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MicroRNA-210 as a novel blood biomarker in acute cerebral ischemia

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1. ABSTRACT

MicroRNA-210 (miR-210), a master and pleiotropic hypoxia-microRNA, plays multiple roles in brain ischemia. However, miR-210 expression and its function in humans have not been explored. The aim of our study is to evaluate the correlation of blood miR-210 with clinical findings in acute ischemic stroke. Blood samples were obtained from stroke patients (n=112) and healthy controls (n= 60). MiR-210 was measured at within 3, 7 and 14 days after stroke using a quantitative PCR technique. Stroke severity and clinical outcome were evaluated by NIHSS and modified Rankin Score. Both blood and brain miR-210 in ischemic mice was examined and the correlation was investigated. Compared to healthy controls, blood miRNA-210 was significantly decreased in stroke patients (0.93 vs. 1.36; P=0.001), especially at 7 days (0.56 vs. 1.36; P=0.001) and 14 days of stroke onset (0.50 vs. 1.36; P=0.001). The cut off point of miR-210 in diagnosis was 0.505 with 88.3% sensitivity. MiR-210 level in stroke patients with good outcome was significantly higher than patients with poor outcome (1.2 vs. 0.44; P=0.012). The correlation between blood and brain miR-210 in ischemic mice was positive (R^2=0.57, P=0.001). Blood miR-210 is a novel sensitive biomarker for clinical diagnosis and prognosis in acute cerebral ischemia.

2. INTRODUCTION

Insufficient blood flow to the brain, known as brain ischemia, leads to poor oxygen supply and consequential death of brain tissue with a wide range of pathophysiological processes. MicroRNA (miRNA), a large conserved family of noncoding short RNAs, is a novel therapeutic tool for cerebral ischemia injury. Evidence demonstrated that miRNAs involve in a variety of physiological and pathological cellular processes, including cell proliferation, differentiation, apoptosis, neuronal development, and injured tissue repairing and remodeling (1-3). The length of miRNAs is only 18-25nts and readily cross the blood-brain barrier (BBB) to the ischemia area through vector (4-5). MiRNA can hybridize to partially complementary binding sequences that are typically localized in the 3'untranslated regions (3'UTR) of target mRNAs, resulting in either degrading mRNAs or inhibiting protein translation (6). At present, about 800 human miRNAs have been detected and they may regulate 1/3 human mRNA transcripts. One miRNA can regulate multiple targets (7). As a biomarker, miRNA is relatively stable and detectable in peripheral blood (8). Selected blood miRNA may correlate with miRNA changes in the brain; blood miRNA may reflect ischemic injury and repair in brain (9-10). MiRNAs may provide a useful blood

biomarker in diagnosis and prognosis and have a promising function therapy of stroke (11-19).

MiRNA-210 (miR-210), a master and pleiotropic hypoxiamir, is activated by hypoxia inducible factor-alpha for the hypoxic induction (20). It is the only miRNA that is up-regulated under hypoxia in several cell types *in vitro* (21-25). Over-expression of miR-210 is sustained though the change of osmotic stress, growth factor deprivation, acidosis and oxidative stress (26). Studies have identified approximately 35 specific transcripts as direct targets of miR-210 in many cellular processes (21, 27-28), which include angiogenesis via EFNA3 (26), ELK3; migration/adhesion via HOXA3, APC, CLASP2, P4HB, PTPN1, MDGA1, and MIDI1P1; protein modification/transports via PTAR1and TNPO1; chromosome structure/inactivation via CBX1, XIST, NIPBL, and SMCHD1; nucleic acid binding via RAD52, CPEB2; development via HOXA1, HOXA3; mitochondrial/metabolism via ISCU, ATP11C (22-23, 25); differentiation via EFNA3, BDNF, ACVR1b (26, 29); cell survival/proliferation via ACVR1β, E2F3, MNT.

MiR-210 plays multiple crucial roles in the cellular regulation in response to low oxygen including ischemic brain injury. Recent study suggested that miR-210 was expressed in both brain and blood of rat middle cerebral artery occlusion (MCAO) model (9-10). However, miR-210 regulation and function in stroke patients are not explored. The aim of our study is to evaluate the relationship between blood miR-210 and clinical findings in patients with acute ischemic stroke. We expect miR-210 as a novel blood biomarker for the diagnosis and prognosis in clinical acute cerebral ischemia. The study may be also useful to establish the protocol and evaluate the effect of miRNA treatment in future.

3 MATERIALS AND METHODS

All studies involving patients were approved by the Institutional Review Board of the Shanghai Jiao Tong University, Shanghai, and patients gave informed consent. Procedures for the use of laboratory animals were approved by the University's Institutional Animal Care and Use Committee, Shanghai, China.

3.1. Study subjects

Patients with acute ischemic stroke were recruited from the department of neurology in Ruijin Hospital, Shanghai, China. They were evaluated and entered into an ongoing prospective registry. A routine examination was performed, which includes blood glucose, lipid, homeostasis function, blood pressure, ECG, cervical Doppler vascular ultrasonography, cardiac ultrasonography, MRI and MR angiography. The diagnosis of acute ischemia stroke was made based on patient history, neurological deficit, lab examination, MRI diffusion feature and MRA result. Patients with history of tumor, acute infectious disease, immunity disease, blood disease, renal or liver failure or recurrent stroke were excluded. We collected the blood samples available from September 2009 to June 2010 for analysis. According to TOAST criteria, the study subjects were classified by etiologic diagnosis as large artery atherosclerotic stroke (LAAS), cardio-embolic infarct (CEI), small artery lacunar stroke, other special etiologies or undetermined etiology. Based on Oxford Community Stroke Project (OCSP) criteria, they were classified as total anterior circulation infarct (TACI), partial anterior circulation infarct (PACI), posterior circulation infarct (POCI) or lacunar cerebral infarct (LACI). The gravity and the outcome were further evaluated by National institutes of health scales score (NIHSS) and modified rankin score (mRS. Vascular risk factors including hyperglycemia, hypertension, hyperlipidemia, atrial fibrillation, coronary artery disease and myocardial infarction were recorded on the basis of the international standard criteria. Control blood samples were obtained from healthy volunteers. The mean age is matched with the study group.

3.2. Mouse model of permanent middle cerebral artery occlusion (pMCAO)

Adult male ICR mice (Sileike, Shanghai, China) weighing 25-30 g body weight were used for pMCAO. The method for mouse pMCAO model was described previously (30). Briefly, mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). The rectal temperature was maintained at 37±0.5□ with a heating pad. Left MCA was permanently occluded by a 6-0 monofilament nylon suture through the external carotid artery into the internal carotid artery to the origin of MCA. The success of MCAO was confirmed by the measurement of surface cerebral blood flow in the ipsilateral hemisphere. Sham group was performed with the same surgical procedure except the inserting suture.

3.3. Blood sample collection

Blood sample of patients (4 ml) was collected into EDTA containing tube (BD Vacutainer, Plymouth, UK) by venipuncture on the administration day. The blood sample was fractionated by centrifuging at 1500 *g* for 10 minutes at room temperature. Red blood cells were lysed by red blood cell lysis buffer and discarded; the leucocytes were collected and immediately processed for the total RNA extraction. Blood of mice (200 μl) was collected into EDTA containing tube at 1, 7, and 14 days after MCAO in mice and immediately prepare the total RNA extraction by the TRIzol LS reagent.

3.4. Brain tissue sample collection

The MCAO mice were sacrificed at 1, 7, and 14 days after ischemia (n=3 per group). The brain was divided into ipsilateral and contralateral hemispheres. The ischemic cortex then was transferred into a test tube containing TRIzol reagent on ice.

3.5. RNA extractions

Total RNA in blood or brain tissue was isolated using TRIzol or TRIzol LS reagent (Invitrogen, Carlsbad, USA) according to the manufacture's protocol. The integrity and concentration of total RNA were quantified using a NanoDrop 1000 spectrophotometer (Thermo

Table 1. Demographic and clinical characteristics in acute ischemic stroke patients and normal controls

	Healthy control	Acute ischemic stroke			
		<3days	7days	14 days	Total
Sample size	60	55	31	26	112
Race (yellow)	100%	100%	100%	100%	100%
Ethnicity (Han)	100%	100%	100%	100%	100%
Age (years)	67(64,73)	67(57,74)	69(53,76)	68(56,78)	68(57,76)
Gender (M/F)	31/29	37/18	23/8	18/8	78/34
Hypertension	-	81.8%	80.6%	88.4%	83.0%
Diabetes	-	14.9%	21.4%	16%	17%
Hyperlipidaemia	-	50%	43.3%	48%	47.6%
Cardiac disease	-	3.6%	6.5%	7.7%	5.4%
NIHSS score	-	5 (0, 10)	5 (1,7)	5 (3,10)	5 (1,10)
mRS score >2	-	30 (54.5%)	19 (61.3%)	20 (76.9%)	69 (61.6%)
≤2	-	25 (45.5%)	12 (38.7%)	6 (23.1%)	43 (38.4%)
TOAST LAAS	-	22 (40%)	13 (41.9%)	15 (57.9%)	50 (44.6%)
CEI	-	2 (3.6%)	2 (6.5%)	2 (7.7%)	6 (5.4%)
Lacunar	-	31 (56.4%)	16 (51.6%)	9 (34.6%)	56 (50.0%)
OPCS TACI	-	2 (3.6%)	2 (6.5%)	2 (7.7%)	6 (5.4%)
PACI	-	18 (32.7%)	10 (32.3%)	11 (42.3%)	39 (34.8%)
POCI	-	19 (34.5%)	9 (29.0%)	3 (11.5%)	31 (27.7%)
LACI	-	16 (29.1%)	10 (32.3%)	10 (38.5%)	36 (32.1%)

Scientific, Utah, USA). Samples with OD260/280 ratio in the range of 1.7-2.0 are used.

3.6. Reverse transcriptions (RT)

Using a specific miR-210 and endogenous control U6 stem-loop primer, reverse transcription was performed according to the manufacture's protocol of the TaqMan miRNA RT Kit (Applied Biosystems, Foster City, USA). A total RNA (10 ng) was reverse transcripted to cDNA with 1mM dNTPs (with dTTP), 50 U Reverse Transcriptase 1 μl, 4 U RNase inhibitor in the presence of specific miRNA-210 or U6 stem loop reverse transcriptase primers in a 15 μl system buffered by RT Buffer and DEPC water, following the thermal cycle program of 16□ for 30 minutes, 42□ for 30 minutes and 85□ for 5 minutes, cDNA was stored at -20□.

3.7. Real time quantitative PCR

The real time quantitative PCR was performed by a fast real-time PCR system (7900HT, ABI, USA) using a TaqMan® miRNA assay kit. The reaction volume is 20 μl containing the components as listed: miRNA-210 or U6 RT reaction product (1.33 μl), 20×TaqMan® MicroRNA assay (miRNA-210 or U6) 1 μl, TaqMan 2×universal PCR master mix 10 μl, DEPC water 7.67 μl. A 96-well plate was then running following the protocol as 95□ for 10 minutes, followed by 43 cycles of 95□ for 15 seconds and 60□ for 1 minute. Finally, the relative miR-210 level was normalized to the endogenous control U6 expression for each sample in triplicate and was calculated by the $2^{-\square Ct}$ method (31).

3.8. Statistical data analysis

Data was first examined to definite normal distribution or not using Kolmogorov-Smirnov test. For the data with a non-normal distribution, the comparison of two independent groups was analyzed by nonparametric Mann-Whitney U test. Spearman correlation between two variables was performed. The data was presented as median (lower Quartile; upper Quartile). Conversely□the normal distribution data was analyzed using the student's *t*-test and Pearson's correlation. Data was presented as mean±SD. Cut off points were determined by receiver operator characteristic (ROC) analysis. The sensitivity and specificity of miRNA, the areas under curve (AUC) and the 95% confidence intervals were calculated. Pearson ChiSquare was analyzed for categorical variables. Statistical data analysis was performed by SPSS 17.0 software. The value $P<0.05$ was considered as significant.

4. RESULTS

4.1. Clinical Characteristics

The clinical characteristics of patients and normal controls are shown in Table 1. The median age and race/ethnicity were not different (P = 0.401) between the stroke patients and the controls. A greater percentage of risk factors in stroke patients was found including hypertension 83%, diabetes 17%, hyperlipidemia 47.6% and cardiac diseases 5.4%. Among stroke patients, according to TOAST classification, 112 were classified as LAAS 50, CEI 6 and Lacunar 56. A smaller amount of CEI is because that these patients were triaged to the department of cardiology in our hospital. According to OPCS classification, 112 were classified as TACI 6, PACI 39, POCI 31, LACI 36 based on MRI imaging. Based on the time point of blood samples collection, 112 were divided as <3 days (n=55), 7 days (n=31), 14 days (n=26). There is no difference in TOAST subtype, OPCS subtype, NIHSS score and mRS score (P=0.457, 0.535, 0.274, 0.154) at the different time points among the patients, respectively.

4.2. Changes of blood miR-210 in acute ischemic stroke patients

To investigate whether miR-210 is involved in acute stroke, we first examined blood miR-210 levels in patients (n=112) and controls (n=60). We found that the blood miR-210 was decreased in acute ischemic stroke compared to the controls [Figure 1A 0.93 (0.26, 1.94) vs. 1.36 (0.79, 2.85), P=0.001]. ROC analysis showed that the diagnostic cut off point of miR-210 was 0.505 [Figure 1B AUC 0.659 (0.578, 0.740), P=0.001]. The blood miRNA-210 level <0.505 had 88.3% sensitivity and 41.1% specificity for the onset of stroke.

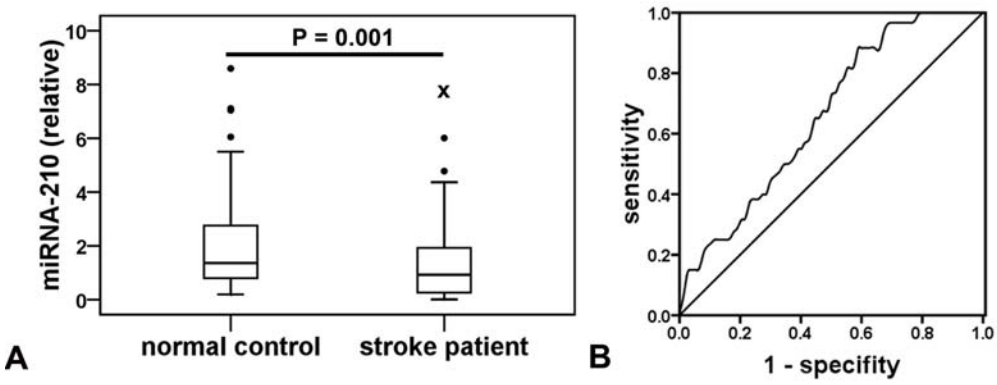

Figure 1. Changes of blood miR-210 in acute ischemic stroke patients. (A) Blood miRNA-210 level is down-regulated in stroke patients compared with the healthy control. (B) ROC curve analysis demonstrates that the cut off point for the diagnosis of stroke is 0.505 with 88.3% sensitivity and 41.1% specificity. Area under the curve is 0.659 (95%CI 0.578, 0.740; P=0.001).

To explore the dynamic changes during stroke, we further analyzed blood miR-210 levels at < 3 days (n=55), 7 days (n=31) and 14 days (n=26) after stroke. Compared to the controls [1.36 (0.79, 2.85), n=60], blood miR-210 decreased gradually at < 3 days [1.14 (0.37, 2.55), P=0.069], 7 days [0.56 (0.12, 1.71), P=0.001] and 14 days [0.50 (0.22, 1.61), P=0.001], respectively (Figure 2).

4.3. The relationship between blood miR-210 and the outcome of ischemic stroke

To examine the relationship between blood miR-210 and the outcome of ischemic stroke, we compared blood miR-210 level in patients with good outcome (mRS score ≤2, n=43) and poor outcome (mRS score >2, n=69). We found that miR-210 level was higher in stroke patients with good outcome than those with poor outcome [Figure 3A, 1.2 (0.56, 2.36) vs. 0.44 (0.16, 1.57), P=0.012]. The ROC analysis showed that its prognostic cut off point is 0.46 [Figure 3B, AUC 0.642 (0.539, 0.744), P=0.012]. Blood miR-210 < 0.46 suggested a poor outcome after stroke (sensitivity 83.7% and specificity 50.7%).

4.4. The relationship between blood miR-210 and ischemic stroke subtype

To evaluate the relationship between blood miR-210 and the stroke subtype, we compared miR-210 level in patients with different TOAST types (Figure 4A) and different OPCS types (Figure 4B). We demonstrated that there were no changes of miR-210 level in TOAST types such as LAAS [0.87 (0.19, 2.08), n=50], CEI [1.05 (0.37, 1.60), n=6] and small artery lacunars stroke [median 0.93 (0.30, 1.95), n=56], (P=0.929). Similarly, we found that there were no changes of miR-210 level in different OPCS types including TACI [0.65 (0.11, 1.54), n=6], PACI [1.2 (0.18, 2.44), n=39], POCI [1.14 (0.44, 2.66), n=31] and LACI [0.49 (0.24, 1.34); n=36], (P=0.165).

4.5. The relationship between blood miR-210 and brain miR-210 in pMCAO mouse

To examine the correlation of miR-210 level in blood and brain tissue, we performed pMCAO mice model to obtain both blood and brain tissue samples in the same animal. Compared to the control, the changes of miR-210 in blood and ischemic hemisphere were parallel at 1, 7 and 14 days after MCAO. MiR-210 was elevated after one day of MCAO, and gradually decreased after 7 and 14 days of MCAO (Figure 5A). The significant positive correlation was found between the blood miR-210 and brain miR-210 (Figure 5B R^2=0.57, P=0.001).

5. DISCUSSION

In present study, we examined blood miR-210 in acute ischemic stroke patients. We have demonstrated for the first time that 1) blood miR-210 expression gradually decreases in patients at < 3 days, 7 days and 14 days; 2) miR-210 level is correlated to the outcome of stroke: the higher miR-210 level, the better outcome; 3) miR-210 is not correlated to TOAST/OPCS classification. In addition, animal experiment demonstrated that blood and brain miR-210 level is parallel, suggesting the changes of blood miR-210 may represent changes in brain miR-210. Our findings provided evidence that blood miR-210 is a novel biomarker for ischemic stroke diagnosis and prognosis.

The pathologic processes of stroke can be separated into early and late stages. During early stage, ROS, excited amino acid and pro-inflammatory mediators are released within hours after ischemia, and further lead to the BBB disruption, brain edema, neuronal death and apoptosis within hours to days (32). Brain angiogenesis, neurogenesis and neurovascular remodeling begin about 7 days after stroke and are further detectable at 14 days (33). Evidence suggested that miR-210 has the anti-apoptosis and pro-angiogenesis properties (12, 21). We hypothesized that miR-210 involved in both early and late stages of acute ischemic injury. Our study demonstrated that blood miR-210 level decreased significantly in patients compared with controls, especially at 7 and 14 days after stroke. The dynamic blood and brain miR-210 level were detected in our pMCAO mouse model. MiR-210 expression was initially elevated at 1 day after ischemia in both blood and brain, and decreased gradually at 7 and 14 days. The animal data agrees with the temporal changes of miR-210 in reported cell culture results, which suggested that miR-210 was elevated immediately in response to hypoxia and

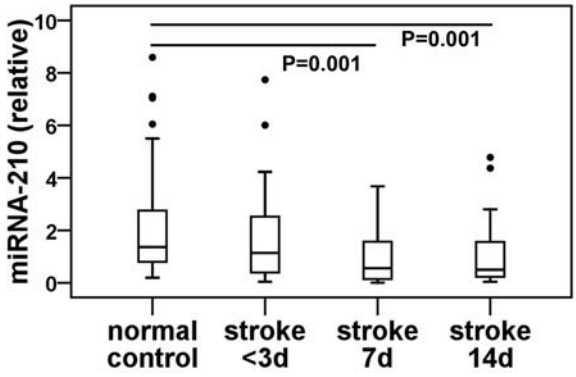

Figure 2. Blood miR-210 level is significantly reduced in ischemia stroke patients compared to healthy normal controls in the first 7 days and 14 day following stroke.

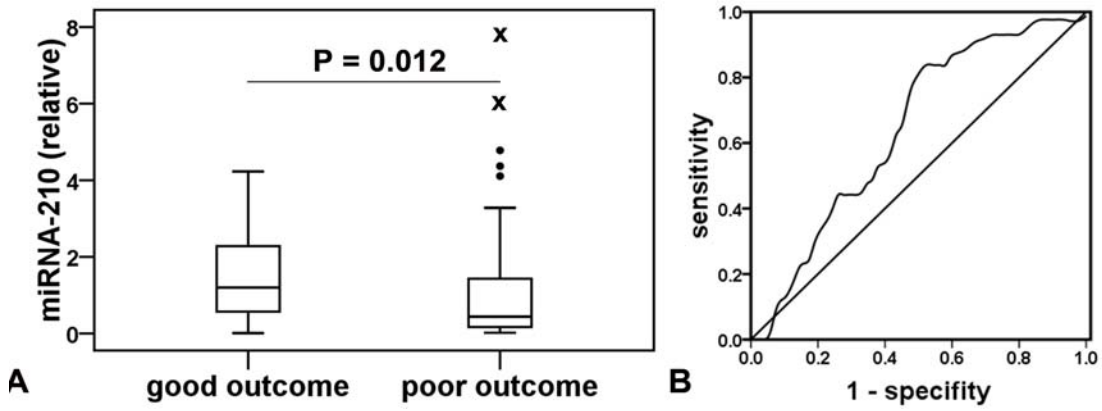

Figure 3. The relationship between blood miR-210 and the outcome of ischemic stroke. (A) Blood miRNA-210 level is significantly elevated in ischemia stroke patients with good outcome compared to those with poor outcome. (B) ROC curve analysis demonstrates that the cut off point for the prognosis of stroke is 0.46 with 83.7% sensitivity and 50.7% specificity. Area under the curve is 0.642 (95%CI 0.539, 0.744; P=0.012).

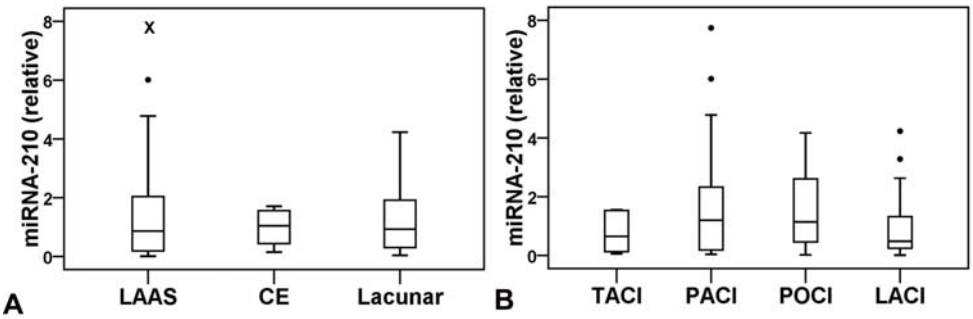

Figure 4. The relationship between blood miR-210 and ischemic stroke subtype. (A) Blood miRNA-210 level is not regulated in ischemia stroke patients with different TOAST subtype (P=0.929). (B) Blood miRNA-210 level is not regulated in ischemia stroke patients with different OPCS subtype (P=0.165).

subsequently decreased several days later (26). The unmatched miR-210 level during acute early phase between patients and mice is possibly due to the relative wide time window in patients. The concordance of lower miR-210 levels at 7 and 14 days between patients and animal suggested that the repair function of miR-210 was diminished at late stage after ischemia. Therefore, supplementing miRNA as potential therapy may foster better repair of cerebral ischemic injury. While stroke patients in our cohort had more clinical risk factors such as hypertension, diabetes and heart disease, that may potentially confound the results, the concordance of

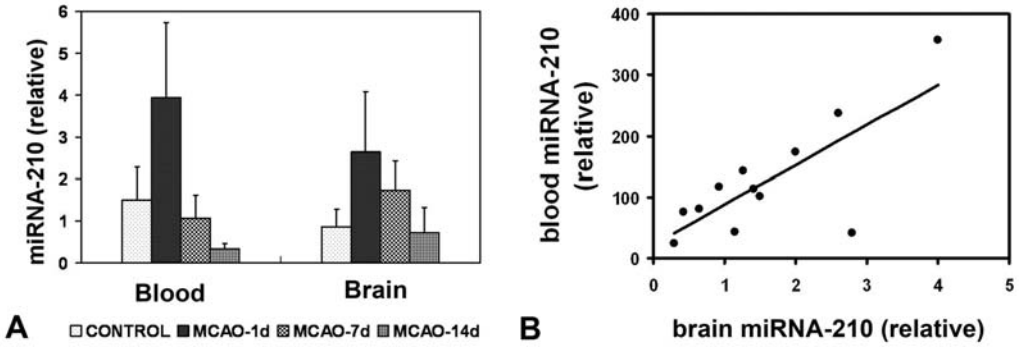

Figure 5. Blood and brain miR-210 in pMCAO mouse. (A) Both blood and brain miR-210 levels were elevated at 1 day after ischemia, and gradually decreased at 7 days and 14 days. (B) The positive relation between blood miRNA-210 and ischemic cortex miRNA-210 level was detected (R^2=0.57, P=0.001).

temporal profile of MiR-210 in both human blood and animal models strongly support its direct role in ischemic progression and repair. The ROC curve analysis suggested that miR-210 had a high sensitivity of 88.3% for the diagnosis of stroke in patients. It is a useful blood biomarker for the diagnosis of stroke. Clinically, the diagnosis of acute ischemic stroke is not difficult because of the developing neuroimaging technique especially MRI. However, it is too expensive for wide use. Developing a non-invasive high sensitive blood biomarker to screen and to follow up the brain ischemia patients is feasible and valuable.

Evidence suggests that miR-210 has a neuroprotection and restoration feature (12, 26). We demonstrated that blood miR-210 level was higher in stroke patients with a good outcome than those with a poor outcome. High miR-210 level represents a better prognostic and a mild neurological impairment after brain ischemia. The ROC curve analysis suggested that miR-210 had a high sensitivity of 83.7% for the prognosis of stroke. It is a useful blood prognostic biomarker in stroke to predict the outcome of the patients after brain ischemia. In general, most biomarkers, which related to the outcome of stroke, consisted of three main parts: ischemia damage cascades markers, neuronal; glial and axonal damage markers and homeostasis/coagulation markers (34-36). However, the majority of biomarkers reflects only the injury of ischemic brain; few are related with the evaluation of the repair. The outcome of stroke is not only influenced by the reduction of the ischemia damage in early stage of stroke, but also the restorative interventions of ischemic brain such as angiogenesis and neurogenesis in later stage. As a repair related biomarker, miR-210 is useful to evaluate the potential overall recovery of ischemic tissue and to guide the therapy. In addition, this biomarker in nucleic acid level was possibly more sensitive and comprehensive in stroke than protein biomarkers because of its multi-target property and small size (37).

According to TOAST/OPCS criteria, stroke patients can be divided into subtypes based on different etiology and lesion position. However, no changes of blood miR-210 level were found among different stroke subtype patients, suggesting that miR-210 was not related in the pathological process of the emboli, thrombus and atherosclerosis formation. Though miR-210 is tissue specific and conserved, the samples origin from blood can not distinguish the different positions of brain ischemia lesion.

In our study, a significant positive correlation was detected between blood and brain miR-210 in MCAO mice model. The result demonstrated that by detecting blood miR-210 level in patients, we can monitor the brain miR-210 change and evaluate injury and repair of the ischemic brain tissue. Recent data suggested that miR-210 may be a new promising molecule for the restoration of ischemic injury and might be useful for stroke therapy in the future. However, the clinical application of miRNA requires a stable and easily obtained parameter to evaluate and monitor the therapy efficacy. Non-invasive blood biomarkers are crucial as clinical exam maybe subjective and neuroimaging may not be feasible as it poses more risk in moving gravely ill patients to the scanners. It is challenging to find out a stable blood biomarker. Our study suggested that blood miR-210 can reflect the changes in brain. If the management of miR-210 for stroke launches, the time, dose and effect of drug administration may be monitored by blood miR-210. In addition, *in vitro* and *in vivo* experiment demonstrated that blood circulating miRNA is extraordinary stable. Neither acid nor heat can change its level. The temporal profile and the correlation between blood and brain miR-210 in stroke patient and pMCAO mouse model established by our experiments contribute to the establishment of a protocol and to the evaluation of the effect of miRNA treatment in the future.

In summary, blood miR-210 in acute ischemic stroke patients is not only useful in diagnosis and prognosis, but also in prediction of response of stroke patients to therapy. The further study of miR-210 and other miRNAs may shed new light in stroke therapy and management.

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Abbreviations: AUC, area under the curve; CEI, cardio-embolic infarct; LAAS, large artery atherosclerotic stroke; LACI, lacunar cerebral infarct; MCAO, middle cerebral artery occlusion; MRS, modified rankin score; NIHSS, national institutes of health scales score; OCSP, Oxford community stroke project classification; PACI, partial anterior circulation infarct; POCI, posterior circulation infarct; ROC, receiver operator characteristic; TACI, total anterior circulation infarct; TOAST, Trial of Org 10172 in Acute Stroke Treatment

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