

## Review Article

# MicroRNA: Not Far from Clinical Application in Ischemic Stroke

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Ischemic stroke predominates in all types of stroke and none neuroprotective agents success in the clinical trial. MicroRNAs are small endogenous noncoding RNA molecules that act as negative or positive regulators of gene expressions by binding completely or partially to complementary target sequences in the mRNAs. The genes which could be modulated by microRNAs play a role in the etiology and pathophysiology ischemic stroke. Therefore, microRNAs may have function on ischemic stroke. A lot of previous studies have investigated the roles of microRNAs in the ischemic stroke. This mini review would highlight the recent progress of microRNAs on the ischemic stroke. Accumulating evidence demonstrated that microRNAs contributed to the etiology of ischemic stroke and modulated the pathophysiological process such as brain edema, local inflammation, and apoptosis in the brain tissues after stroke. And we also discussed the potential application of microRNAs in ischemic stroke such as a biomarker of stroke and drug target. In conclusion, microRNAs play an important role in stroke etiology, pathophysiology, diagnosis, and therapy for ischemic stroke. It needs further research to investigate the biological function in ischemic stroke before it enters the clinical practice.

## 1. Introduction

During the last decades, developing a neuroprotective agent to treat stroke was a nightmare for industry because more than 1700 agents failed [1]. However, due to high mortality and morbidity of stroke, 80% of which are ischemic stroke, looking for a novel therapeutics is an urgent thing [2, 3]. In the recent years, much evidence demonstrates that microRNA may be both potential target and therapeutics for ischemic stroke. microRNA (miRNA) is a small noncoding RNA with 18–25 nucleotides functioning in transcriptional and post-transcriptional regulation of gene expression [4]. It was firstly found by Lee et al. in 1993 in *elegans* [5]. Until now, there are thousands of microRNAs in the human beings, animals, and even plants [6]. They play essential roles in multiple physiology processes, such as neuronal development and angiogenesis and also in many pathophysiological processes, such as inflammation after stroke [7]. At the mean time, microRNAs are also influenced by environmental issues and regulated by other molecules. Twenty years after discovery, miRNA was finally used to treat human disease in one clinical trial. Miravirsen, a locked nucleic acid-modified DNA phosphorothioate antisense oligonucleotide that sequesters

mature miR-122 in a highly stable heteroduplex, was used to treat patients with hepatitis C virus [8]. Therefore, we highlighted the roles of miRNA in the ischemic stroke to find the novel potential way for stroke treatment.

## 2. Biogenesis and Physiological Function of miRNA

miRNAs are obtained from their own genes or introns and most of their genes may lie in the introns of protein and non-protein coding genes [9]. These genes are initially transcribed by RNA polymerase II via binding to their promoters to form pri-miRNA which has a stem-loop precursor in the 3'UTR. The resulting transcript is capped with a specially modified nucleotide at the 5' end, polyadenylated with a poly (A) tail and spliced [10]. The pri-miRNA is recognized by a nuclear protein known as DiGeorge Syndrome Critical Region 8 (DGCR8) and cut by the enzyme Drosha. After the stem-loop is cleaved, it is termed as pre-miRNA which contains six miRNAs. The pre-miRNA is then transported into cytoplasm and cleaved by Dicer enzyme to form functional miRNA with about 22 nucleotides in length [11].

When the mature miRNA is generated, it forms the RNA-induced silencing complex (RISC) containing Dicer and many associated proteins such as Argonaute protein, human immunodeficiency virus transactivating response RNA binding protein (TRBP), and protein activator of the interferon induced protein kinase (PACT) [12]. Then the RISC could bind to these complementary sequences in the target genes to modulate the expressions of these genes. Almost one-third of human coding genes are regulated by miRNAs. In the plant cells, miRNA could be completely complementary to the binding sequences while miRNA more often has only partly the right sequence of nucleotides to bond with the target mRNA in animals [13]. However, nucleotides 2–7 of the partially complementary miRNAs (called as seed region) still have to be perfectly complementary. Moreover, miRNAs from animals are usually complementary to a site in the 3' untranslated region (3'UTR) whereas plant miRNAs are usually complementary to coding regions of mRNAs [14]. It was firstly found that miRNA downregulated the expressions of its target genes; recently, miRNA could up-regulate the expression of its target genes, such as miR369-3 which associates with adenylate/uridylylate-rich elements (AREs) to initiate the transcription of the target gene [15].

### 3. Detection of miRNA

Unlike other types of mRNA, miRNA is stable for long while in the serum [16]. It provides the base for the transmission. In some small pilot studies, researchers use some specific miRNAs as the marker of diseases for miRNAs represents the physiology or pathophysiology situation in the body [13]. How to detect the miRNA in the serum or tissues is the key of clinical application and research. Currently, there are some common ways to measure the expression of miRNA, northern blots, hybridization in situ, microarray, reverse transcription PCR, real-time PCR, and Taqman miRNA assays. The major advantages and disadvantages of these technical tools were summarized in another review [17].

### 4. miRNA and Stroke Pathophysiology

Above all the known miRNAs, 70% are found in the brain tissues and many of them are only found in brain tissues, such as miR-9, miR-124a, miR-124b, miR-135, and miR-219 [18]. These miRNAs regulate the neuron development process, such as neurogenesis, synaptic formation, and stem cell differentiation [19]. They also play important roles in the pathophysiological process such as self-neuroprotection and influence the outcomes of neurological disorders [20]. Here we discussed the roles of miRNAs in the etiology, the pathophysiology, the biomarkers, and the therapeutics.

**4.1. miRNA Contributes to the Stroke Etiology.** The atherosclerosis is the major cause of ischemic stroke, either in carotid or intracranial artery [21]. The foam cells in the plaque create a necrotic center which could lead to thrombus occluding the vessels and causing a stroke. The foam cells are obtained from the macrophages absorbing the oxidized-low density

lipoprotein (LDL). miRNA functions both in macrophages and oxidized-LDL [22]. miR-126 could target the 3'UTR of vascular cell adhesion molecule 1 (VCAM-1) and modulate its expression [23]. The VCAM-1 containing six or seven immunoglobulin domains is expressed in the activated endothelial cells to promote the adhesion of macrophages to vascular endothelium which plays a role in the development of atherosclerosis [24]. In the atherosclerosis, the low level of miR-126 leads to the high expression of VCAM1 which in turn promotes the atherosclerosis. On the other side, miR-125a-5p targeted the oxysterol binding protein-related protein 9 (ORP9) and decreased the level of oxidized-LDL which inhibited the generation of foam cells. When using the specific antagonist of miR-125a-5p, it increased the number of foam cells and also promoted the overexpression of oxidized-LDL receptor 1 [25].

Shearing force is the leading cause of atherosclerosis in the arterial bifurcation [26]. However, the underlying mechanism is unclear. Recently, it was found that shearing force could induce the overexpression of miR-21 in the endothelium. Overexpression of miR-21 could decrease the expression of phosphate and tensin homolog (PTEN) which is proved to inhibit the intimal hyperplasia of carotid artery [27]. Besides that, miR-21 stimulated the growth of vascular smooth muscle cell in vessels after injury [28].

Angiogenesis within the plaque increases the risk of plaque hemorrhage and rupture which could lead to severe vascular events [29]. A lot of miRNAs play a role in this process. miR-122 increases the growth and migration of vascular endothelial cells via downregulating the expression of signal transducer and activator of transcription 5a (STAT5a) [30]. And miR-221/miR-222 promoted the growth of artery via downregulating c-Kit [31]. The miRNAs which effected the angiogenesis were summarized by others [32, 33].

**4.2. miRNA and Apoptosis.** Ischemia causes the breakdown of blood brain barrier (BBB) which would let many substances enter the brain and aggravate the brain edema. Recently, researchers found that miR-15a could directly target Bcl-2 gene and decrease its expression. Bcl-2 is an antiapoptosis gene [34]. The inhibition of Bcl-2 induced the apoptosis of vascular endothelial cells. The expression of miR-15a was under control of peroxisome proliferator-activated receptor  $\delta$  (PPAR $\delta$ ) and the inhibitor could reverse the effect of miR-15a and demonstrate neuroprotection in vivo [35]. Besides miR-15a, 3'UTR of Bcl-2 also has the binding site of miR-497 which could be up-regulated by ischemia. Overexpression of miR-497 amplified the ischemic injury via inhibition of Bcl-2 while antagomir-497, its specific inhibitor, could reduce the infarct volume, decrease the mortality, and improve the neurological function in experimental stroke [36].

miRNAs are not always bad guys in ischemic stroke and many of them showed neuroprotection in vitro and in vivo. Ji et al. found that miR-21 was overexpressed in the penumbra when compared to the same area in the contralateral hemisphere [28]. Moreover, the overexpression of miR-21 in vitro could protect neurons against the ischemic insult [37]. The reason was that miR-21 could target and

decrease the expression of Fas ligand which is proved to be an inducer of cell death [38]. When the miR-21 was inhibited, the cell death was increased.

**4.3. miRNA and Local Inflammation after Ischemic Stroke.** Inflammation plays a vital role in the pathogenesis of ischemic stroke [39]. Inflammatory responses to cerebral ischemia include rapid activation of resident inflammatory cells, production of inflammatory mediators, and translocation of intercellular nuclear factors [40–43]. The activated inflammatory process would increase the astrogliosis which could exacerbate the inflammatory process further. One study showed that miR-125b played a role in the astrogliosis via binding to the 3'UTR of cyclin-dependent kinase inhibitor 2A (CDKN2A) and decreasing its expression [44]. In normal situation, CDKN2A is a negative regulator of cell growth and inhibits the growth of astrocytes. Application of antagomir-125b, the inhibitor of miR-125, increased the astroglial growth in ischemic stroke.

As to inflammatory cytokines, miRNA also modulates their expressions in the ischemic stroke brain tissues. Interferon  $\beta$  is an anti-inflammation cytokine and could prevent the neuron from ischemic injury for it could decrease the infarct volume by 30% [45]. Some miRNAs such as miR-26a, miR-34a, miR-145, and let-7b have their targeting site in the 3'UTR of interferon  $\beta$ . And they regulated the expression of interferon  $\beta$  to influence the outcome of ischemic stroke [46].

**4.4. miRNA and Cerebral Edema.** Ischemic stroke leads to many kinds of cerebral edema such as vasogenic and cytotoxic edema [47]. Cerebral edema is an excess accumulation of fluid in the intracellular or extracellular spaces of the brain and aquaporin controls the water balance. Therefore, the disturbance of aquaporin would lead the unbalance of water. miR-320a was found to downregulate the expression of aquaporin 1 and 4 which play essential roles in the water reabsorption. In ischemic brain tissues, miR-320a was increased and when it was inhibited, the cerebral edema was decreased [48].

**4.5. miRNA and Ischemic Preconditioning (IPC).** IPC is an intrinsic process whereby repeated short episodes of ischemia protect the cells against a subsequent ischemic insult [49]. Though it demonstrates neuroprotection in many studies, its underlying mechanism is still unclear [50]. It was found that ischemic preconditioning increased the expressions of miR-200 family (included miR-200a, miR-200b, and miR-429) in the brain tissues. Then overexpression of these miRNAs increase the survival rate of neurons treated by oxygen glucose deprivation. miRNA-200 increased the stability of hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) via binding to prolyl hydroxylase 2 (PHD2) which is shown to promote the degradation of HIF-1 $\alpha$ . HIF-1 $\alpha$  is a nuclear factor which controls many antiapoptotic gene expressions. Therefore, miR-200/PHD2/HIF-1 $\alpha$  plays a role in the ischemic preconditioning [51]. Besides miR-200, another miRNA, the effect of miR-132 on ischemic preconditioning was evaluated later. It was found that in the cortex with ischemic preconditioning,

there were overexpression of methyl-CpG binding protein 2 (MeCP2) and low expression of miR-132. MeCP2 has a binding site of miR-132 in its 3'UTR and previous research showed that MeCP2 is a key protein in the ischemic preconditioning [52].

## 5. miRNAs as Biomarkers of Ischemic Stroke

Since miRNAs were associated with ischemic stroke pathophysiological process, they might be used as biomarkers to diagnosis the occurrence of stroke. Jeyaseelan et al. tested the miRNAs in the brain tissues and serum and found that 24 h and 48 h after ischemic stroke, there were significant differences of miRNAs compared to the baseline. Some miRNAs like miR-298, miR-155, and miR-362-3p may increase or decrease by more than 2-fold. More importantly, it was found that the miRNAs in the serum were in proportion to that in brain tissues [53]. This result was further confirmed by another study [54]. These data implied that miRNA could be used as a diagnostic tool in ischemic stroke.

Ischemic stroke is not a single disease but a syndrome. The outcomes of stroke varies from different causes. Makeing the subtypes of ischemic stroke is important to predict the prognosis. miRNA was proved to be a useful tool to differentiate the different subtypes in one study. miRNA had different profiles between small vessels and large vessels related stroke [54]. Another study demonstrated that the peripheral blood miRNAs and their profiles can be developed as biomarkers in diagnosis and prognosis of cerebral ischaemic stroke [55]. The dysregulated miRNAs have been detectable even after several months from the onset of stroke in what is usually regarded as neurologically stable patients [55].

## 6. Drug Target of Ischemic Stroke

As we mentioned, miRNA contributed to the pathophysiological process. And more importantly, the changes of miRNA happened early or immediately after ischemic stroke and these changes would affect the local inflammation, neuron apoptosis, and brain edema via changing the expressions of target genes. Therefore, if restoring the expressions of miRNA, it would help to treat stroke and improve the prognosis of stroke patients. Some miRNAs were up-regulated while some downregulated; hence, the overexpressed miRNA need to be treated by inhibitor while the low expressed miRNAs need to be increased.

**6.1. Increased Levels of miRNA to Treat Stroke.** Currently, there are two common ways to increase the level of miRNA, miRNA mimic, and vectors containing the miRNA genes. miRNA mimic means a double-stranded or single-stranded oligonucleotide or analog thereof with a substantially similar base composition as a particular miRNA. Usually, its 5'-end bears a partially complementary motif to the selected sequence in the 3'UTR unique to the target gene as an endogenous miRNA and produces posttranscriptional repression [56]. Unlike endogenous miRNAs, miR-Mimics act in a gene-specific fashion. The miR-Mimic approach

belongs to the “miRNA-targeting” and “miRNA-gain-of-function” strategy and is primarily used as an exogenous tool to study gene function by targeting mRNA through miRNA-like actions in mammalian cells [57]. miR-21 was shown to protect neurons against the insult of OGD and miR-21 mimics also demonstrated the similar neuroprotection [28].

Another traditional way to improve the expression of specific gene is to insert the gene fragment into a vector such as plasmid and viral vector under a common promoter and introduce this recombinant vector into the cells [58]. Zhao et al. used lentiviral vector to overexpress miR424 to treat experimental stroke. They found that pre- and posttreatment with Lenti-miR-424 both decreased cerebral infarction size and brain edema after middle cerebral artery occlusion. Meanwhile, this recombinant virus inhibited neuronal apoptosis and microglia activation, including suppressing ionized calcium binding adaptor molecule-1 immunoreactivity and protein level and reduced tumor necrosis factor- $\alpha$  production [59]. Liu et al. inserted the gene fragment of miR-17-92 into the plasmid and transduced the recombinant plasmid either into cultured ischemic neural progenitor cells or into the subventricular zone (SVZ) of ischemic animals significantly increased cell proliferation in stroke [60]. And in another study, an adeno-associated viral vector (AAV) was used to overexpress miR-223 to investigate its role in stroke. The result showed that miR-223, as a major regulator of the expression of GluR2 and NR2B, has a therapeutic role in stroke [61].

**6.2. Inhibition of miRNA Treating Stroke.** After stroke, expressions of many genes are inhibited due to the high level of miRNAs. Therefore, downregulation of these miRNAs may be the therapeutic targets for ischemic stroke. There are many tools to decrease the level of miRNA such as antisense oligonucleotide (ASO) and antagomirs. ASOs are single strands of DNA or RNA that are complementary to a specific sequence. The antisense RNA could prevent translation of certain mRNA strands by binding to them and antisense DNA can target its complementary (coding or noncoding) RNA to form the DNA/RNA hybrid which can be degraded by the enzyme RNase H [62]. Antagomirs are a novel class of chemically engineered oligonucleotides that they prevent other molecules from binding to a desired site on an mRNA molecule. They are designed to have a sequence that is complementary to an mRNA sequence (3'UTR) that serves as a binding site for microRNA. Upon binding, Blockmirs sterically block microRNA from binding to the same site, which prevents the degradation of the target mRNA via RISC [63]. Mir-145 is one of the miRNAs that showed significant upregulation from 3 h to 3 days of reperfusion after transient MCAO and bioinformatics analysis with RegRNA and Miranda showed that rat miR-145 sequence has a complementary 8-bp targeting site in the 3'-UTR of rat SOD2. Whereas infusion of an antagomir-145 that targets mir-145, the area that showed SOD2 immunoreactivity was much bigger with more numbers of neurons showing intense SOD2 immunostaining. Antagomir-145 also decreased cortical infarcts [64]. Another study used antagomir-181 to treat

stroke and found that reduced levels of miR-181 were associated with reduced injury by increasing GRP78 protein levels [65]. Other antagomirs were developed and investigated such as antogomir-let7f which targets insulin-like growth factor-1 [66] and antogomir-29c which inhibits DNA methyltransferase 3a [67]. And the clinical trial also used antogomir to treat the HCV patients and got the positive results. All these results implied that antogomirs via downregulating the key miRNA may be the next generation of therapeutics for ischemic stroke [8].

Due to the efficiency of inhibition, ASO may be a potential therapeutic in stroke area [68]. The atherosclerosis in carotid artery is the high risk factor of stroke. The expression level of miR-21 in dedifferentiated vascular smooth muscle cells was significantly higher in balloon injury vessels. Modulating an aberrantly overexpressed miR-21 via ASO had a significant negative effect on neointimal lesion formation [28].

## 7. Perspectives

In the last decades since the first miRNA was identified, people have made large progress on the nature of miRNA and also the relation of miRNA and stroke. Current evidence indicates that miRNAs play roles in stroke etiology and pathophysiology and may act as biomarkers and drug targets or therapeutics. However, it is far away from the clinical application due to some severe problems waiting to solve.

First problem is the unspecific and minimal effect of miRNA. miRNA modulate their target genes via complementary to the seed sequence. However, most of seed sequences only have 7 or 8 nucleotides. Hence, one mRNA has multiple miRNA targets while one miRNA could bind to its binding site in multiple mRNAs. This will pose two questions. One is that only one miRNA could not effectively down- or up-regulate the genes which means the low biological effect. Another is that the nonspecific target of miRNA which would cause severe adverse effects due to the normal genes was disturbed.

The second problem is how to bring the miRNA into the brain. According to the Stroke Treatment Academic Industry Roundtable (STAIR) [1], to develop a new neuroprotection agent, the route of drug delivery need to be considered due to the existence of blood brain barrier (BBB) which prevents most of exogenous substances from entering the central nerve system [69]. Intravenous delivery is easy, safe, cheap, and is widely used in the clinical practice. However, the major advantage is the poor distribution in the brain due to BBB which is intact in the initial several hours after stroke onset [70]. In the preclinical studies, people choose to directly inject the antagomirs or miRNA-Mimic [59] into the brain to avoid this problem [71]. Obviously, it would be limited in the animal models for the secondary injury of the surgery and the expensive fees. In the recent years, a novel drug delivery that targets the brain comes up which is intranasal delivery. Although the exact mechanism of intranasal delivery is not entirely understood, a great deal of evidence demonstrated that the pathways involve olfactory

nerve pathways, trigeminal nerve pathways, vascular pathways and lymphatic pathways [72]. As to miRNA, one study used intranasal delivery of AM206, a neutralizing inhibitor of miR-206 (antagomir), to treat Alzheimer's disease (AD). The results showed that intranasally administered AM206 reached the brain and increased memory function in AD mice [73].

In conclusion, miRNAs play an important role in stroke etiology, pathophysiology, diagnosis, and therapy for ischemic stroke. It needs further research to investigate the biological function in ischemic stroke before it enters the clinical practice.

## Authors' Contribution

Yun Li and Yahong Liu contributed equally to this work.

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