Circulating MicroRNAs as Potential Noninvasive Biomarkers of Spontaneous Intracerebral Hemorrhage

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■ BACKGROUND: **Spontaneous intracerebral hemorrhage (ICH) is a common and severe neurological disorder that has been associated with high rates of mortality and morbidity. It is urgent to find new biomarkers for the early diagnosis and prevention of ICH. In recent years, microRNAs (miRNAs) have been proved to play an important role in vascular damage and inflammation in cerebrovascular diseases, including ICH. In the peripheral blood, circulating miRNAs will be present at a remarkably steady level. In the present study, we explored the circulating plasma microRNA (miR)-181b, miR-223, miR-155, and miR-145 as new potential biomarkers for the diagnosis of ICH.**

■ METHODS: **The plasma samples from 106 patients with ICH and 50 patients without ICH (control group) were collected and subjected to quantitative real-time polymerase chain reaction analyses for the expression levels of circulating miR-181b, miR-223, miR-155, and miR-145.**

■ RESULTS: **The expression levels of plasma circulating miR-145 ($P < 0.001$), miR-223, and miR-155 were increased in patients with ICH compared with those in the control group ($P < 0.05$). However, the expression of plasma circulating miR-181b was decreased in patients with ICH compared with that in the control group ($P < 0.001$). Receiver operating characteristic curve analyses were performed to determine the diagnostic sensitivity and specificity of miR-145 and miR-181b to detect ICH. The area under the curve for miR-145 was 0.766 (95% confidence interval, 0.689–0.838) and for miR-181b was 0.78 (95% confidence interval, 0.70–0.86), suggesting that circulating miR-145 and miR-181b can be used to differentiate patients with ICH from those without ICH.**

■ CONCLUSION: **Our results have shown that measurement of circulating miR-181b, miR-223, miR-155, and miR-145 in plasma samples could serve as a potential noninvasive tool for ICH detection.**

INTRODUCTION

Spontaneous (nontraumatic) intracerebral hemorrhage (ICH), or hemorrhagic stroke, is a common and severe neurological disorder that has been associated with high mortality and morbidity. The rates have not changed for the past 30 years. ICH accounts for 15% of all strokes. At 1 year, the mortality ranges from 40% to 65%, depending on the location of the hemorrhage. One half of the deaths will occur within the first 2 days.[1] At 12 months, only ~50% of patients can be expected to be independent. The incidence of hemorrhage increases exponentially with age and has been greater in men than in women.[2] The main cause of primary spontaneous ICH has most

Key words
- Circulating
- Diagnosis
- Intracerebral hemorrhage
- MicroRNA
- Plasma

Abbreviations and Acronyms
ACE: Angiotensin-converting enzyme
CT: Computed tomography
EC: Endothelial cell
ICH: Intracerebral hemorrhage
MRI: Magnetic resonance imaging
miRNA: MicroRNA
mRNA: Messenger RNA
MiR-181b: MicroRNA-181b
MiR-145: MicroRNA-145
MiR-155: MicroRNA-155
MiR-223: MicroRNA-223
qRT-PCR: Quantitative real-time polymerase chain reaction
VSMC: Vascular smooth muscle cell

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often been hypertension (80% of cases). Less often, the hemorrhage will have been caused by atherosclerosis, blood disorders, inflammatory changes in the cerebral vessels, intoxication, avitaminosis, and other causes. ICH can occur from diapedesis or rupture of the vessel.[3] Prolonged arterial hypertension contributes to the formation of lipogialinosis and, subsequently, fibrinoid necrosis of perforating artery walls, characterized by the absence of anastomoses with other vessels. With an increase in arterial pressure, the walls of these vessels will rupture, with the formation of hematomas (putamenal, cerebellar, and subcortical) or hemorrhagic soaking of blood components of the thalamic and stem brain regions through the pathologically altered vascular walls.[4] After ICH in the perihematomal region, the blood flow will decrease. In addition, owing to the reduced metabolism, the region will not experience ischemia. The decay products of hemorrhage will cause the development of cytotoxic and, after a violation of the blood–brain barrier, vasogenic edema. An inflammatory reaction, apoptosis, and necrosis of the nervous tissue will develop in the perihematomal region. The formation of perihematomal edema will enhance compression and dislocation of the brain.[5]

MiRNAs are endogenous noncoding RNAs of 18–22 nucleotides that regulate gene expression at the post-transcriptional level through interaction with 3′-untranslated regions of the target messenger RNAs (mRNAs).[6] MiRNAs regulate an array of biological processes such as cellular differentiation, proliferation, development, and apoptosis and, thus, play a role in modulating physiological processes. In human biological fluids, such as blood, miRNAs are present at a surprisingly stable level. During the past decade, circulating miRNAs have been emerging as attractive potential biomarkers in various diseases, including ICH.[7] Circulating miRNAs have adequate biological and physicochemical properties to be useful as novel biomarkers in clinical practice. First, circulating miRNAs are highly stable and have a long half-life in the sample (plasma or serum). Second, they can be obtained using minimally invasive techniques. Third, circulating miRNA profiles can show high specificity when stratified by tissue type and disease. Fourth, circulating miRNA profiles will be affected in situations of cellular stress and pathophysiologic conditions such as ICH. Finally, circulating miRNAs can be quantified profitably, efficiently, and relatively rapidly in current clinical laboratories with high sensitivity and specificity using quantitative real-time polymerase chain reaction (qRT-PCR). A role for different miRNAs could connect the disparate theories regarding the pathological derangements of ICH. Studies have suggested a role for miRNAs in the occurrence of vascular damage, neuronal damage, and inflammation. These processes have been linked with the progression of ICH. However, advances regarding the presence of dysregulated miRNAs in ICH have remained sparse. Endogenous miR-181b, miR-223, miR-145, and miR-155 have been found to be key regulators of vascular damage, neuronal damage, and inflammation (**Table 1**).[8-16] Circulating miR-145, miR-181b, miR-223, and miR-155 can be released into the blood (plasma or serum) in response to vascular injury, neuronal damage, and inflammation. Thus, the present study examined the expression profile of these circulating miRNAs in the plasma of patients with ICH. This profile will serve as a reference and contribute to the knowledge pertaining to circulating miR-181b, miR-223, miR-155, and miR-145 in ICH.

METHODS

Patients and Healthy Controls

The ethics committee of the First Affiliated Hospital of the Harbin Medical University approved the study protocol, which was implemented in accordance with the principles of the Declaration of Helsinki. All the participants had provided written informed consent. Blood samples and clinical data were collected from 106 patients with ICH who had undergone treatment at the Department of Neurosurgery at the First Affiliated Hospital of Harbin Medical University in China from January 2016 to December 2017. ICH was diagnosed from the clinical symptoms, neurological examination findings, and results from computed tomography (CT) or magnetic resonance imaging (MRI) of the brain, defined according to the World Health Organization criteria. Clinical severity was assessed at admission using the National Institutes of

Table 1. MicroRNAs Possibly Involved in Pathology of Intracerebral Hemorrhage

MicroRNA	Gene Target	Effect of MicroRNA	Reference
miR-181b	NF-κB	Decreases leukocyte infiltration in vascular endothelium; decreases vascular inflammation	8
	TGF-β, pSMAD2/3	Development of vascular stiffness	9
	SRF	Critical modulator of VSMC phenotype and proliferation in response to vascular injury	10
miR-145	CD40	Critical modulator of VSMC phenotype and proliferation in response to vascular injury	11
	NF-κB	Decreases vascular inflammation	12
	Nurr1, TNF-α	Increases neuron cell death	13
miR-223	NLRP3	Encodes a key component of NLRP3 inflammasome; decreases inflammation	14
	STAT3	Promotes anti-inflammatory response	15
miR-155	SOCS-1, SHIP-1, C/EBP-β	Promoted neuronal cell death and proinflammatory microglia activation	16

miR, microRNA; NF-κB, nuclear factor-κB; TGF-β, transforming growth factor-β; SRF, serum response factor; VSMC, vascular smooth muscle cell; STAT3, signal transducer and activator of transcription 3; SOCS-1, suppressor of cytokine signaling 1; SHIP-1, SH-2 containing inositol 5' polyphosphatase 1; C/EBP-β, CCAAT/enhancer-binding protein-β.

Health stroke scale score. We excluded patients with other cardiovascular diseases, immune diseases, previous surgery, injuries, organ failure, tumors, or secondary ICH (i.e., hemorrhage resulting from aneurysm, vascular malformation, hemorrhagic infarction, or during anticoagulant treatment). We also excluded patients with intraventricular hemorrhage and/or infection in their medical history because these diseases could influence the miRNA levels in our patients. Healthy volunteers ($n = 50$) without a history of cerebrovascular, other cardiovascular disease, and/or cancer were recruited from the physical examination center as healthy controls. The clinical characteristics of the patients with ICH are summarized in Table 2.

Plasma Preparation

The blood samples from the patients with ICH were drawn within 24 hours after admission to the hospital and before surgery. The samples for all healthy controls were collected after the volunteers had fasted 8–10 hours. All plasma samples were extracted from EDTA-K3 tubes and centrifuged, as described previously.[17] After the first centrifugation for 10 minutes at 1600*g*, the supernatants were carefully removed and transferred to a new tube, followed by centrifugation at 16,000*g* for 10 minutes to remove any residual blood cells. The plasma was then divided into small aliquots and snap frozen at −80°C.

RNA Isolation and qRT-PCR Detection

Total RNA was isolated from 200-µL plasma samples using the miRNeasy Serum/Plasma Kit for purification of total RNA, including miRNA (Qiagen USA, Germantown, Maryland, USA) and QIAzol Lysis Reagent (Qiagen USA) according to the manufacturer's instructions. The RNA concentration ranged from 50 to 100 ng/µL, and the ratio of optical density 260 and optical density 280 absorbance for each sample was 1.8–2. All isolated RNA was stored in a −80°C freezer until used. Reverse transcription of the extracted total RNA into complementary DNA was performed using the Transcriptor First Stand cDNA Synthesis Kit (Roche, Penzberg, Germany). qRT-PCR was performed using the Fast Start Universal SYBR Green Master (Roche) according to the manufacturer's instructions. MiR-16 was used as the endogenous reference gene. The sequence of all primers used in the present study is provided in Table 3. The primers for each miRNA were obtained from Invitrogen (Thermo Fisher Scientific, Carlsbad, California, USA).

Table 2. Characteristics of Patients with Intracerebral Hemorrhage

Characteristic	Patients ($n = 106$)
Age (years)	56.3 ± 7.2
Male sex	71 (67.0)
History of hypertension	51 (48.1)
History of diabetes	18 (17.0)
First systolic BP (mm Hg)	169.7 ± 27.4
Heart rate (bpm)	79.7± 18.0
Temperature (°C)	36.5 ± 0.2
Initial hematoma volume (mL)	16.4 (7.9–38.3)
IVH	27 (25.5)
Anticoagulant therapy	12 (11.3)
Antiplatelet therapy	20 (18.9)
Glucose (mmol/L)	6.67 (5.80–8.88)
Admission INR	1.1 (1.0–1.1)
Admission PT (seconds)	11.7 (11.2–12.2)
Admission APTT (seconds)	24.3 (21.6–26.9)

Data presented as *n* (%), mean ± standard deviation, or median (interquartile range).
BP, blood pressure; IVH, intraventricular hemorrhage (on presentation); INR, international normalized ratio; PT, prothrombin time; APTT, activated partial thromboplastin time.

Statistical Analysis

The relative levels of circulating miR-181b, miR-223, miR-155, and miR-145 were quantified using the $2^{-\Delta\Delta Cq}$ method. The receiver operating characteristic curve was applied to analyze the diagnostic values of circulating miR-181b and miR-145. The Student *t* test, analysis of variance, χ^2 test, or the Mann-Whitney *U* test was applied, as appropriate. A probability of $P < 0.05$ or $P < 0.001$ was considered to indicate statistical significance. The statistical analyses were performed using SPSS, version 22.0, software (IBM Corp., Armonk, New York, USA), and the graphs were generated using Prism, version 7.0 (GraphPad, San Diego, California, USA).

RESULTS

We compared the expression of circulating miR-181b, miR-223, miR-155, and miR-145 in the plasma samples derived from 106 patients with ICH and 50 controls using qRT-PCR. Our results showed that the expression of circulating miR-181b was lower in the patients with ICH compared with those in the control group (1.03 ± 0.1 vs. 2.09 ± 0.3; $P < 0.001$; Figure 1B). In addition, the results showed that the expression of circulating miR-223 (8.42 ± 1.6 vs. 2.76 ± 0.4; $P < 0.05$) and miR-155 (5.19 ± 0.1 vs. 1.81 ± 0.2; $P < 0.05$) was greater in the patients with ICH compared with their expression in the control group (Figure 1A, C, D). For circulating miR-145, the expression was significantly greater in the patients with ICH compared with the expression in the control group (16.19 ± 2.75 vs. 2.10 ± 0.4; $P < 0.001$; Figure 1D). The receiver operating characteristic analysis for miR-181b and miR-145 revealed that the area under the curve was 0.78 (95% confidence interval, 0.70–0.86) and 0.766 (95% confidence interval, 0.68–0.83), respectively. This finding showed that miR-181b and miR-145 had considerable accuracy in discriminating the plasma of patients with ICH from that of the control group (Figure 2A, B).

DISCUSSION

In the present study, we found that the expression of 4 circulating miRNAs in the patients with ICH exhibited a unique pattern of change compared with the expression in the control group. We have demonstrated that the expression of circulating miR-223, miR-155, and miR-145 in plasma was increased in the patients with ICH compared with their expression in the control group. However, the expression of plasma circulating miR-181b was

Table 3. Sequence of All Primers

miRNA	Primer Sequence (5′-3′)
miR-155	RT: CCTGTTGTCTCCAGCCACAAAAGAGCACAATATTTCAGGAGACAACAGGACCCCTA; forward: CGCCGTTAATGCTAATCGTGA; reverse: CAGCCACAAAAGAGCACAAT
miR-223	RT: CCTGTTGTCTCCAGCCACAAAAGAGCACAATATTTCAGGAGACAACAGGAACTCAG; forward: GCGGCCGTGTATTTGACAAG; reverse: CAGCCACAAAAGAGCACAAT
miR-145	RT: CCTGTTGTCTCCAGCCACAAAAGAGCACAATATTTCAGGAGACAACAGGAGGGATT; forward: CGGGCGTCCAGTTTTCCCAGG; reverse: CAGCCACAAAAGAGCACAAT
miR-181b	RT: CCTGTTGTCTCCAGCCACAAAAGAGCACAATATTTCAGGAGACAACAGGACCCACC; forward: CGCCGAACATTCATTCATTGCTGTC; reverse: CAGCCACAAAAGAGCACAAT
miR-16	RT: CCTGTTGTCTCCAGCCACAAAAGAGCACAATATTTCAGGAGACAACAGGCGCCAAT; forward: CGGGCTAGCAGCACGTAAAT; reverse: CAGCCACAAAAGAGCACAAT

miRNA, microRNA; RT, reverse transcription; miR, microRNA.

decreased in patients with ICH. To the best of our knowledge, the present study was the first comprehensive study on the expression and clinical significance of the plasma levels of circulating miR-181b, miR-223, miR-155, and miR-145 in patients with ICH. The area under the curve for the prediction of ICH was 0.78 and 0.76 for circulating miR-181b and miR-145 levels, respectively.

The transition of vascular smooth muscle cells (VSMCs) from a state of differentiation to a state of proliferation contributes to the

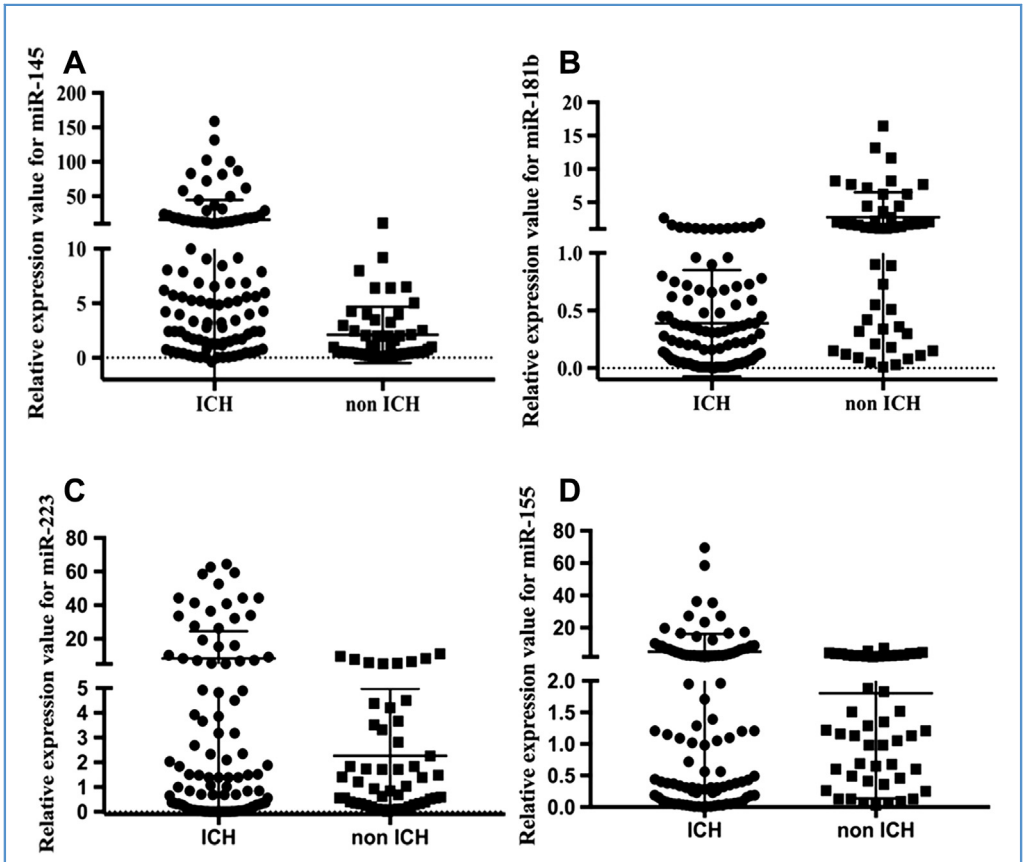

Figure 1. Circulating micro-RNA (miR) in plasma in the validation population. Box plots show expression of (**A**) miR-145, (**B**) miR-181b, (**C**) miR-223, and (**D**) miR-155 measured using quantitative real-time polymerase chain reaction in patients with spontaneous intracerebral hemorrhage ($n = 106$) and control group ($n = 50$).

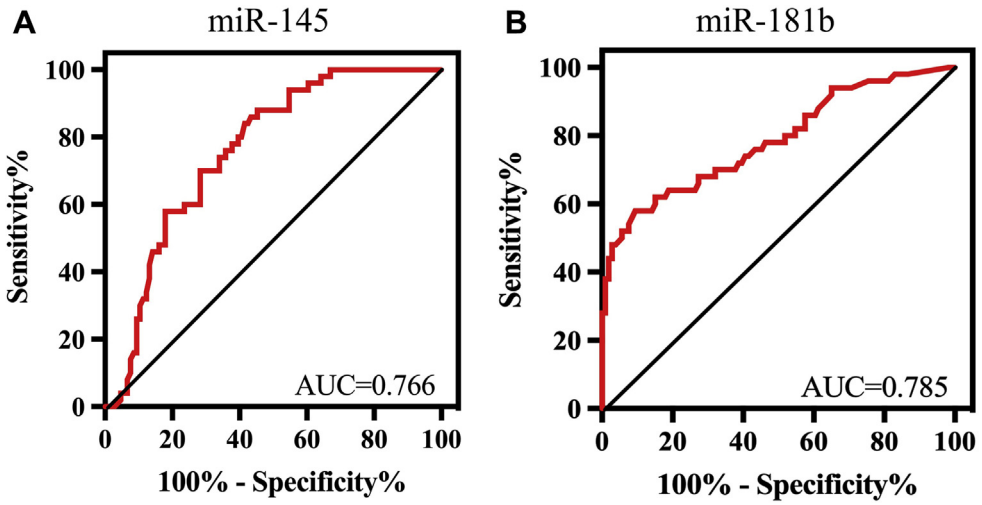

Figure 2. Evaluation of circulating (**A**) microRNA (miR)-145 and (**B**) miR-181b in plasma for the diagnosis of spontaneous intracerebral hemorrhage. Receiver operating characteristic curve comparing circulating miR-181b and miR-145 expression in plasma between patients with intracerebral hemorrhage and control group.

pathogenesis of hypertension and atherosclerosis. The miR-145 effects on VSMC differentiation, manifesting as a modulator of the smooth muscle cell phenotype in response to injury, are a consequence of targeting several genes such as Kruppel-like factor-4 and Kruppel-like factor-5.[18,19] A change in the expression of miR-145 in the presence of atherosclerosis will reduce or increase the formation of neointima and, thus, itself can influence the progression of atherosclerosis.[20] Nitrogen oxide serves a key role in the cardiovascular system, including dilating blood vessels to relieve hypertension. Wang and Jin[21] examined the expression of miR-145 and reported that it negatively regulates the production of nitrogen oxide through targeting SLC7A1 in vitro and in vivo. Santovito et al.[22] found that miR-145 was overexpressed in atherosclerotic plaques in patients with hypertension but not in patients without significant hypertension. Angiotensin-converting enzyme (ACE) plays a fundamental role in the development of hypertension and atherosclerosis.[23] Endothelial ACE expression will be downregulated in response to shear stress. The exact mechanism is not fully understood; however, the final effect will be an increase in miR-145 expression in the endothelial cells (ECs), significant mediators of the effects of ACE.[24] According to our data, it is possible that the increased expression of miR-145 might be a compensatory response to chronic hemodynamic stress resulting from high blood pressure in conjunction with atherosclerosis of the cerebral vessels. MiR-145 is required for stretch-induced VSMC differentiation and acquisition of contractile phenotype.

MiR-223 has a close relationship with cerebrovascular diseases. Wang et al.[25] reported that the expression of miR-223 was increased in the circulating blood of patients with acute ischemic stroke. MiR-223 was upregulated significantly in mice that had undergone middle cerebral artery occlusion.[25] Chen et al.[26] showed that increased blood exosomal miR-223 is associated with acute ischemic stroke. The results reported by Feng et al.[27] suggested that miR-223 might suppress proliferation of cortical neurons treated with an oxygen–glucose deprivation and simulated reperfusion model via inhibiting insulin-like growth factor 1 receptor expression.[27] In addition, existing data have indicated that miR-223 will both positively and negatively affect neuroinflammatory cascades. MiR-223 reduced the neurotoxicity after global ischemia and excitotoxic injury by enhancing the degradation of mRNA-encoding glutamate receptors.[28] Our results were also consistent with a previous study of inflammation-related miR-223 expression, which showed that a complex inflammatory response will be present after experimental ICH. Yang et al.[29] reported that miR-223 is a crucial regulator of microglial activation, inflammation, and neuron injury after ICH by directly targeting NLRP3 in vitro and in vivo. These results indicated that miR-223 is a novel inflammatory regulator in ICH.[29] Shan et al.[30] reported that miR-223 has antiproliferative and proapoptotic effects on ECs, as shown by decreased proliferation but increased apoptosis in ECs with overexpression of miR-223. This finding is also consistent with its cellular functions in VSMCs.[30] We believe that ICH induced increases in EC apoptosis, accompanied by an increase in the expression of circulating miR-223 in plasma.

The expression of miR-155 has been detected in the vascular endothelium of the brain and microglia.[31,32] The downregulation of miR-155 leads to the increased expression of mTOR, SMAD2, and SMAD3 and, thus, activation of mTOR and transforming growth factor-β signaling cascade and transcription of genes responsible for endothelial morphogenesis.[33] Systemic inhibition of miR-155 after experimental cerebral ischemia supports the vascular integrity, neuronal survival, and reduction of neuroinflammation.[34]

The critical role of the miR-181 family in vascular inflammation has been documented. Sun et al.[35] reported that miR-181b serves as a potent regulator of downstream nuclear factor-κB signaling in the vascular endothelium by targeting importin-α3, a protein

required for nuclear translocation of nuclear factor-κB. MiR-181b regulates atherosclerotic inflammation and vascular endothelial function through Notch1 signaling pathway, in which overexpression of miR-181b protected endothelial cell function by inhibiting Notch1 expression.[36] MiR-181b has neuroprotective effects that alleviate neurological injury in ICH. Wang et al.[37] demonstrated that miR-181b mimics the significantly attenuated apoptosis of neurons and caspase-3 activity in vitro. In addition, their data demonstrated that overexpression of miR-181b attenuated the neuronal necrosis and apoptosis induced by erythrocyte lysates. In vivo, downregulated miR-181b will increase the heat shock protein A5 level and result in significant elevations of proinflammatory cytokines, brain edema, and neurological injury after ICH.

Endothelial dysfunction and arterial stiffening play major roles in cerebrovascular diseases. Hypertension and increased vascular stiffness are common with aging. A broad consensus has been reached that hypertension is the most important risk factor for ICH. Several studies have suggested an association between vascular stiffening and hypertension.[38] Arterial stiffening results from many disease states such as hypertension and atherosclerosis.[39] Arterial stiffening plays a key role in the pathophysiological mechanism of the cerebrovascular system, predicting the incidence of complications, with a prognostic value in patients with spontaneous ICH.[40] Hori et al.[9] identified the potential role of miR-181b in the development of vascular stiffness, which ultimately was associated with a greater systolic blood pressure via activation of transforming growth factor-β. In addition, miR-181b expression will decrease with an increase in age in vivo.[9] The results suggested that miR-181b is not only an active participant in the ICH pathogenesis but also a biomarker for ICH, as shown in our study.

The emerging roles of circulating miRNAs in the pathogenesis of ICH reviewed in the cited studies imply the application of circulating miRNAs as novel biomarkers for the early noninvasive diagnosis and prediction of ICH.[41,42] At present, the diagnosis of ICH relies on imaging techniques such as MRI or CT. Unlike tumors, which have many specific and nonspecific blood markers that can be used to both diagnose and assess the severity of the malignancy, no biomarkers have been established for patients with ICH. However, if MRI and/or CT studies are either unavailable or have shown no obvious acute abnormalities, an accurate and reliable blood miRNA test could assist in the early diagnosis of stroke and the prediction of likely mortality and morbidity or the prognostic outcomes of patients with ICH. The specific circulating miRNA expression profile could constitute the fingerprint of a pathophysiological or diseased condition. The understanding of ICH and its process-specific miRNAs and the regulatory mechanisms of the miRNAs has led to the clarification of the roles of circulating miRNAs, which could result in a promising screening tool for the faster diagnosis and more accurate prediction of ICH.

The present study had a number of limitations. First, the possible clinical implications of the circulating miR-181b, miR-145, miR-223, and miR-155 patterns and concentrations in the detection, diagnosis, and prognosis of patients with ICH remain to be elucidated using a larger number of patient samples. Second, we tested for changes in circulating miR-181b, miR-145, miR-223, and miR-155 only at the acute phase of ICH. We did not monitor the levels in the chronic phase and were unable to evaluate the prognostic value of these 4 circulating miRNAs. In addition, correlations are possible between brain edema size and other markers such as D-dimer, treatment, associations between the circulating miRNA profiles, and gene expression profiles, as well as ICH grade and clinical outcome. All these factors are goals for future research. In addition, the functions and mechanisms of miR-181b, miR-145, miR-223, and miR-155 in ICH require further investigation to determine whether miR-181b, miR-145, miR-223, and miR-155 could be used as potential therapeutic targets.

CONCLUSION

Our study has demonstrated that the expression of circulating miR-181b, miR-145, miR-223, and miR-155 in the plasma of patients after ICH is increased compared with their expression in the control group. Moreover, the expression of circulating miR-181b in the plasma of patients after ICH was decreased compared with that in the control group. These findings suggest that miRNAs could play important roles in ICH. Further studies of these 4 circulating miRNAs might enhance our understanding of the miRNA-based mechanisms of ICH and provide candidate targets for future clinical applications.

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