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INVITED REVIEW

Blood microRNAs as potential diagnostic markers for hemorrhagic stroke

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Abstract
Proper medical treatment of a stroke victim relies on accurate and rapid differentiation between ischemic and hemorrhagic stroke, which in current practice is performed by computerized tomography (CT) or magnetic resonance imaging (MRI) scans. A panel of microRNAs could be an extremely useful clinical tool for distinguishing between hemorrhagic and ischemic stroke. This review has shown that blood miRNA profile can distinguish hemorrhagic from ischemic stroke in patients and in experimental animal models. It also seems likely they can differentiate between intracerebral and subarachnoid hemorrhage stroke. The miRNA profile in cerebrospinal fluid could be a useful diagnostic tool for subarachnoid hemorrhagic stroke. Decreased or increased miRNA levels may be needed either as prevention or treatment of stroke. Administration *in vivo* of miR-130a inhibitor or miRNA mimic (miR-367, miR-223) in an intracerebral hemorrhage animal model improved neurological outcomes.

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Introduction
Strokes can be broadly classified as ischemic or hemorrhagic. Hemorrhagic strokes account for about 20% of all strokes and are divided into categories depending on the site and cause of bleeding. In intracerebral hemorrhage (ICH), bleeding occurs from a ruptured blood vessel within the brain. Hypertension, excessive alcohol intake, and advanced age are all important risk factors. Ischemic strokes can convert to an ICH (Berger et al., 2001), and may be associated with infective endocarditis (Morris et al., 2014). A subarachnoid hemorrhage (SAH) involves bleeding from a damaged blood vessel causing blood to accumulate at the surface of the brain. Most often, a SAH happens because of a leaking saccular aneurysm. Hemorrhagic stroke is life threatening with up to 50% of all people with ICH dying, many within the first two days. Surgical removal of the hematoma as an early-stage treatment for ICH may improve long-term prognosis (Morgenstern et al., 1998), but no effective targeted therapy for hemorrhagic stroke exists yet. ICH is more likely to result in death or major disability than ischemic stroke or SAH. The sudden buildup of pressure outside the brain in SAH may cause loss of consciousness or death.

Proper medical treatment of a stroke victim relies on accurate and rapid differentiation between ischemic and hemorrhagic stroke. Not only do ischemic and hemorrhagic stroke have completely divergent therapeutic options, the treatment itself can convert ischemic stroke to hemorrhagic stroke (Zhang et al., 2014). Clinically, it is therefore crucial to monitor and distinguish ischemia *versus* hemorrhage stroke within the first week of symptom onset to prevent adverse outcome. Also it is important to distinguish between ICH and SAH as this will influence possible treatment. In current practice, diagnosis of hemorrhage *versus* ischemia stroke is performed by computerized tomography (CT) or magnetic resonance imaging (MRI) scans. There is a need for a reliable, relatively inexpensive method for differentiating between ischemic and hemorrhagic stroke in patients - potentially a point-of-care assay that can be performed on a daily basis within the first week of stroke onset. Most biomarkers associated with stroke and proposed as diagnostics in the emergency room for acute stroke are blood-borne proteins of tissue injury such as C-reactive protein, matrix metallopeptidase 9, D-dimer, S100β protein, and B-type natriuric peptide (Lopez et al., 2012).

MicroRNAs are small non-coding RNAs of approximately 22 nucleotides long, involved in the regulation of gene expression, thus controlling a range of physiological and pathological functions such as development, differentiation, apoptosis and metabolism (Ambros, 2004). It has been shown that serum or plasma miRNAs are stable and indicative of the disease state (Chen et al., 2008). Recently much interest has developed in the use of circulating cell-free miRNAs as novel markers in the clinical diagnosis of disease especially in cancer (Ho et al., 2010). This article reviews recent human and animal studies of miRNAs as biomarkers of hemorrhagic stroke, and whether specific miRNAs, or a combination, can be used to distinguish between ischemic and hemorrhagic stroke.

MicroRNAs in Hemorrhagic Stroke

Leung et al. (2014) compared miR-124-3p and miR-16 plasma levels in 93 stroke patients, median age 72 years, 51% male. Ischemic stroke was diagnosed in 74 patients and 19 patients were diagnosed with hemorrhagic stroke, with blood samples being collected within 24 hours of symptom onset. Twenty-three age- and sex-matched healthy individuals were recruited as controls. Hypertension was a major stroke risk factor in the patients. Hemorrhagic stroke patients had higher median plasma miR-124-3p levels than ischemic stroke patients and healthy controls (1.94 and 2.55 fold change, respectively). The median plasma level of miR-16 was increased in ischemic stroke patients compared with hemorrhagic stroke patients and healthy controls (1.24 and 1.35 fold change, respectively). This study did not indicate the number of hemorrhagic stroke patients diagnosed with ICH or SAH, and therefore it is not known whether plasma levels of miR-124-3p and miR-16 can differentiate between these two categories. The findings from ICH and SAH studies are summarized in **Tables 1** and **2**, respectively.

Intracerebral hemorrhage

Human studies

Four clinical studies were found. They indicated that serum miR-130a, or a panel of blood specific miRNAs, could distinguish ICH patients from controls. Additionally, plasma miR-29c and miR-122 could distinguish between hematoma enlargement group and non-hematoma enlargement group of ICH patients.

Animal studies

Three studies had been performed with ICH rats and one study with ICH mice. Inhibition of miR-130a or enhancement of miR-367 or miR-223 had positive outcome by inhibiting inflammation (**Table 1**).

Subarachnoid hemorrhage

Human studies

Four clinical studies were found. Blood miR-132 and miR-324 could differentiate SAH patients with delayed cerebral infarction and SAH patients with non-delayed cerebral infarction from controls. Also a panel of specific miRNAs in cerebrospinal fluid could distinguish SAH patients from controls, and SAH patients with no vasospasm from SAH patients with vasospasm.

Animal studies

One study in SAH rats showed miR-30a and miR-143 might be useful biomarkers for SAH (**Table 2**).

Targeting MicroRNAs as a Novel Therapeutic Approach

Both increased and decreased miRNA levels may be needed either as prevention or treatment of hemorrhagic stroke. Using an experimental model of ICH, injection of miR-130a inhibitor into the right lateral ventricle before ICH induction in male rats significantly reduced endogenous expression of miR-130a, decreased brain edema, and alleviated brain-blood barrier disruption at 1 day after ICH. Neurological function was significantly improved (Wang et al., 2016). Also in a mouse model of ICH, intracerebroventricular injection of miR-367 mimic significantly increased the miR-367 level *in vivo*, and significantly inhibited interleukin-1 receptor-associated kinase 4 (IRAK4), nuclear factor-κB (NF-κB), p65, interleukin 6 (IL-6), IL-1β and tumor necrosis factor-alpha (TNF-α) expression in brain tissues after ICH, indicating that miR-367 could inhibit inflammatory response *in vivo*. A miR-367 mimic significantly decreased brain edema and neurological injury (Yuan et al., 2015; **Figure 1**). Overexpression of mir-223 following intracerebroventricular injection of miR-223 mimic in ICH mice resulted in reduced brain edema, and improved neurological functions. MiR-223 significantly inhibited caspase-1 p20, NLRP3, TNF-α, IL-1β, and IL-6 expression in brain tissues after ICH, showing that miR-223 could inhibit inflammatory response *in vivo* (Yang et al., 2015).

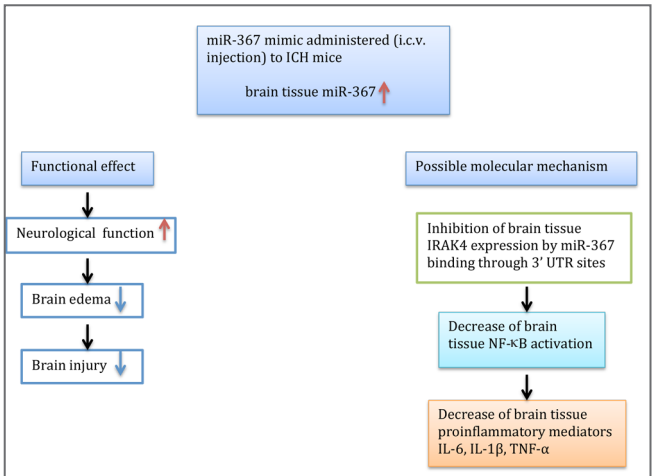

Figure 1 Possible molecular mechanism of miR-367 mimic-mediated support of post-stroke recovery in intracerebral hemorrhage (ICH) mice.

Upregulation of brain tissue miR-367 results in decreased brain edema and brain injury, and improved neurological function in treated animals. Possible molecular mechanisms mediating these processes could be associated with inhibition of brain tissue interleukin (IL)-1 receptor-associated kinase 4 (IRAK4) expression and decreased brain tissue nuclear factor-κB (NF-κB) activation by miR-367 mimic, leading to a decrease in proinflammatory mediators interleukin 6 (IL-6), IL-1β, and tumor necrosis factor-alpha (TNF-α).

Future Perspectives

Numerous circulating miRNAs have been reported to have a potential value in diagnosis of hemorrhagic stroke, with considerable variation in findings. Most of the studies had performed RT-PCR to validate changes in miRNAs detected by microarray analysis. In several studies, changes in specific miRNAs were confirmed in experimental hemorrhagic stroke in healthy rats or mice. The findings of previous clinical studies need to be repeated in other hospital centers and include both male and female patients. Also experimental animal studies should be performed with hemorrhagic stroke rats or mice of both sexes, and to use antagomirs or mimics

Table 1 MicroRNAs in intracerebral hemorrhage (ICH) patients and experimental animal models

Reference	No. of ICH patients or animals; ages, gender; risk factors	Comparison	Changes in miRNAs in ICH patients or animals	Functional outcomes	Conclusion
Human studies					
Guo et al. (2013)	15 patients; 53 ± 2 years of age, 53% male; hypertension and smoking	8 healthy controls; 30 ± 3 years of age; 50% male	Microarray analysis showed 30 miRNAs in blood plasma were upregulated (changed ≥ 2-fold) in a sex-independent manner. Among these upregulated miRNAs in ICH, 13 miRNAs were not changed in ischemic stroke patients. By RT-PCR, miR-29b, -365, -27a, -150*, -34c-3p, and -24 showed the same trend to microarray data.	The most significantly over-represented biological processes associated with the deregulated miRNAs were inflammatory disease and inflammatory response.	A separate validation cohort showed that the plasma levels of miR-27a, -365, -150* and -34c-3p were significantly increased in ICH patients, whereas the change of miR-29b did not reach significance. These miRNAs enable ICH to be distinguished from ischemic stroke.
Wang et al. (2016)	50 patients; mean age 60 years, 70% male: 32 had deep ICH and 18 had lobar ICH; 54% being treated with antihypertensives	15 healthy controls	Blood samples collected from patients at days 0–1, 2–3, 5–8, and 9–14 after symptom onset and from controls. RT-PCR showed that miR-130a expression was elevated compared to controls.	Serum miR-130a level was significantly correlated with perihematomal edema volume at days 0–1, 2–3. Serum miR-130a levels were associated with clinical outcome NIHSS scores at day 14 and modified Rankin Scale scores at day 90 only in patients with deep hematoma.	Serum miR-130a would serve as a useful biomarker for ICH in human patients.
Zheng et al. (2012)	79 patients admitted within 6 hours of symptom onset: 30 with hematoma enlargement within 24 hours after hospitalization; mean age 58 years, 87% male; 49 without hematoma enlargement, mean age 53 years, 65% male; hypertension and smoking	30 healthy controls, mean age 55 years, 87% male, hypertension and smoking	Blood samples taken from patients immediately after admission. Overall 30 differentially expressed (DE) miRNAs were identified among patients with or without hematoma enlargement (HE). 19 DE miRNAs were upregulated in the HE group compared with the non-HE group, and 11 miRNAs were significantly downregulated. Plasma levels of miR-29c and miR-122 were higher in the HE group than in the non-HE group based on microarray profile (fold change = 1.97, 1.76, respectively). From RT-PCR measurements, miR-29c was upregulated in the HE group compared with the non-HE group by 2.07-fold ($P = 0.001$) and to the normal control group by 2.36-fold.	A subset of 10 miRNAs (miR-1249, -574-5p, -1290, -522, - 130a*, -1202, -71-2*, -586, -122, -29c) allowed discrimination of HE patients from controls	A broad range of biological processes or molecular function categories were enriched among the target gene list including cell apoptosis, collagen biosynthesis, blood vessel development and morphogenesis, cell-cell or cell-substrate adhesion, extracellular matrix constituent and binding, metallopeptidase activity, ion channel and transporter activity, water homeostasis, and inflammatory response.
Zhu et al. (2015)	33 patients, 39% male, median age 57 years, presenting within 24 hours of onset of a primary basal ganglia hemorrhage and absence of coma, hypertension	15 healthy controls, 53% male, median age 58 years, hypertension	Blood samples were collected from patients on admission and from controls at study entry.	55 DE miRNAs exhibiting a fold change ≥ 1.5 were identified among patients and controls, of which 54 were downregulated and one was upregulated. Of these, miR-126, -146a, -let-7a, and -26a (0.39-fold, 0.33-fold, 0.46-fold, 0.31-fold, respectively) were significantly decreased in the ICH patients compared with the controls. In RT-PCR validation, miR-126, 146a, -let-7a, and -26a were all present at significantly lower levels in patients relative to controls, with average fold-changes of 0.63, 0.64, 0.50 and 0.54, respectively.	Some genes targeted by miR-126, -146a, -let-7a and -26a are involved in perihematomal edema formation after ICH. The biological processes associated with these genes included the innate immune response, leukocyte activation (miR-126, -146a) response to oxidative stress (miR-146a), programmed cell death (miR-let-7a), and smooth muscle cell proliferation (miR-26a). Only the level of serum miR-126 was significantly correlated with relative perihematomal edema development.

Table 1 Continued

Reference	No. of ICH patients or animals, ages, gender, risk factors	Controls	Changes in miRNAs in ICH patients or animals	Functional outcomes	Conclusion
Animal studies					
Liu et al. (2010)	6 male Sprague-Dawley rats with ischemia caused by middle cerebral artery occlusion. 6 received intracerebroventricular injections of lysed blood, 6 received fresh autologous tail vein blood, 6 received thrombin. Also 6 were injected subcutaneously with kainic acid to induce severe, prolonged generalized seizures.	6 that had not been experimentally treated in any way were used as controls.	At 24 hours after each operation or assignment as a control, blood was collected from the tail vein of all animals into EDTA tubes. Animals were then killed, the brain was removed, and the hippocampus dissected as rapidly as possible.	In the brain, 24 miRNAs were upregulated > 2-fold by brain ischemia, 17 by brain hemorrhage (lysed blood), and 13 by kainate seizures. In addition, 13 miRNAs were downregulated > 2-fold by brain ischemia, 12 by brain hemorrhage (lysed blood), and 18 by kainate seizures. A Venn diagram of these results showed that one miRNA (miR-542-3p) was upregulated and four miRNAs (miR-155, -362-3p, -122, and -450a-5p) were downregulated in all three conditions. There were 8 miRNAs upregulated for brain hemorrhage that were not upregulated in brain ischemia, and 6 miRNAs downregulated for brain hemorrhage that were not downregulated in brain ischemia. In blood, 10 miRNAs were upregulated > 2-fold by brain ischemia, 20 by brain hemorrhage (lysed blood), and 10 by kainate seizures. In addition, 65 miRNAs were downregulated > 2-fold by brain ischemia, 21 by brain hemorrhage (lysed blood), and 21 by kainite seizures. A Venn diagram showed that five miRNAs (miR-96, -152, -298, -333, and -505) were upregulated and seven miRNAs (miR-125a-5p, -130b, -142-3p, -330, 342-5p, -685, and -347) were downregulated in all three conditions. There were 12 miRNAs upregulated for brain hemorrhage that were not upregulated in brain ischemia, and 7 miRNAs downregulated for brain hemorrhage that were not downregulated in brain ischemia.	The top five ranked biologic functions that overlapped for both brain and blood included cell cycle, cell death, cancer, cell morphology, and organismal development. While this study has shown that miRNAs can differentiate hemorrhagic stroke from ischemic stroke, the identities of the specific miRNAs in blood and brain were not reported.
Yuan et al. (2015)	Newborn (< 24 hours old) Sprague-Dawley rats; primary hippocampal microglia isolated from glial cultures on day 10. Microglia (1×10^5) stimulated with erythrocyte lysates for 48 hours. Mouse models of ICH prepared by injecting autologous nonanticoagulated blood into the caudate nucleus.	Microglia (1×10^5) stimulated with phosphate buffered saline for 48 hours.	Erythrocyte lysates downregulated miR-367 expression but upregulated IRAK4 expression in primary microglia.	MiR-367 mimic significantly increased the miR-367 level *in vivo*, and significantly inhibited IRAK4, NF-κB, p65, IL-6, IL-1β and TNF-α expression in brain tissues after ICH. Also miR-367 mimic significantly decreased brain edema and neurological injury in ICH mice.	MiR-367 could inhibit inflammatory response *in vivo*, ameliorate the neurological symptoms, and improve brain function after ICH.
Yang et al. (2015)	Adult male C57BL/6 ICH mice		Erythrocyte lysis significantly increased mRNA and protein levels of TNF-α, IL-1β, and IL-6, which are typical proinflammatory cytokines released by microglia cells exposed to erythrocyte lysis.	Intracerebroventricular injection of miR-223 mimic in ICH mice resulted in reduced brain edema, and improved neurological functions. MiR-223 significantly inhibited caspase-1 p20, NLRP3, TNF-α, IL-1β, and IL-6 expression in brain tissues after ICH.	MiR-223 could inhibit inflammatory response *in vivo*.
Wang et al. (2016)	Adult Sprague Dawley male ICH rats	Sham-operated controls	Within 1 day after ICH, miR-130a level in perihematomal tissues was significantly increased and reached a peak, which was almost 2.5 times that of sham-operated animals. Afterwards the level of miR-130a declined with time, returning to normal by day 7. A similar profile of miR-130a was observed in the serum.	MiR-130a inhibitor was injected into the right lateral ventricle before ICH induction and significantly reduced endogenous expression of miR-130a, decreased brain water content, and alleviated brain-blood barrier disruption at 1 day after ICH. Neurological function was significantly improved after inhibition of miR-130a	

EDTA: Ethylenediamine tetraacetic acid; IL-6: interleukin 6; IRAK4: interleukin-1 receptor-associated kinase 4; NF-κB: nuclear factor-kappaB; NIHSS: NIH Stroke Scale; RT-PCR: reverse transcription polymerase chain reaction; TNF-α: tumor necrosis factor-alpha.

Table 2 MicroRNAs in subarachnoid hemorrhage (SAH) patients and experimental animal models

Reference	No. of SAH patients or animals, ages, gender, risk factors	Controls	Changes in miRNAs in SAH patients or animals	Functional outcomes	Conclusion
Human studies					
Su et al. (2015)	20 SAH patients with delayed cerebral infarction (DCI), mean age 59 years, 55% male; 20 SAH patients without DCI, mean age 59 years, 35% male; hypertension	20 healthy family members as controls	Peripheral blood samples collected on day 7 after onset of SAH. By microarray analysis, 81 miRNAs were upregulated and 18 were downregulated. In RT-PCR analysis, miR-132 presented a 9.5-fold upregulation in SAH patients with DCI and 3.4-fold upregulation in non-DCI group. For miR-324, there was a 4924-fold upregulation in SAH patients with DCI and 4545-fold upregulation in non-DCI group. The trends of change in these two miRNAs were found to be consistent with what was shown in the microarray analysis. There were no significant differences in fold changes between SAH patients with and without DCI. Compared to healthy control, miR-132-3p was 9.5-fold upregulated in SAH DCI group and 3.4-fold upregulated in SAH non-DCI group.	The dysregulated miRNAs in the patient poled sample were shown to be associated with prion disease, ErbB signaling pathway, transforming growth factor-β (TGF-β) signaling pathway, neurotrophin signaling pathway, and dopaminergic synaptic processes.	There were no significant differences in fold changes for miR-132 and miR-324 between SAH patients with and without DCI.
Liu et al. (2014)	6 patients with ruptured intracranial aneurysms, ages in the ranges 45–50 and 60–65 years, 17% male, hypertension and smoking, intracranial aneurysmal tissues were removed during surgery, hypertension	6 traumatic patients undergoing craniotomy treatments, normal superficial temporal arteries were collected; hypertension	By microarray analysis, 157 miRNAs were identified as DE in the aneurysmal tissue compared to normal arteries (fold change ≥ 2), including 72 upregulated and 85 downregulated.	The changed miRNAs included endothelium-enriched miRNAs such as members of let-7 family, miR-17, -23b, -126, -24-1, -222, and vascular smooth muscle-enriched miRNAs such as miR-143 and -145. Also, miR-1, -10a, -125b and -26a, which were implicated in modulating vascular smooth muscle cell functions such as proliferation, apoptosis and shift of phenotype, were changed. In contrast, miRNAs involved in monocyte and macrophage functions such as miR-155, -146a, -223, -124a were not significantly changed.	The changed miRNAs were associated with several biological processes related to aneurysm formation including inflammation, dysregulation of extracellular matrix, smooth muscle proliferation, programmed cell death, and response to oxidative stress.
Stylli et al. (2016)	9 patients with aneurysmal SAH and no cerebral vasospasm, median age 51 years, 56% male; 10 patients with an aneurysmal SAH and vasospasm, median age 56 years, 30% male	4 control patients, median age 56 years, 25% male	CSF was collected on a daily basis: for the patients, the period ranged from 1 to 18 days (mean 6.9 days). CSF was centrifuged to remove any contaminating blood cells	By microarray analysis, a total of 265 miRNAs were DE. Of these, 36 miRNAs exhibited significant fold change. 18 of the significantly DEmiRNAs were located within a single comparison group. They included miR-137, -320e, -346, -514-3pm, -521, -624-3p, -708-5p, -1244, -2117, -4521 (Comparison 3) and miR-566 (Comparison 6). Comparison 1, no SAH *vs*. SAH no vasospasm; Comparison 3, no SAH *vs*. SAH vasospasm; Comparison 6, SAH no vasospasm *vs*. SAH vasospasm.	MiR-451a showed significant increased fold changes in a number of group comparisons: 72-fold (Comparison 1), 59-fold (Comparison 3). These two comparisons were between non-SAH and SAH groups, indicating a potential major regulatory role of this miRNA in the pathophysiologic processes involved in aneurysmal SAH.
Powers et al. (2016)	8 patients with aneurysmal SAH, ages ranged 30–74 years, no male		CSF was collected from days 3–12 post hemorrhage and not centrifuged. 52 miRNAs were detected in CSF and fold expression relative to the first time point (day 3) characterized. Hierarchical clustering revealed two large clusters of miRNAs, one that contained miRNAs known to be present in blood and decreased in abundance over tine (Group 1), and a second that showed increased abundance over time (Group 2). In Group 1, the overall downward trend was typified by the abundance of miR-92a and let-7b. In Group 2, the overall increase over time was typified by the abundance of miR-491. These trends were confirmed by RT-PCR with samples collected on days 3, 5 and 11.	The second cluster of miRNAs may be released into the CSF by the brain itself as a result of the initial SAH.	Temporal changes in the release and abundance of specific miRNAs into the CSF by the brain may provide insight into the role of miRNAs in brain injury and the brain's response.
Animal studies					
Muller et al. (2015)	Adult male Sprague-Dawley SAH rats	Sham group as controls	MCAs and basilar artery were dissected.	Of the 482 miRNAs expressed in both sham-operated animals and animals subjected to SAH, four miRNAs (miR-30a, -143, -191* and -223) showed statistically significant changes in expression between the experimental groups. RT-PCR assays confirmed that miR-30a and miR-143 exhibited significantly altered expression levels after SAH when compared to sham animals. Both miR-30a and miR-143 were significantly upregulated at 1 and 6 hours post-SAH as compared to sham. The increases were approximately 2-fold for miR-30a at both time points and 3- and 4-fold, respectively, for miR-143. For both miR-30a and miR-143, the SAH-induced upregulation was transient because expression levels at 24 hours post-SAH did not differ from sham. The other two miRNAs identified in the miRNA screen, miR-191* and miR-223 showed no significant alteration in expression between the sham and the other groups.	MiR-30a and miR-143 might be useful biomarkers for SAH.

CSF: Cerebrospinal fluid; MCAs: middle cerebral arteries; RT-PCR: reverse transcription polymerase chain reaction.

to decrease or increase miRNAs, respectively. As most hemorrhagic stroke patients have existing comorbidities and are aged ≥ 50 years, confirmation studies should involve animal models with hypertension, hyperlipidemia, diabetes mellitus, and aging. The clinical study by Leung et al. (2014) and the experimental animal study by Liu et al. (2010) have shown that miRNA profile can distinguish hemorrhagic stroke from ischemic stroke. The reported miRNA profiles in the studies reviewed would suggest that they can differentiate between ICH and SAH, and clinical studies should be performed to confirm this.

Conflicts of interest: *None declared.*

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