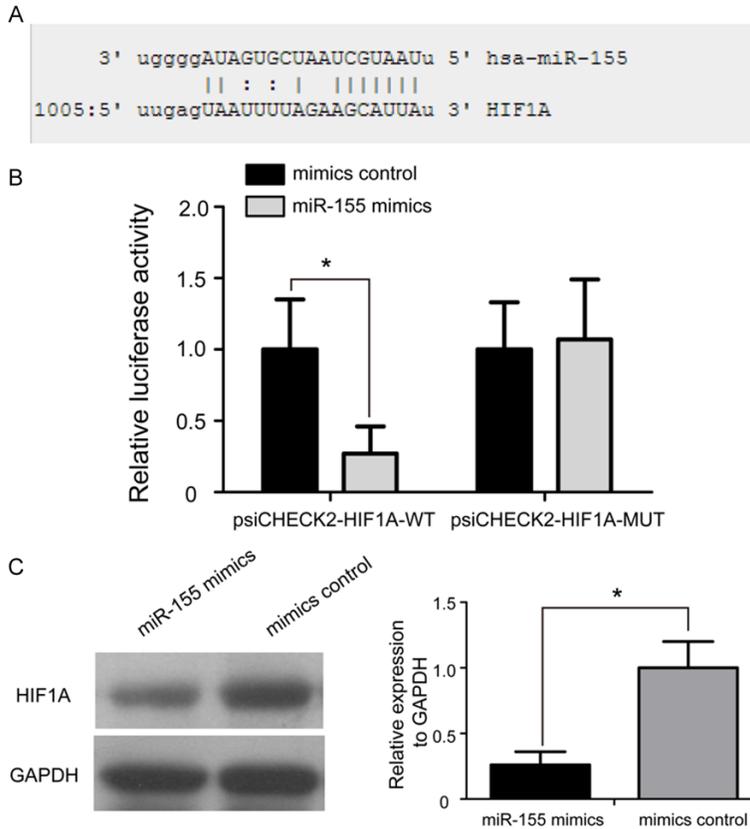


Figure 2. HIF1A was a direct target gene of miR-155. A. The predictable binding sites between miR-155 and HIF1A mRNA 3'-UTR region was shown. B. miR-155 mimics inhibited the relative luciferase activity in the psiCHECK2-HIF1A-WT reporter plasmid treated HEK-293T cell, whereas, the relative luciferase activity was not decreased in psiCHECK2-HIF1A-MUT reporter plasmid transfected HEK-293T cell. WT: wild type; MUT: mutant. *P<0.05. C. Western blotting analysis was used to detect the expression levels of HIF1A expression in HEK-293T cell after transfected with miR-155 mimics or mimics control.

expression, alone or in combination, were all significantly associated with high total cholesterol (P<0.05), high LDL (P<0.05) and low HDL (P<0.05) of AIS patients.

In additional, multivariate logistic regression analysis demonstrated that high miR-155 expression (Table 4, P<0.05), low HIF1A mRNA expression (Table 4, P<0.05), and combined expression of miR-155 and HIF1A mRNA (Table 4, P<0.05) were all significant and independent predictor for determining the presence of AIS.

Discussion

AIS represents a big public health problem, which brings tremendous hurt and strong economic burden to patients. All humanities hard work to identify the molecular mechanisms

causing cerebral injury and explore novel biomarker for treatment [13, 15]. Previous studies have demonstrated that miRNAs expression levels was significantly altered in response to AIS injury in patients serum [19, 20], suggesting that miRNAs may be potential new biomarkers for diagnosis and prognosis judgment and therapeutic targets for AIS.

It is widely acknowledged that miRNAs released from circulating cells or damaged cells lead to increased serum miRNA expression levels [13, 21]. Recently, sequencing for profiling expression of miRNAs in serum of AIS patients showed many miRNAs were aberrantly expressed, including, miR-23b, miR-106b, miR-130a, miR-155, and miR-425 [16, 17]. Here, we analyzed the expression levels of these miRNAs and found that the miR-155 expression was significantly up-regulated in serum of AIS patient compared with healthy control.

miR-155 is located on human chromosome 21q21.3, which has been reported to serve as a diagnostic and prognostic biomarker in many cancers [22]. For instance, Some studies have well demonstrated that miR-155 can be as a good serum diagnostic and prognostic biomarker for colorectal cancer [23] and hematological malignancies [24]. Up-regulation of a potential prognostic biomarker, miR-155, enhances cell proliferation in patients with oral squamous cell carcinoma [25]. miR-155 plays a vital role in prognosis in individuals with monoclonal B-cell lymphocytosis and patients with B chronic lymphocytic leukemia [26]. High expression of miR-155 is as-soci-ated with the aggressive malignant behavior of gallbladder carcinoma [27]. In our research, we found that AIS led to significant downregualtion of the serum miR-155 and the significance of dramatic changes were investigated.

miR-155 and HIF1A associated with AIS

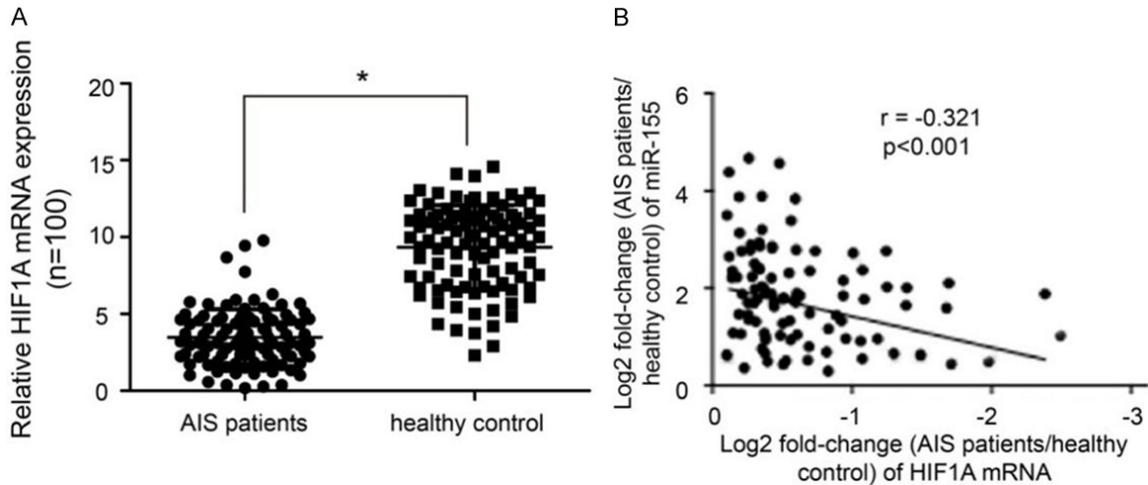


Figure 3. HIF1A was down-regulated and negative correlated with miR-155 expression in serum of AIS patients. A. RT-qPCR showed that HIF1A mRNA expression was significantly down-regulated in serum of AIS patients compared to healthy control. GAPDH was used as an internal control. * $P < 0.05$. B. Expressions of HIF1A mRNA was significantly negative correlated with miR-155 in serum of AIS patients.

Table 3. Associations of miR-155 and/or HIF1A mRNA expression in serum of AIS patients with clinical characteristics

Clinical characteristics	High miR-155		Low HIF1A mRNA		High miR-155 and low HIF1A mRNA	
	Correlation coefficient (r)	P value	Correlation coefficient (r)	P value	Correlation coefficient (r)	P value
Total cholesterol (mg/dL)	0.271	0.002*	0.172	0.001*	0.345	0.045*
HDL (mmol/L)	-0.238	0.033*	-0.457	0.009*	-0.312	0.023*
LDL (mmol/L)	0.234	0.001*	0.263	0.038*	0.409	0.007*

* $P < 0.05$.

Table 4. Multivariate logistic regression analysis for presence of AIS patients

	Multivariate logistic regression		
	OR	95% CI	P
Total cholesterol (mg/dL)	0.895	0.212-4.368	0.009*
HDL (mmol/L)	0.126	0.000-2.374	0.035*
LDL (mmol/L)	0.259	0.006-3.295	0.001*
High miR-155	5.352	1.674-15.930	0.001*
Low HIF1A mRNA	1.363	0.079-5.428	0.010*
High miR-155 + low HIF1A mRNA	4.478	2.480-10.039	0.001*

OR: odds ratio. * $P < 0.05$.

HIF1A acts as a mainly regulator of cellular and systemic homeostatic response to hypoxia by activating transcription of many genes [28]. Studies have found that HIF-1 plays a key role in many ischemic diseases [29]. Knockdown of HIF1A leads to worsening of neurological functions after stroke. In this study, we found that HIF1A was a direct target gene of miR-155. The

mRNA expression of HIF1A was significantly down-regulated in serum of AIS patients compared with healthy control. Meanwhile, its expression was significantly negative correlated with miR-155 in serum of AIS patients. These results indicated that miR-155 and HIF1A mRNA may associate with AIS.

Furthermore, we evaluated the associations of miR-155 and/or HIF1A mRNA expression with various clinical characteristics of AIS patients. Our data showed that high miR-155 expression and low HIF1A mRNA expression, alone or in combination, were all significantly associated with high total cholesterol, high LDL and low HDL of AIS patients. More interestingly, miR-155 and/or HIF1A mRNA expression were all significant and independent predictor for determining the presence of AIS.

In conclusion, our study showed up-regulation of serum miR-155 and/or down-regulation HIF1A mRNA was independent predictor of AIS. The findings suggested that the combination of miR-155 and its target gene HIF1A may be a promising AIS biomarker. We can utilize these findings for the development of new strategies for AIS patients to diagnose, treatment and prognosis.

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Disclosure of conflict of interest

None.

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