Identification of miRNA-21 and miRNA-24 in plasma as potential early stage markers of acute cerebral infarction

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Abstract. This study investigated the role of microRNA-21 (miR-21) and microRNA-24 (miR-24) in the pathological processes that follow cerebral ischemic injury and examined the potential use of miR-21 and -24 in stroke diagnostics as sensitive plasma biomarkers. An oxygen-glucose deprivation (OGD) model was constructed using mouse N2A neuroblastoma cells (N2A). Western blot analysis and quantitative polymerase chain reaction (qPCR) were employed to detect protein and miRNA expression levels. miR-21 and miR-24 were analyzed in the plasma from 68 patients with acute cerebral infarction (ACI) and 21 healthy individuals. In the present study, it was identified that plasma miR-21 and miR-24 were lower in ACI patients than in the controls ($P<0.05$). A positive correlation was demonstrated between plasma miR-21 and miR-24, and a negative correlation was revealed between miR-21, miR-24 and the National Institutes of Health Scales Score (NIHSS) within the first day following stroke. In addition, the expression of miR-21 and miR-24 was upregulated by 3.3- and 4.9-fold, respectively, when the reoxygenation time persisted up to 24 h following 3 h of OGD. The expression of Bcl-2 was upregulated following gain of miR-21 function, while X-linked inhibitor of apoptosis protein (XIAP) was downregulated after gain of miR-24 function in N2A cells. The data suggested that miR-21 may have an antiapoptotic effect in N2A neuroblastoma cells following OGD and reoxygenation, while miR-24 may have a pro-apoptotic effect. Therefore, these microRNAs may be potential therapeutic targets for the treatment of post-ischemic injury and may act as diagnostic markers during the early stage of ACI.

Introduction

MicroRNAs (miRNAs), that are abundantly expressed in the brain, have emerged as key mediators of post-transcriptional gene silencing in ischemic stroke biology (1). Several studies investigating successfully implemented miRNA-based therapeutics have provided new treatment strategies for ischemic stroke, by means of regulating large sets of genes in numerous associated pathways (1).

Tissue-specific miRNAs have been demonstrated to serve as diagnostically sensitive plasma biomarkers in tissue injury (2). A number of miRNAs, including miR-124 and miR-210, have been investigated for their role as potential diagnostic markers in cerebral ischemia (3,4). It has been reported that miR-21 and miR-24 are upregulated in stroke patients (5), and that they can also be detected in the brain of rats subjected to middle cerebral artery occlusion (MCAO), a common animal model in cerebral ischemia (CI) research (6)

Overexpression of miR-21 protects against ischemic neuronal death by downregulating FASLG (7). miR-24 suppresses cardiomyocyte apoptosis, in part by directly repressing Bim, a novel member of the Bcl-2 family, which positively regulates apoptosis (8). miR-23a is part of the same cluster as miR-24 and by regulating X-linked inhibitor of apoptosis protein (XIAP), contributes to sex differences in responses to cerebral ischemia (9). Furthermore, miR-497 promotes ischemic neuronal death by negatively regulating anti-apoptotic proteins, Bcl-2 and Bcl-w (10), and Bcl-2 is also a potential target of miR-21 (11,12)

In the present study, it was hypothesized that miR-21 and miR-24 have important roles in ischemic stroke, but the mechanism underlying this effect, whether it involves targeting Bcl-2, Bim and XIAP, remains unclear. Furthermore, whether these miRNAs may serve as non-invasive plasma biomarkers has not been investigated. In the present study, the difference of plasma miR-21 and miR-24 in acute cerebral infarction (ACI) and healthy controls was investigated, and the correlations between plasma miR-21, miR-24 and NIHSS scores were analyzed. Furthermore, we investigated the expression of miR-21, miR-24 and apoptosis/autophage-associated proteins in N2A cells following oxygen-glucose deprivation (OGD) and reoxygenation, and the effects on protein Bcl-2, XIAP following the induction of a gain of function in miR-21 and miR-24.

Materials and methods

Study population and ethics. A total of 68 patients with ACI participated in the study, with 21 healthy individuals used

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as the controls. Patients with ACI were recruited from the Department of Neurology at Peking University Third Hospital (Beijing, China). The methods were approved by the local ethics committee of Peking University Third Hospital and all study participants signed informed consent forms. The diagnosis of ACI was conducted based on patient history, lab examination, neurological deficit, magnetic resonance imaging (MRI) and magnetic resonance angiography (MRA) results. Patients with a history of tumor, immune disease, blood disease, acute infectious disease, renal or liver failure were excluded. The severity was further evaluated by NIHSS. Plasma samples were extracted from ethylene diamine tetraacetic acid (EDTA)-containing tubes (BD Biosciences, Franklin Lakes, NJ, USA) and stored at -80˚C. Hemolysis may affect the result, so the sample hemolysis can not be used. The first samples were collected within 24 h following the patients' admission to the hospital and the control plasma samples were obtained from healthy volunteers. The mean age of the control is matched with that of ACI patients.

N2A cell culture and OGD. N2A cells were purchased from the cell resource center of Peking Union Medical College (Beijing, China). An OGD model was utilized to mimic ischemic-like conditions *in vitro* (10) and a Bio-Bag was used to produce anaerobic environment.

Gain of miR-21 and miR-24 function. N2A cells were plated in 6-well plates and treated with miR-21 or miR-24 mimics, inhibitor and no template control (NTC). The cells were harvested following 24 or 48 h to detect miRNA or protein expression levels.

Western blotting. Total protein was isolated from N2A cells. Samples of 30-60 μg protein were subjected to 10-12% SDS/polyacrylamide gel (SDS/PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (0.45 μm). The membranes were blocked in 5% skimmed milk (dissolved in 1X TBS Tween-20 buffer) for 1 h at room temperature. Following this, the membrane was incubated with the primary antibody overnight at 4˚C then washed with TBST three times at 10 min intervals, followed by incubation with secondary antibody at room temperature for 2 h and another three washes with TBST. The membrane was scanned with LI-COR Odyssey (800 nm; LI-COR, Inc., Lincoln, NE, USA). All primary antibodies were purchased from Cell Signaling Technology, Inc. (CST, Boston, MA, USA; 1:1,000 in 5% BSA). Mouse anti-GAPDH antibodies were purchased from CoWin Biotech Co., Ltd. (Beijing, China; 1:5,000 in 5% BSA). The secondary fluorescein-conjugated antibody was purchased from LI-COR, Inc. (1:10,000, 5% skimmed milk).

RNA isolation. Total RNA was isolated from N2A cell culture and plasma by TRIzol and TRIzol LS (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA; no. 15596-026, no. 10296-028) respectively, according to the manufacturer's instructions. In particular, the EDTA anticoagulant plasma was separated by centrifugation for 10 min at 3,625 x g, then 750 μl TRIzol LS was added to 250 μl plasma followed by 5 min incubation at room temperature and 10 μl cel-mir-39 (1 μM) was added to each sample as an internal calibrator (13). The miRNA sequences are summarized in Table I.

Table I. miRNA sequences.

miRNA	Sequence
hsa-mir-21-5p	UAGCUUAUCAGACUGAUGUUGA
hsa-mir-24-3p	UGGCUCAGUUCAGCAGGAACAG
cel-miR-39	UCACCGGGUGUAAAUCAGCUUG

Reverse transcription (RT) and quantitative (q)PCR. Total RNA extracted from N2A cell cultures was reversely transcribed using TaqMan miRNA RT kit (Applied Biosystems, Carlsbad, CA, USA). PCRs were then conducted using the TaqMan® miRNA assay kit (Applied Biosystems). The relative miRNA levels were normalized to endogenous U6 expression for each sample (10). RT primers were dissolved in nuclease-free water and mixed in equal concentration to generate a RT primer mix. RT of plasma RNA was conducted according to the manual for the reverse transcription system (RevertAid First Strand cDNA Synthesis kit, no. 1621). qRT-PCR was performed using the SYBR Green PCR Master Mix kit (CWBIO, no. CW0956) and was processed in 96-well plates on a 7500HT analyzer (Applied Biosystems). Expression values were normalized using the mean threshold cycle (Ct) obtained from the spiked-in controls cel-mir-39 (calculation formula: 2 exp (mean Ct spiked-in controls - Ct target miRNA) and log transformed (14).

The TaqMan® miRNA assay (Applied Biosystems) uses gene-specific stem loop reverse-transcription primers and TaqMan® probes to detect mature miRNA transcripts. This method is highly specific but very expensive. So, we detected plasma miRNA using SYBR qRT-PCR (15-17), which produced a good amplification and melting curve (data not shown). Table II summarizes the sequences of RT and PCR primer used.

Statistical data analysis. Statistical analysis was performed by SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). For the data with a non-normal distribution, the comparison of two independent groups was analyzed by non-parametric Mann-Whitney U test. Spearman correlation between the two variables was performed. A value of $P<0.05$ was considered to indicate a statistically significant result. The data with a non-normal distribution was presented as the median value (lower quartile; upper quartile). The relative plasma miRNA was presented as box-and-whisker plots. The normal distribution data was analyzed using the Student's t-test. Quantitative data for the cells were expressed as the mean ± SD based on at least three separate experiments of triplicate samples. ImageJ software was used to analyze selected bands in the western blot analysis. Differences among groups were statistically analyzed by the one-way analysis of variance (ANOVA) test.

Results

Patient clinical characteristics. Table III summarizes the demographic and baseline clinical characteristics of the patients that participated in the study. The median age was not different ($P=0.095$) between the stroke patients and the controls. However, the stroke patients exhibited an increased percentage

Table II. Sequences of RT primers and PCR primers.

miRNA	RT primer	F/R	PCR primer
has-mir-21-5p	***TCAACA	F	GCCGCTAGCTTATCAGACTGA
		R	GTGCAGGGTCCGAGGT
has-mir-24-3p	***CTGTTC	F	GGTGGCTCAGTTCAGCAG
		R	GTGCAGGGTCCGAGGT
cel-miR-39	***CAAGCT	F	GCGTCACCGGGTGTAAATC
		R	GTGCAGGGTCCGAGGT
U6	CGCTTCACGAAT	F	GCTTCGGCAGCACATATACTAAAAT
	TTGCGTGTCAT	R	CGCTTCACGAATTTGCGTGTCAT

***mean sequence of 'GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC'. RT, reverse transcription; F, forward; R, reverse.

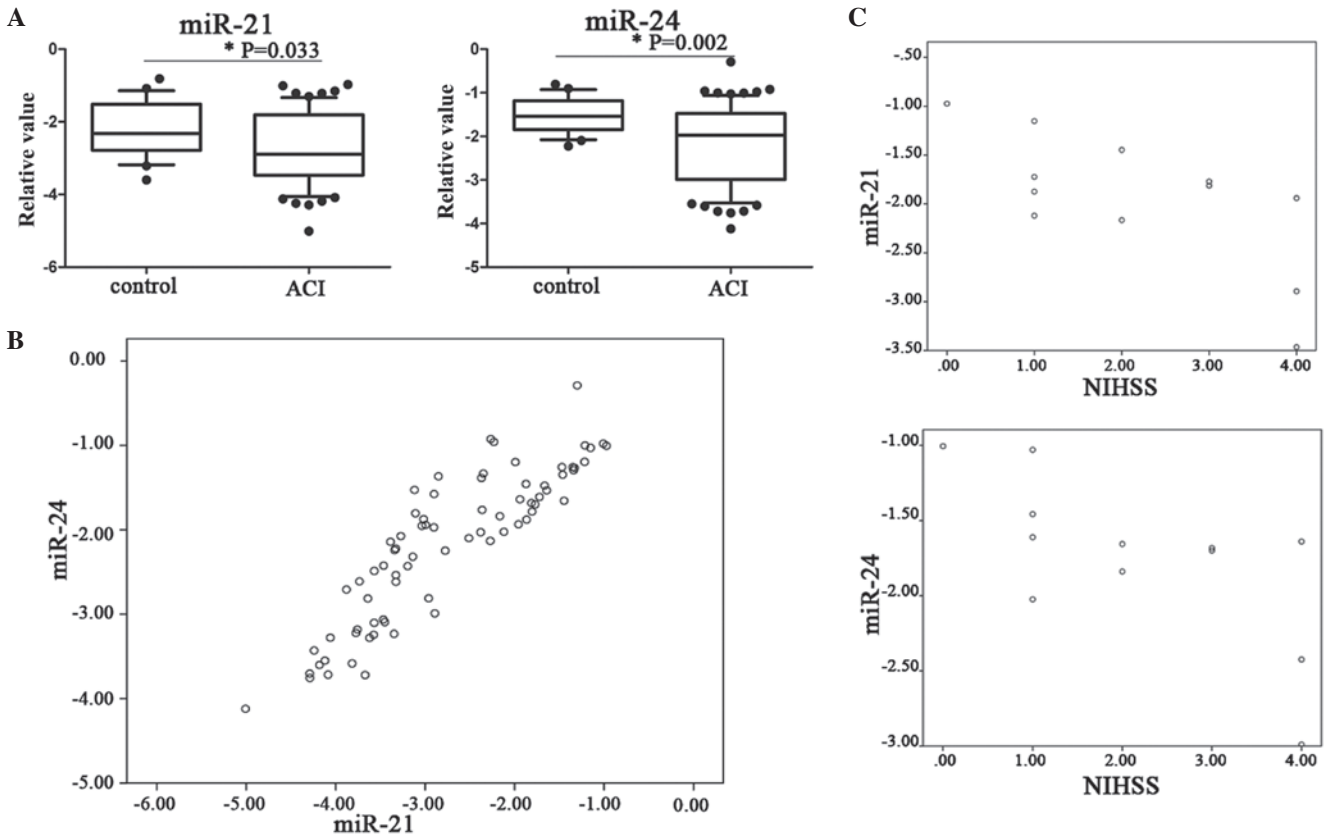

Figure 1. (A) Changes in plasma miR-21 and miR-24 in acute ischemia stroke patients. Plasma miR-21 and miR-24 levels are downregulated in ACI patients compared with the healthy control (*P<0.05). The line within each box denotes the median. The horizontal borders of each box denote the 25th and 75th percentiles and the limits of the vertical lines ('whiskers') denote the 10th and 90th percentiles. (B) The expression of miR-21 and miR-24 was positively correlated in the plasma of ACI. r=0.884, P<0.01; the value P<0.01 was considered as significant. (C) The expression levels of miR-21 and miR-24 were negative related to NIHSS within first day of onset. r=-0.703, P=0.011; r=-0.694, P=0.012; the value P<0.05 was considered as significant. NIHSS, National Institutes of Health Scales Score; ACI, acute cerebral infarction.

of the risk factors, compared with the controls, including hypertension 54.4%, diabetes 17%, hyperlipidemia 11.7% and history of cardiovascular and cerebrovascular disease 24%.

Expression of miR-21 and miR-24 in plasma of stroke patients. In the present study, it was not possible to use any specific miRNA or set of miRNAs as endogenous controls in plasma/serum (18), so synthesized cel-miR-39 (40 pmol/l) was added, as internal calibrators, as described previously (13).

To investigate whether miR-21 and miR-24 are involved in acute ischemic stroke, plasma miR-21 and miR-24 levels were examined in the patients and controls. It was identified that plasma miR-21 and miR-24 levels were decreased in acute ischemic stroke compared with the controls (P<0.05; Fig 1A).

It was identified that plasma miR-21 and miR-24 levels were positively correlated (r=0.884, P<0.01;Fig. 1B). To examine the associations between plasma miR-21 and miR-24 and the outcome of ACI, the correlations between the expression of miR-21, miR-24 and NIHSS score were examined. We identified a significant negative correlation within the first day after onset (r=-0.703, P<0.05; r=-0.694, P<0.05, respectively; Fig 1C).

miR-21 and miR-24 are upregulated in N2A following OGD and reoxygenation. In the present study, the change in plasma levels of miR-21 and miR-24 were observed, we then investigated their change in cells. The expression levels of miR-21 and miR-24 were upregulated ~3.3- and 4.9-fold, respectively, when the recovery time persisted up to 24 h following 3 h of OGD (Fig. 2).

Apoptosis of N2A culture cell is enhanced while autophagy is downregulated following OGD. Caspase-3 cleavage is an indicator of apoptosis. OGD exposure for 3 h does not result in visible caspase-3 cleavage, but the cleaved product became visible following 24 h of reoxygenation. The expression of anti-apoptotic protein XIAP was downregulated as the recovery time prolonged, and the expression of LC3-II and Beclin1 was downregulated, implying a decrease in autophagy (Fig. 3A.)

miR-21 enhances Bcl-2 expression, while miR-24 suppresses XIAP expression. miR-21 targets the Bcl-2 gene, an important anti-apoptotic protein (11,12). In the present study, it was observed that ectopic expression of miR-21 moderately increases the Bcl-2 protein level in N2A cells. Ectopic expression of miR-24 also moderately decreases the expression levels of XIAP (Fig. 3B).

Table III. Demographic and clinical characteristics in acute ischemic stroke patients and healthy controls.

Characteristic	Control	ACI
Sample size	21	68
Age	58 (54,67)	64 (55,76)
Gender (M/F)	10/9	45/23
Hypertension	-	37 (54.4%)
Diabetes	-	17 (25%)
Hyperlipidaemia	-	8 (11.7%)
History of CVD and CVA	-	24 (35.3%)
Smoking	-	18 (26.5%)
Alcohol	-	8 (11.8%)

CVD, cardiovascular disease; CVA, cerebrovascular disease; ACI, acute cerebral infarction; M, male; F, female.

Discussion

The complex and intricate signaling pathways activated by cerebral ischemia are not completely understood, but recent studies have implicated miRNAs as important in the pathological processes that cause ischemic injury (4,7,9,10). miRNA has been identified as a circulating biomarker in numerous diseases (2,19-21), including ischemic stroke (3,4), but whether miR-21 and miR-24 may be utilized as diagnostic biomarkers in cerebral ischemia, has not been investigated.

The results from the present study demonstrated that the differences in plasma miR-21 and miR-24 between ACI and control were statistically significant (P<0.05; Fig. 1A). It was identified that miR-21 and miR-24 were positively correlated, which suggests their release may be stimulated at the time of ischemic insult, or occur following the resultant cerebral injury (Fig. 1B).

It is well established that the integrity of the blood-brain barrier (BBB) is disrupted in stroke (22), which provides an opportunity for miRNA to be released into the plasma. Stroke therapeutics is currently limited by a lack of accurate and reliable blood biomarkers, the identification of which would facilitate early diagnosis and risk prediction. The results from the present study and others alike, have identified that miR-21 and miR-24 may have opposing effects. So, we hypothesized that the ratio of miR-21/miR-24 may be more useful as an indicator of ACI, than just one miRNA alone. However, we identified a positive correlation between miR-21 and miR-24 (Fig. 1B) and a negative correlation between miR-21 and miR-24 with the NIHSS score on first day onset (Fig. 1C), but not the ratio of miR-21/miR-24 (data not shown).

Following this, we continued by investigating the role of these two miRNAs in ischemic stroke. The results demonstrated that miR-21 and miR-24 in the N2A culture cell were upregulated 3.3- and 4.9-fold, respectively, at the 24 h recovery time point following 3 h of OGD. Their change may have important role in the OGD and reoxygenation injury of N2A cells.

Since miR-24 inhibits apoptosis in mouse cardiomyocytes, in part, by direct repression of Bim (8), we investigated whether elevated miR-24 may repress Bim and have a protective role in CI. However, the results identified that the expression of Bim in N2A culture cells was too low to be detected by western blot analysis (data not shown). This result suggests that Bim may have limited involvement in OGD and reoxygenation injury of N2A cells.

miR-23a, encoded by the same cluster as miR-24, which regulates XIAP, contributes to sex differences in response to cerebral ischemia (9). XIAP is also a target of miR-24 (23), so the present study aimed to investigate the role of XIAP in our OGD model and whether miR-24 may be important in CI by directly targeting XIAP. It was identified that the expression of XIAP was reduced during the recovery time extension following 3 h OGD. The expression of XIAP was downregulated following the addition of an miR-24 mimic to the N2A cells, compared with application of an miR-24 inhibitor and NTC. These data indicated that miR-24 may be a pro-apoptotic factor during cerebral ischemia.

Bcl-2, an important anti-apoptotic protein within the intrinsic apoptotic pathway, is one of the potential targets of miR-21. However, it has been identified that miR-21 positively regulated the expression of Bcl-2, which contradicted our existing understanding of the miRNA (11,12). miR-21 is known to be a strong antiapoptotic factor (24, 25) and is neuroprotective against ischemic neuronal death by downregulating FASLG (7). Our results revealed that miR-21 expression was

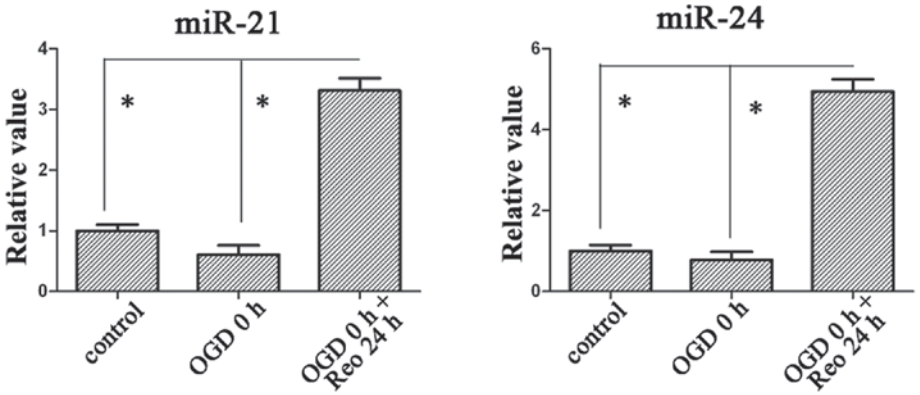

Figure 2. Expression of miR-21 and miR-24 in N2A cells following OGD. The expression of miR-21 and miR-24 was detected using Taqman methods, and were upregulated 3.3- and 4.9-fold, respectively, on Reo 24 h after 3 h of OGD. OGD, oxygen-glucose deprivation; Reo, reoxygenation.

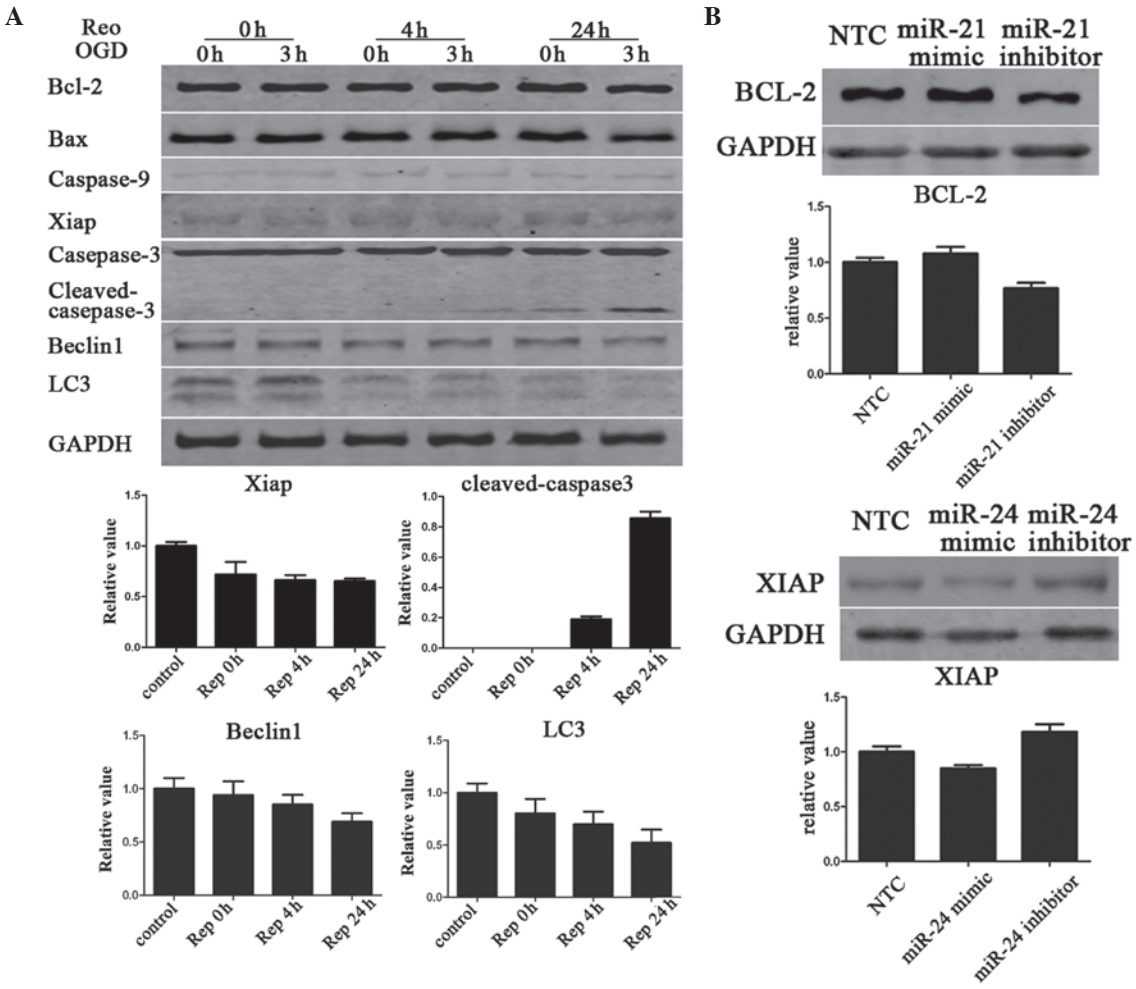

Figure 3. (A) The exchange of apoptotic and autophagy pathway proteins in N2A cells following OGD and Reo. The expression of XIAP was decreased as the Reo time prolonged following 3 h of OGD, while cleaved caspase-3 was increased. The expression of Beclin1 and LC3-II were decreased as the Reo time prolonged following 3 h of OGD. (B) Expression of Bcl-2 and XIAP in N2A cells transfected with mimics, inhibitor and negative control. OGD, oxygen-glucose deprivation; Reo, reoxygenation.

elevated in N2A following 24 h of reoxygenation. Previously, Yin *et al* identified that miR-497 promoted ischemic neuronal death by negatively regulating antiapoptotic proteins, Bcl-2 and Bcl-w, and the protein levels of Bcl-2 and Bcl-w were significantly decreased following OGD (10). By contrast, we did not observe any significant changes in Bcl-2 following OGD. However, the expression of Bcl-2 was slightly increased after adding the miR-21 mimic to the N2A cells, compared with the miR-21 inhibitor and NTC. These data indicate that miR-21 may be a protective and anti-apoptotic factor in cerebral ischemia. The results from the present study are consistent with the data from a previous study (7). These findings enrich our

understanding of the impact of miR-21 and miR-24 expression in ACI, and the mechanisms underlying this effect. Therefore, miR-21 and miR-24 may be an attractive therapeutic molecule for the treatment of stroke

In conclusion, the present study identified that miR-21 and miR-24 have different effects in the pathogenesis of CI, and may be possible plasma markers in early stage of ACI. These miRNAs are therefore potential therapeutic targets for the development of novel diagnostic and treatment strategies in CI.

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