NEURAL REGENERATION RESEARCH
September 2016, Volume 11, Issue 9
www.nrronline.org

INVITED REVIEW

Blood microRNAs as potential diagnostic and prognostic markers in cerebral ischemic injury

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How to cite this article: *Martinez B, Peplow PV (2016) Blood microRNAs as potential diagnostic and prognostic markers in cerebral ischemic injury. Neural Regen Res 11(9):1375-1378.*

Abstract

MicroRNAs are a family of small, genome-encoded endogenous RNAs that are transcribed but are not translated into proteins. They serve essential roles in virtually every aspect of brain function, including neurogenesis, neural development, and cellular responses leading to changes in synaptic plasticity. They are also implicated in neurodegeneration and neurological disorders, in responses to hypoxia and ischemia, and in ischemic tolerance induced by ischemic preconditioning. In recent developments, miRNA expression profiling has been examined in stroke, and these studies indicate that miRNAs have emerged as key mediators in ischemic stroke biology. Both increased and decreased miRNA levels may be needed either as prevention or treatment of stroke. Novel approaches are being developed to get miRNA related therapeutics into the brain across an intact blood-brain barrier, including chemical modification, use of targeting molecules and methods to disrupt the blood-brain barrier.

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doi: *10.4103/1673-5374.191196*

Accepted: *2016-09-08*

Key Words: *blood microRNAs; diagnostic biomarkers; prognostic biomarkers; cerebral ischemic injury; ischemic stroke; human patients; rat and mouse models*

Introduction

Stroke is one of the leading causes of death worldwide and a major cause of long-term disability. The main type of stroke is ischemic stroke (80%) and it is sub-classified as thrombotic or embolic in nature. A thrombotic stroke or infarction occurs when a clot forms in an artery supplying the brain and accounts for approximately 50% of all strokes, whereas an embolic stroke is the result of a clot formed elsewhere in the body and subsequently transported through the bloodstream to the brain. The only US FDA-approved intervention for acute ischemic stroke is intravenous administration of tissue plasminogen activator, which acts by dissolving the blood clot (or recanalization) within 4.5 hours of stroke onset and thereby improving blood flow to the part of the brain being deprived of blood. Owing to the short time window, only a small number of patients receive this therapy.

MicroRNAs (miRNAs) are a family of small, genome-encoded endogenous RNAs that are transcribed but are not translated into proteins. They serve essential roles in virtually every aspect of brain function, including neurogenesis, neural development, and cellular responses leading to changes in synaptic plasticity. They are also implicated in neurodegeneration and neurological disorders, in responses to hypoxia and ischemia, and in ischemic tolerance induced by ischemic preconditioning. Complex interplay among multiple pathways including excitotoxicity, mitochondrial dysfunction, ionic imbalance, oxidative stress, and inflammation are involved in the mechanism of cerebral ischemia/reperfusion injury. These processes lead to both necrotic death in the anoxic core and delayed apoptosis-mediated cell death in the surrounding penumbra. While the fate of neurons in the anoxic core is likely fixed relatively soon after the initial insult, the cells in the peri-ischemic penumbra represent targets for rescue from delayed cell death. In more recent developments, miRNA expression profiling has been examined in stroke, and these studies indicate that miRNAs have emerged as key mediators in ischemic stroke biology.

MicroRNAs in Acute and Chronic Phase of Ischemic Stroke

Yang et al. (2016) reported that the circulating levels of miR-107, -128b, and -153 were significantly elevated in ischemic stroke patients (61 ± 11 years of age; 68% male) in comparison to healthy volunteers (56 ± 4 years of age; 60% male), with the peripheral blood samples collected from patients within 24 hours of hospital admission. The circulating miRNAs were only measured in the acute phase, and although the impact of age, smoking and hyperlipidemia on these was ruled out, it did not rule out the interference of other factors such as hypertension and diabetes mellitus. Also while these circulating miRNAs might be used as potential non-invasive biomarkers for the diagnosis of ischemic stroke, the prognostic values of the circulating miRNAs for ischemic stroke could not be evaluated without the data at the chronic phase of stroke and the functional outcomes. Another study has shown that the highest expression of circulating miR-125b-2*, -27a*, -422a, -488 and -627 occurred within the acute phase of ischemic stroke irrespective of age or severity or confounding metabolic complications with blood collected from patients (60 ± 1 years of age) within 1 to 7 days

of stroke (Sepramaniam et al., 2014). Patients from outpatient clinics in recovery phases from 2 to 24 months from stroke onset were also included in this study. Cluster analysis showed segregation among patients within 6 and 24 months of recovery and these clusters were distinct from acute patients (within 1 to 7 days) indicating temporal regulation of miRNAs during the progression of stroke pathogenesis and with the 'recovery' patients clustered closer with the healthy controls suggesting a tendency for the miRNA expression to return to normalcy (Sepramaniam et al., 2014). Using a rat middle cerebral artery occlusion (MCAO) stroke model, animals were sacrificed over a period of 0 to 72 hours following MCAO and brain samples collected. The highest expression of miR-125b-2*, -27a*, -422a, -488 and -627 occurred in the acute phase (0 to 24 hours) in the brain and in their corresponding blood, confirming that upregulation of expression of these miRNAs is a consequence of acute cerebral ischemia (Sepramaniam et al., 2014). He et al. (2016) identified 16 miRNAs as significantly differentially expressed in the peripheral blood of ischemic stroke patients (2 men and 3 women, 50 to 83 years of age), including 13 upregulated and 3 downregulated miRNAs. Among these miRNAs, the most significantly upregulated miRNA was miR-145 and the most significantly downregulated miRNA was miR-122. However, it was not indicated at what time the blood samples were collected from stroke onset, and the comparison used 5 other individuals as normal samples and were diagnosed as having other cerebral diseases that resulted from injury or anxiety.

In a study of chronic stroke patients 18 to 49 years of age and for which blood samples were collected within 6 to 18 months from the index stroke, it was shown that among the 157 miRNAs identified for stroke samples, 138 miRNAs were highly expressed (upregulated, fold change > 1.0) and 19 miRNAs were poorly expressed (downregulated, fold change < 1.0). Among the highly expressed miRNAs observed for stroke, 17 miRNAs could be identified as highly expressed in the subtypes large-vessel atherosclerosis, small-vessel disease, and cardioembolism (Tan et al., 2009). The miRNA profile of small artery stroke samples showed a distinctly different pattern from that of the large artery stroke samples. Seventy-nine miRNAs could be identified as differentially regulated among these two subtypes. Of these, in the small artery stroke samples, 71 constituted the highly expressed miRNAs (fold change > 1), 7 were found to be expressed at the basal level (fold change 1.0–1.04) and 1 to be poorly expressed (fold change 0.92). An opposite trend was observed in the large artery stroke samples. Of the 79 miRNAs, 77 were downregulated, 1 was undetectable and 1 was upregulated. Of the highly upregulated miRNAs (fold change > 1.5) in small artery stroke samples, 7 miRNAs (miR-130b, -29b, -301a, -339-5p, -532-5p, -634, -886-5p) showed more than 2-fold change. More miRNAs were downregulated in all good outcome (modified Rankin scale < 2) stroke samples compared to normal controls, irrespective of stroke subtype (Tan et al., 2009). For ischemic stroke patients 18 to 49 years of age without any or minimal risk factor, and blood collected between 2 and 24 months from stroke onset, 4 miRNAs (miR-25*, -34b, -483-5p, -498) were downregulated (Tan et al., 2013). These four mi-RNAs could prove to be specific for stroke pathogenesis in low risk stroke patients. In a previous study on young stroke patients with existing risk factors, only miR-25* was found to be expressed but was upregulated (Tan et al., 2009).

A study by Zeng et al. (2011) has shown that miR-210 measured in blood from ischemic stroke patients within 3, 7 and 14 days after stroke was significantly decreased compared to controls especially at 7 and 14 days of stroke onset. There were no changes of miR-210 for different stroke subtypes such as large artery atherosclerotic stroke, cardioembolic stroke, small artery lacunar stroke. The miR-210 level in stroke patients with good outcome (mRS ≤ 2) was significantly higher than patients with poor outcome (mRS > 2). A positive correlation was found between blood and brain miR-210 in MCAO stroke mice. This study suggests that miR-210 is a sensitive biomarker for diagnosis and prognosis in acute cerebral ischemia. Li et al. (2015) examined the levels of three mi-RNAs, miR-185, -146a, and -145, in the blood of ischemic stroke patients ≥ 50 years of age in the acute (1 to 5 days) or subacute phase (6 to 30 days). More of these patients had smoking and hypertension as risk factors when compared to healthy controls. The miR-145 levels in stroke patients were similar to controls, whereas miR-146a and miR-185 levels were downregulated compared with controls. The miR-146a levels were downregulated in the acute phase but upregulated in the subacute phase. For miR-185, the levels were downregulated in both the acute and subacute phases. Among various miRNAs, miR-146a and miR-185 are inflammation-related miRNAs. However, in an earlier study, circulatory miR-145 expression was increased in ischemic stroke patients 18 to 49 years of age compared to healthy control subjects (Gan et al., 2012). It is not clear at what time the blood samples were collected from stroke onset and the difference between this study and that of Li et al. (2015) may be due to different methodologies or existing risk factors.

Jickling et al. (2014) found that eight miRNAs were differentially expressed in blood collected 28 hours from stroke onset between acute stroke patients and vascular risk factor controls, also matched for age and gender (63 years of age, 50% female). Six miRNAs had decreased expression (miR-122, -148a, -let-7i, -19a, -320d, -4429) and two had increased expression (miR-363, -487b). These miRNAs are either known to regulate or are predicted with high probability to regulate the expression of several genes in pathways associated with ischemic stroke including NF-κB signaling, toll-like receptor signaling, leukocyte extravasation, interleukin signaling, transforming growth factor-β signaling, and the prothrombin activation pathway. In MCAO rat models, miR-122 decreased in blood after MCAO and a miR-122 mimic elevated miR-122 in blood 24 hours after MCAO (Liu et al., 2015). Intravenous but not intracerebroventricular injection of miR-122 mimic decreased neurological deficits and brain infarction, attenuated ICAM-1 expression, and maintained vessel integrity after MCAO. The miR-122 mimic also downregulated direct and indirect target genes in blood after

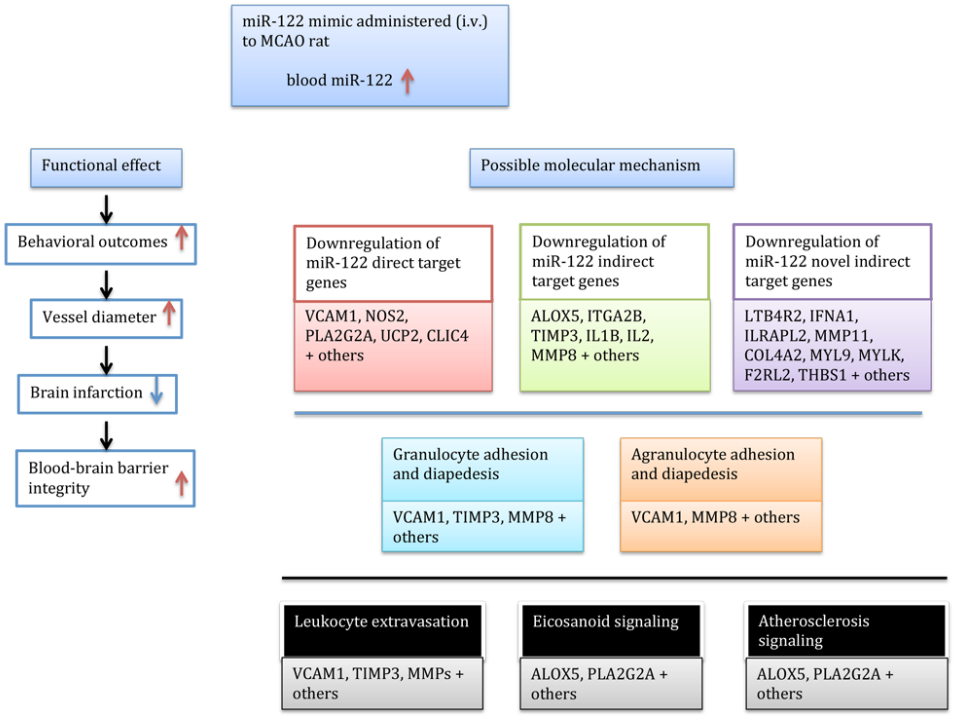

Figure 1 Possible molecular mechanism of miR-122 mimic-mediated support of poststroke recovery.
Upregulation of miR-122 results in stabilization of blood-brain barrier integrity, maintenance of vessel diameter, decreased brain infarction, and improved behavioral outcomes in treated animals. Possible molecular mechanisms mediating these processes could be associated with downregulation of miR-122 direct and indirect target genes by miR-122 mimic (upper panel) that are predicted to affect cell adhesion, diapedesis (middle panel), leukocyte extravasation, eicosanoid and atherosclerosis signaling (lower panel).

MCAO which are predicted to affect cell adhesion, diapedesis, leukocyte extravasation, eicosanoid and atherosclerosis signaling (Liu et al., 2015; **Figure 1**). Jia et al. (2015) examined nine previously reported stroke-associated miRNAs (miR-21, -23a, -29b, -124, 145, -210, -221, -223, -483-5p) in 146 acute ischemic stroke patients and 96 control participants without prior history of stroke, myocardial infarction or peripheral vascular disease. Blood was collected from each patient within 24 hours of stroke onset or control group. Serum miR-145 was significantly increased and serum miR-23a and -221 were decreased in patients. Serum miR-145 was strong positively correlated with plasma high sensitivity-C-reactive protein and moderate positively correlated with serum interleukin-6. Plasma high sensitivity-C-reactive protein and interleukin-6 are pro-inflammation markers in brain injury.

Transient focal ischemia in male rats by MCAO within 24 hours of reperfusion resulted in miR-19b, -290, and -292-5p to be highly expressed, whereas miR-103 and -107 were poorly expressed, in peripheral blood (Jeyasseelan et al., 2008). An entirely new group of miRNAs was observed in the 48-hour reperfusion blood samples. Among the 14 miRNAs that appeared at both time points, miR-150, -195 and -320 showed an opposite trend in expression at 24 and 48 hours, whereas miR-103, -107 and -191 showed almost the same level of downregulation at both time points. Fifty-six common miRNAs were present in brain samples of the rats subjected to MCAO, 32 miRNAs were found to be present in the 24-hour samples only and 8 miRNAs were present exclusively in the 48-hour samples. Upregulation was observed for miR-138 and -145 at 24 and 48 hours of reperfusion, respectively. The let-7 family was upregulated in the 48-hour-reperfused MCAO brain samples together with miR-150 and -125a (Jeyasseelan et al., 2008). A study by Dharap et al. (2008) profiled miRNAs in the brain of adult male spontaneously hypertensive rats as a function of reperfusion time after transient MCAO. Of the 238 miRNAs evaluated, 8 showed increased and 12 showed decreased expression at least at 4 out of 5 reperfusion time points studied between 3 and 72 hours compared with sham. MiR-206, -145, -290 showed increased expression while miR-218, -27a, -137 exhibited decreased expression, and miR-let-7a did not show any change in expression at 24-hour reperfusion compared to sham. Antagomir-mediated prevention of miR-145 expression led to an increased protein expression of its downstream target superoxide dismutase-2 in the postischemic brain of male MCAO rats (Dharap et al., 2008).

MiRNAs may have an important role in neuroprotection brought on by ischemic preconditioning. Lee et al. (2010) showed that ischemic preconditioning was induced in mice by MCAO for 15 minutes, and two miR families, miR-200 and miR-182, were selectively upregulated at 3 hours after ischemic preconditioning. The miR-200 family was neuroprotective mainly by downregulating prolyl hydroxylase 2 levels and increasing hypoxia-inducible factor-1α.

Targeting MicroRNAs as a Novel Therapeutic Approach

Both increased and decreased miRNA levels may be needed either as prevention or treatment of stroke. Novel approaches are being developed to get miRNA related therapeutics into the brain across an intact blood-brain barrier, including chemical modification, use of targeting molecules and methods to disrupt the blood-brain barrier. The potential for antagomir therapy has been shown in the context of acute

ischemic stroke in mice in which regulation of miR-181 affects the extent of brain injury. Knockdown of miR-181 by intracerebroventicular infusion of its antagomir effectively decreased the infarction size and protected the penumbra (Ouyang et al., 2012). Yin et al. (2010) showed intracerebroventricular administration of miR-497 antagomir resulted in smaller infarct area following MCAO in mice. Antagomir-based therapy has demonstrated gender-specific miRNA interactions whereby intracerebroventricular infusion of let7f antagomir promoted neuroprotection in MCAO female rats but not MCAO males (Selvamani et al., 2012). Intravenous injection of a specific miR-155 inhibitor at 48 hours after MCAO in male mice was found to support brain microvasculature, reduce brain tissue damage, and improve the animal functional recovery (Caballero-Garrido et al., 2015).

Future Perspectives

Numerous circulating miRNAs have been reported to have potential value in diagnosis of ischemic stroke, but no miRNAs have yet been clearly identified. Inconsistencies have been found in miRNA expression levels between different array platforms, highlighting the current technical challenges and limitations of miRNA studies. A recent rigorous study revealed inherent problems within and between the different assays (Git et al., 2010). This study emphasizes the complexities and limitations of new technologies for miRNA studies and the need to validate changes in miRNA expression using multiple approaches. It is critical to identify and validate miRNA/mRNA target pairs. As proposed by Kuhn et al. (2008), (1) miRNA/mRNA target interaction must be verified, (2) the miRNA and predicted mRNA target must be coexpressed, (3) a given miRNA must have a predictable effect on target protein expression, and (4) miRNA-mediated regulation of target gene expression should equate to altered biological function. In the next decade of miRNA studies, efforts are needed to advance and evolve the tools necessary for analysis and validation of miRNAs, as these tools will be essential in establishing direct correlations between miRNA-mediated post-transcriptional gene expression and disease (Saugstad, 2013).

In several of the ischemic stroke studies reviewed confirmation of changes in specific miRNAs was confirmed by MCAO in young healthy male rats or mice. Also most of the stroke studies did not indicate the number of male and female patients enrolled in the studies, impeding any efforts to address the importance of gender-specific differences in miRNAs and their contributions to ischemic stroke and/or responses to antagomirs or mimics. In future studies, confirmation of an upregulation or downregulation of individual miRNAs needs to be obtained using both males and females in a rat or mouse stroke model, and also to use antagomirs or mimics to decrease or increase miRNAs, respectively. Finally, as most stroke patients have existing comorbidities and are aged ≥ 50 years, confirmation studies should involve animal stroke models with hypertension, hyperlipidemia, diabetes mellitus and also include aged animals.

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