

## MicroRNAs in Ischemic Stroke: From Animal Studies to Preclinical Research

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### ABSTRACT

**INTRODUCTION:** MicroRNAs (miRNAs) constitute a class of endogenous small non-coding RNAs. Expanding evidence indicates that a set of miRNAs, expressed in a spatially and temporally controlled manner in the brain, are involved in neuronal

development, differentiation and synaptic plasticity.

**AIM:** The aim of our review was to summarize the role of miRNAs in the development of ischemic stroke based on animal and human and preclinical treatment studies.

**CONCLUSION:** Disregulated miRNAs have revealed their complex role in pathophysiological processes in both animal and human research, and have also shown their potential role in disease diagnosis, and use as drug targets in ischemic stroke. Although miRNAs represent a potentially powerful treatment paradigm, much work remains to be done in describing individual specific miRNA-mRNA interactions, understanding the regional and cell type specific distribution of miRNAs within the nervous system, and in developing organ-targeted delivery systems to optimize the therapeutic potential of miRNAs.

**Key words:** Ischemic stroke; MicroRNA; Animal model; Human study; Preclinical research; Pathophysiology; Treatment

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### INTRODUCTION

Although microRNAs (miRNAs) were discovered in the early 90's, their presence in humans was confirmed in the beginning of the 21st century<sup>[1,2]</sup>. They are endogenous, noncoding, single-stranded small RNAs (approx of ≈22 nucleotides) and play an important role in gene regulation with an entirely novel level of control by either degrading mRNAs or arresting their translation<sup>[3]</sup>. MiRNAs hybridize to partially complementary binding sequences that are typically localized in the 3'untranslated regions (3'-UTR) of target mRNAs, resulting cleavage or translational repression in a sequence-specific manner<sup>[4]</sup>. They seem to regulate the expression of at least one-third of the human genome and play a critical role in various biological processes, including cell differentiation, apoptosis, development, angiogenesis and metabolism<sup>[4,5]</sup> MiRNAs are abundantly expressed

in the nervous system and have been implicated in a variety of human neurological diseases<sup>[4-6]</sup>.

Stroke, which is mainly ischemic, is one of the leading causes of death and the leading cause of disability worldwide. Ischemic stroke (IS) is a complex genetic disorder caused by a combination of multiple genetic and environmental factors<sup>[7]</sup>. Currently, thrombolytic therapy (both intravenous and interventional) within a narrow time window is the only acute therapeutic intervention for ischemic stroke, and vast majority of the patients falls out of the treatment due to strict inclusion criteria and limited time window<sup>[4]</sup>. Regarding secondary prevention therapy, patients will receive therapies depending on etiology, however, the etiology of approximately one third of all stroke cases remains unknown even after a precise and advanced diagnostic workup<sup>[8]</sup>. The use of biomarkers would lowering the rate of cryptogenic strokes and also could contribute to speed up the diagnostic process prescribing the most appropriate primary and secondary treatments in order to minimize therapeutic risks and to avoid recurrences<sup>[8]</sup>. Due to the enormous research effort in this field, several pathophysiological aspects of cerebrovascular diseases have been elucidated. Nevertheless, many mechanisms still need to be clarified, thus preventing the development of efficient diagnostic and therapeutic strategies for a relevant number of patients<sup>[4]</sup>.

Increasing evidence shows that miRNA-mediated regulation may be important in the occurrence and development of neurological diseases and may function as a novel biomarker and tool for clinical therapy. The aim our review was to summarize the current evidence (based on experimental and clinical data) of microRNAs in the development of ischemic stroke and collecting articles focusing on potential treatment targets.

The aim of our review was to collect articles based on the etiology and clinical importance of miRNAs in acute ischemic stroke. We searched PubMed, MEDLINE, and the Cochrane Library restricted to English language publications up to December 2019. We used these search items in the following combinations: microRNA, experimental stroke, ischemic stroke, animal study, basic research, clinical study, outcome, mortality, death. After reviewing the abstracts, we obtained and reviewed the full text and reference lists of relevant articles.

## ANIMAL STUDIES

In their very first study, Jeyaseela *et al.* miRNA profiling (screening) were performed on rat brains subjected to middle cerebral artery occlusion (MCAo) and reperfusion for 24 or 48 hours<sup>[10]</sup>. They have identified the expression of 114 miRNAs and among them, 106 and 82 transcripts were detected in the 24-hour and 48-hour reperfusion brain slices. Based on their results distinct regulation patterns for 7 clusters of miRNA could be supposed in the background of ischemic injury, and some of them possibly control the expression of 4 genes known to be important in the progression of cerebral ischemia<sup>[10]</sup>.

In their well-conducted study Dharap *et al.* profiled miRNAs in adult rat brain as a function of reperfusion time after transient middle cerebral artery occlusion. 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 3h and 3 days compared with sham<sup>[11]</sup>. This analysis showed that the 8 miRNAs tested can bind to the promoters of many genes that play an important role in normal and ischemic brain<sup>[11]</sup>.

Different regulation patterns can be seen in experimental ischemic and hemorrhagic strokes and drug-induced seizures<sup>[12]</sup>. MiR-298 was the only miRNA that was upregulated in both brain and blood after

almost all experimental conditions. This microRNA was recently shown to recognize specific binding sites in the 30-UTR of b-amyloid precursor protein-converting enzyme-1 (BACE1) mRNA and to regulate BACE1 protein expression in cultured neuronal cells<sup>[12]</sup>.

Cerebral vascular endothelial cells (CECs) are the basic components of the blood-brain barrier (BBB) and play a critical role in maintaining cerebral homeostasis in physiological conditions; ischemia-induced cerebral endothelial injury or death increases vascular permeability and disruption of the BBB, leading to primary brain damage and post-ischemic secondary injury<sup>[13]</sup>. Experimental data suggest that peroxisome proliferator-activated receptor  $\delta$  (PPAR $\delta$ ) activation has a potential neuroprotective role in ischemic stroke<sup>[13]</sup>. In oxygen-glucose deprivation (OGD) and a transient focal cerebral ischemia model PPAR $\delta$  inhibits pro-apoptotic miR-15a expression in a transcriptional manner, and subsequently increases the translation of the anti-apoptotic protein bcl-2, resulting in a reduction of caspase-3 activity, fragmentation of the Golgi apparatus, and cell death in mouse cerebral endothelium after *in vitro* and *in vivo* ischemic insults<sup>[13]</sup>.

In another interesting experimental study two miRNA families, miR-200 and miR-182, were upregulated early after ischemic preconditioning and the miR-200 family was neuroprotective mainly by downregulating prolyl hydroxylase 2 levels<sup>[14]</sup>.

Based on previous results microRNA-21 (miR-21) is seemed to be a strong antiapoptotic factor in some biological systems<sup>[15]</sup>. Overexpression of miR-21 protects against ischemic neuronal death, and that downregulation of the tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) family member, Fas ligand (FASLG), which is an important cell death-inducing ligand whose gene is targeted by miR-21, probably mediates the neuroprotective effect<sup>[15]</sup>.

Stroke-induced cell death in males is initiated by the mitochondrial release of apoptosis-inducing factor, resulting in caspase-independent cell death. In contrast, ischemic cell death in females is primarily triggered by mitochondrial cytochrome c release with subsequent caspase activation. Because X-linked inhibitor of apoptosis (XIAP) is the primary endogenous inhibitor of caspases, its regulation may play a unique role in the response to injury in females<sup>[16]</sup>. miR-23a directly bound the 3' UTR of XIAP, and miR-23a inhibition led to an increase in XIAP mRNA *in vitro*, demonstrating that XIAP is a target for miR-23a. miR-23a levels differed in male and female ischemic brains, providing evidence for sex-specific miRNA expression in stroke<sup>[16]</sup>.

The Notch pathway is a highly conserved regulatory signaling network and has been linked to a variety of pathogenic conditions in human<sup>[17]</sup>. The Notch signaling pathway critically controls stem cell maintenance and cell fate determination, furthermore focal cerebral ischemia activates the Notch signaling pathway in neural progenitor cells localized to the subventricular zone (SVZ) of the lateral ventricle, leading to expansion of neural progenitor cells<sup>[17]</sup>. It has been demonstrated that stroke alters miRNA expression in SVZ neural progenitor cells and miR-124a mediated stroke-induced neurogenesis by targeting the Jagged-1 (JAG)-Notch signaling pathway<sup>[17]</sup>.

Chaperones play a key role in the organization of molecular, organellar, and cellular networks under both physiological and pathological conditions. The heat shock proteins of the 70 kDa molecular weight family (HSP70), including HSP72 (cytosol), GRP75 (mitochondria), and GRP78/BIP (endoplasmic reticulum; ER), are highly evolutionarily conserved; all three of these HSP70 family members are protective in animal models of stroke<sup>[18]</sup>. Recent data showed that miR-181 contributes to cerebral ischemic injury, at early reperfusion miR-181 increases in the injured area destined

to die, but decreases in the area that is potentially salvageable<sup>[18]</sup>. Knockdown of endogenous miR-181 provides protection against ischemia/reperfusion-induced brain cell death by targeting 78 kDa glucose-regulated protein (GRP78), a well-studied and important molecular chaperone and cytoprotective protein<sup>[18]</sup>.

It has been previously shown that enhancement of axonal outgrowth and myelination in the ischemic boundary zone (IBZ, the area that is adjacent to the infarcted area, and potentially salvageable after stroke) leads to improved functional outcomes in animal models<sup>[19]</sup>. Serum response factor (SRF) is a transcription factor that transactivates actin associated genes, and has been implicated in oligodendrocyte (OL) differentiation. In animal models SRF was upregulated in OLs and OL precursor cells (OPCs) after stroke. Moreover, upregulation of SRF was concurrent with downregulation of the miRNAs, miR-9 and the miR-200 family in the ischemic white matter region, the corpus callosum. Inhibition of SRF activation by CCG-1423, a specific inhibitor of SRF function, blocked OPCs from differentiating into OLs. Over-expression of miR-9 and miR-200 in cultured OPCs suppressed SRF expression and inhibited OPC differentiation. Moreover, co-expression of miR-9 and miR-200 attenuated activity of a luciferase reporter assay containing the Srf 3' untranslated region (UTR)<sup>[19]</sup>.

Hibernation torpor provides an excellent model of natural tolerance to ischemia<sup>[20]</sup>. During torpor, hibernating animals lower their energy consumption, blood flow and body temperature to otherwise lethal levels, but because of special adaptive changes, suffer no CNS damage or cellular loss despite levels of brain blood flow that are characteristic of the ischemic core of a stroke<sup>[20]</sup>. The miR-200 family (miR-200a,b,c/miR-141/miR-429) and the miR-182 family (miR-182/miR183/miR-96) were in the brain during the torpor phase raising the possibility that natural tolerance to brain ischemia in hibernators is linked to regulation by microRNAs of a broad range of ubiquitin-like modifiers<sup>[20]</sup>.

Overstimulation of the glutamate receptor (glutamate excitotoxicity) is a major mechanism for neuronal cell death during stroke, central nervous system (CNS) trauma, and chronic neurodegenerative disorders. Excessive calcium influx through the N-methyl-D-aspartate receptors (NMDARs) results in abnormally high intracellular calcium concentrations leading to lethal consequences<sup>[21]</sup>. miR-223 controls the response to neuronal injury by regulating the functional expression of the glutamate receptor subunits (GluR2 and NR2B) in brain. Overexpression of miR-223 lowers the levels of GluR2 and NR2B by targeting 3'-UTR target sites in GluR2 and NR2B, inhibits NMDA-induced calcium influx in hippocampal neurons, and protects the brain from neuronal cell death following transient global ischemia and excitotoxic injury. MiR-223 deficiency results in higher levels of NR2B and GluR2, enhanced NMDA-induced calcium influx, and increased miniature excitatory postsynaptic currents in hippocampal neurons<sup>[21]</sup>.

Glutathione depletion and 12-lipoxygenase-dependent metabolism of arachidonic acid are also known to be implicated in neurodegeneration associated with acute ischemic stroke<sup>[22]</sup>. Neural cell death caused by arachidonic acid insult of glutathione deficient cells was preceded by a 12-lipoxygenase-dependent loss of miR-29b. Based on this study, loss of miR-29b at the infarct site was a key contributor to stroke lesion. Such loss is contributed by activity of the 12-lipoxygenase pathway providing maiden evidence linking arachidonic acid metabolism to miRNA stroke biology<sup>[22]</sup>.

Focal cerebral ischaemia is a powerful stimulus that elicits a specific and dynamic spatio-temporal pattern of gene induction<sup>[23]</sup>. In a well-designed study Gubern *et al.* identified 32 miRNAs with

altered expression in ischaemic brain, most in both the acute and late phases. They have identified miR-347 as a new ischaemia-regulated miRNA, and Acs14 and Arf3 as new ischaemia-regulated genes, as well as demonstrating the involvement of miR-347 in neuronal apoptotic death and the regulation of Acs14, Bnip31 and Phyhip expression<sup>[23]</sup>.

Ischemic stroke causes neuronal cell death that can be separated into two distinct regions, the core and penumbra<sup>[24]</sup>. Neurons within the core die within minutes to hours, the ischemic penumbra, is functionally silent but metabolically active and can account for up to half of the ischemic infarct at early stages<sup>[24]</sup>. Recent data showed that miR-19b, miR-29b-2\* and miR-339-5p are up-regulated following ischemic insults and may be regulating gene expression to control important cellular pathways in the salvageable ischemic penumbra<sup>[24]</sup>.

The receptor p75NTR is a member of the tumor necrosis factor (TNF) receptor superfamily. p75NTR binds to mature and precursor neurotrophins (proneurotrophins) and regulates numerous functions, including neuronal differentiation, neurite outgrowth, and cell death<sup>[25]</sup>. Previous studies have noted the incidental induction of p75NTR in neurons after brain ischemia; however, its specific role in ischemic injury and the identity of the p75NTR ligand mediating potential effects are unknown<sup>[25]</sup>. A recent study identified miR-592 as a key regulator of p75NTR expression and point to a potential therapeutic candidate to limit neuronal apoptosis after ischemic injury<sup>[25]</sup>.

Three specific groups of miRNAs were observed among a total of 346 differentially expressed miRNAs. miRNAs, miR21, -142-3p, -142-5p, and -146a displayed significant upregulation during stroke recovery (48 hrs to 168 hrs) compared with those during acute phases (0 hrs to 24 hrs). On the other hand, an opposite trend was observed in the expression of miR196a/b/c, -224 and -324-3p. Interestingly, miR-206, -290, -291a-5p and -30c-1\*, positively correlated with the infarct sizes, with an initial increase up to 24hrs followed by a gradual decrease from 48 hrs to 168 hrs (R=0.95). Taken together with the expression levels of corresponding mRNA targets, Hedgehog, Notch, Wnt and TGF- $\beta$  signaling pathways seemed to be play significant roles in stroke recovery and especially in neuronal repair<sup>[26]</sup>.

REST (Repressor Element-1 Silencing Transcription factor, also termed NRSF) is a gene silencing transcription factor that is widely expressed during embryogenesis and the late stages of neuronal differentiation and its loss is critical for elaboration of the neuronal phenotype<sup>[27]</sup>. In mature neurons, REST is quiescent, but can be reactivated in selectively-vulnerable hippocampal neurons by ischemic insults<sup>[27]</sup>. The activation of REST is causally related to silencing of miR-132 in selectively-vulnerable CA1 neurons and that REST-dependent repression of miR-132 is critical to ischemia-induced neuronal death in a clinically-relevant model of ischemic stroke *in vivo*. Notably, overexpression of miR-132 in the hippocampal CA1 of living rats affords robust protection against ischemia-induced neuronal death<sup>[27]</sup>.

Multipotent mesenchymal stromal cells (MSCs) have potential therapeutic benefit in many diseases including neurological diseases, injury and stroke<sup>[28]</sup>. Previous studies suggest that exosomes from MSCs mediate the miR-133b transfer to astrocytes and neurons, which regulate gene expression, subsequently benefit neurite remodeling and functional recovery after stroke<sup>[28]</sup>.

## IN VIVO AND IN VITRO TREATMENT STUDIES

Antagomirs are RNA-like oligonucleotides that harbor various

modifications for RNase protection and pharmacologic properties such as enhanced tissue and cellular uptake. They differ from normal RNA by complete 2'-O-methylation of sugar, phosphorothioate backbone and a cholesterol-moiety at 3'-end. Antagomirs efficiently silence miRNAs in most tissues after three injections at 80 mg/kg bodyweight (bw) on consecutive days<sup>[29,30]</sup>. The efficient use of oligonucleotides to target miRNAs for functional studies and therapeutic use will be dependent on characterizations based on miRNA targets in vivo<sup>[30]</sup>.

Insulin-like growth factor-1 (IGF-1) seemed to be neuroprotective to when administered 4 h post stroke in female rats, making this a promising candidate for stroke therapy. IGF-1 therapy is also tolerated well in human patients, however its capacity to promote tumor (including glioblastoma) growth and metastasis, necessitates closely monitoring of its usage<sup>[31]</sup>. Antagomirs to two miRNAs, miR1 and Let7f, with consensus binding sites in the 3' UTRs of multiple IGF signaling pathway components confer neuroprotection in female rats, while antagomir to a brain-specific miRNA not associated with IGF signaling, was not neuroprotective<sup>[31]</sup>.

MiR-181a is highly conserved across most vertebrates, is enriched in the brain and increases during maturation of hippocampal neurons. The overexpression of miR-181a induces drug addiction-related synaptic changes in the hippocampus and increases astrocyte death because of glucose deprivation. MiR-181a upregulated in the infarct core and downregulated in the penumbra after focal ischemia<sup>[32]</sup>. MiR-181a antagomir reduced neuronal injury in vitro and hippocampal neuronal loss in vivo after forebrain ischemia<sup>[32]</sup>. MiR-181a inhibition is thus a potential target in the setting of forebrain or global cerebral ischemia as well as focal ischemia. Based on another study, post-treatment with miR-181a antagomir significantly reduced infarction size, improved neurological deficits and reduced NF- $\kappa$ B activation and numbers of infiltrating leukocytes. Targets affected by miR-181a antagomir administered after stroke onset include X-linked inhibitor of apoptosis protein (XIAP). Posttreatment with miR-181a antagomir significantly improved behavioral outcome assessed by rotarod at one month<sup>[33]</sup>.

Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (NCX) is a plasma membrane transporter that, by regulating Ca<sup>2+</sup> and Na<sup>+</sup> homeostasis, contributes to brain stroke damage<sup>[33]</sup>. Among those genes whose expression is influenced by cerebral ischemia is the sodium-calcium exchanger-1 (NCX1), a ubiquitous plasma membrane protein regulating cellular calcium and sodium homeostasis in the brain<sup>[34]</sup>. Vinciguerra *et al.* demonstrated in a rat model of transient cerebral ischemia that microRNA-103-1 selectively downregulates brain NCX1 and its inhibition by anti-miRNA ameliorates stroke damage and neurological deficits<sup>[34]</sup>.

Computational analysis of potential neuronal targets of miR-200c identifies reelin, an extracellular matrix protein essential for proper neuronal migration in the developing brain and in maintaining synaptogenesis in adulthood. Reelin coordinates neuronal cell survival by inhibiting apoptosis and may play a protective role in the response to cerebral ischemia-reperfusion injury, as reelin-deficient mice are more susceptible to injury after transient cerebral ischemia<sup>[35]</sup>. Pretreatment with miR-200c antagomir decreased post-middle cerebral artery occlusion brain levels of miR-200c, resulting in a significant reduction in infarct volume and neurological deficit. Changes in brain levels of miR-200c inversely correlated with reelin protein expression. Direct targeting was verified with dual luciferase assay. Inhibition of miR-200c resulted in an increase in cell survival subsequent to in vitro oxidative injury. This effect was blocked by knockdown of reelin mRNA, whereas application of reelin protein afforded protection. These findings suggest that the

poststroke increase in miR-200c contributes to brain cell death by inhibiting reelin expression, and that reducing poststroke miR-200c is a potential target to mitigate stroke-induced brain injury<sup>[35]</sup>.

ADAMTS13 (A Disintegrin And Metalloprotease with Thrombospondin type I repeats-13) plays an important role in preventing microvascular thrombosis by cleaving ultra large von Willebrand factor (ULVWF) multimers, the most thrombogenic form of von Willebrand factor (VWF), into smaller less active multimers, reducing potential thrombotic activity. Early studies have shown that ADAMTS13 deficiency aggravates brain injury in a murine model of acute ischemic stroke<sup>[36]</sup>. ADAMTS13 is a target for miR-525-5p<sup>[36]</sup>. In vivo treatment with miR-525-5p agomir effectively decreased ADAMTS13 mRNA and protein levels in the ischemic region. These data suggest that miR-525-5p is an endogenous regulator of ADAMTS13 that improves ischemia/reperfusion (I/R)-induced brain injury and dysfunction<sup>[36]</sup>.

MiR-155 has been implicated in regulating various physiological and pathological processes such as hematopoietic lineage differentiation, immunity, inflammation, cancer, and cardiovascular diseases<sup>[37]</sup>. Intravenous injections of a specific miR-155 inhibitor were initiated at 48 h after mouse distal middle cerebral artery occlusion (dMCAO) in a very recent study, showing that in vivo miR-155 inhibition after ischemia supports brain microvasculature, reduces brain tissue damage, and improves the animal functional recovery<sup>[37]</sup>.

Hypoxia occurs immediately upon cerebrovascular occlusion and contributes to the progression of ischemic cascade. Hence, hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), an essential regulator of hypoxic events could be a useful target for the treatment of ischemic stroke. HIF-1 $\alpha$  mediates important endogenous adaptive mechanisms in order to maintain oxygen homeostasis and also facilitates cellular adaptation to low oxygen conditions by regulating more than 80 downstream genes<sup>[38]</sup>. MiR-335 has recently been identified as a direct regulator of hypoxia inducible factor-1 $\alpha$  and as a potential therapeutic target in cerebral ischemia. MiR-335 and hypoxia inducible factor-1 $\alpha$  mRNA showed an inverse expression profile, both in vivo and in vitro ischemic conditions and miR-335 mimic was found to reduce infarct volume in the early time (immediately after middle cerebral artery occlusion) of embolic stroke animal models while the miR-335 inhibitor appears to be beneficial at the late time of stroke (24 hrs after middle cerebral artery occlusion). Modulation of hypoxia inducible factor-1 $\alpha$  expression by miR-335 also influenced the expression of crucial genes implicated in neurovascular permeability, cell death and maintenance of the blood brain barrier<sup>[38]</sup>.

Recent studies showed that experimental agents may play a beneficial role in the treatment of ischemic stroke via miRNA pathways. Trimetazidine, 1-(2,3,4-trimethoxybenzyl) piperazine dihydrochloride (TMZ), is an anti-ischemic agent, and its anti-ischemic effects have been experimentally assessed in various models including cell cultures, isolated and perfused organs, and also in vivo<sup>[39]</sup>. A recent study demonstrated that miR-21 involved trimetazidine-induced anti-apoptosis in cell culture<sup>[39]</sup>. MicroRNA-155 (miR-155) induces the expression of inflammatory cytokines, and acetylbritannilactone (ABL) exerts potent antiinflammatory actions by inhibiting expression of inflammation-related genes<sup>[40]</sup>. MiR-155 has been shown to mediate inflammatory responses in ischemic cerebral tissue by modulating TLR4/MyD88 and SOCS1 expression, and ABL exerts its antiinflammatory action by suppressing miR-155 expression, suggesting a novel miR-155-based therapy for ischemic stroke<sup>[40]</sup>.

Previous studies have shown that fastigial nucleus stimulation

(FNS) reduces tissue damage resulting from focal cerebral ischemia<sup>[41]</sup>. Using a combination of deep sequencing and microarray with computational analysis, Pang *et al.* identified a novel miRNA in the rat ischemic cortex after 1 h of FNS. This novel miRNA (PC-3p-3469\_406), herein referred to as rno-miR-676-1, was upregulated in rats with cerebral ischemia after FNS. In vivo observations indicate that this novel miRNA may have antiapoptotic effects and contribute to neuroprotection induced by FNS<sup>[41]</sup>.

Repetitive transcranial magnetic stimulation (rTMS) has increasingly been studied over the past decade to determine whether it has a therapeutic benefit on focal cerebral ischemia<sup>[42]</sup>. It has been demonstrated that 10 Hz rTMS can promote adult neural stem cell proliferation in the subventricular zone after focal cerebral ischemia. The protective effect of rTMS was associated with the miR-25/p57-signaling pathway<sup>[42]</sup>. Pre-Implantation factor (PIF) is a conserved, embryo-derived peptide that can be detected in human, mouse, and bovine maternal circulation during pregnancy<sup>[43]</sup>. Recently, a synthetic PIF analog (sPIF) of 15 amino acids (MVRKPGSANKPSDD) was reported to elicit neuroprotective effects in a murine model of experimental autoimmune encephalomyelitis<sup>[43]</sup>. Using murine cell culture combined with a rat hypoxic-ischemic brain injury model, it has been shown that synthetic PIF (sPIF) inhibits microRNA let-7 biogenesis by destabilizing the key microRNA processing protein, KH-type splicing regulatory protein, in a Toll-like receptor 4-dependent manner. This regulation, together with its induced upregulation of the anti-inflammatory cytokine interleukine-10 (IL-10), may contribute to sPIF-mediated neuroprotection in vivo and also may underlie PIF's physiological role in maternal immune modulation and embryo implantation<sup>[43]</sup>.

Finally, direct miR therapy can be beneficial in experimental stroke. MiR-424 is a tumor marker that is involved in cancer cell proliferation, migration, and invasion, with a demonstrated role in cell cycle regulation<sup>[44]</sup>. It can also promote monoblastic cell differentiation modulate vascular endothelial and smooth muscle cell phenotype and angiogenesis, and improve pulmonary hypertension in experimental models. With respect to cerebrovascular disease, studies have shown that the miR-424 level is decreased in the blood and brain tissue of ischemic stroke patients and experimental animals<sup>[44]</sup>. Treatment with miR-424 decreased infarct volume and inhibited neuronal apoptosis after ischemia/reperfusion, reduced reactive oxygen species and malondialdehyde levels in the cortex, and increased the expression and activation of MnSOD as well as the expression of extracellular SOD and the redox-sensitive transcription factor nuclear factor erythroid 2-related factor suggesting that MiR-424 protects against transient cerebral ischemia/reperfusion injury by inhibiting oxidative stress<sup>[44]</sup>.

The viability of miRNAs as therapeutic targets is confirmed by the fact that a phase I clinical trial of an anti-miRNA was successfully concluded (santaris.com). In this case, a locked nucleic acid-based anti-miRNA targeting a liver specific miRNA, miR-122 (miravirsen or SPC3649), is being developed as a hepatitis C therapy. SPC3649 is currently being tested in a Phase IIa clinical trial of previously untreated hepatitis C patients<sup>[45]</sup>. In contrast to antagomir-directed miRNA silencing, miRNA overexpression strategies also exist and may be helpful in certain settings. Systemic delivery of miR-34a in a lipid-based delivery vehicle has been shown to block lung tumor growth in vivo<sup>[45,46]</sup>.

## HUMAN RESEARCH

In their very first study Tan *et al.* investigated ischemic stroke patients aged between 18-49 years, characterized based on World

Health Organization clinical criteria were further classified according to TOAST classification, MiRNAs that are implicated in the endothelial/vascular function, erythropoiesis, angiogenesis and neural function showed differential expression profile as compared to the normal control, and miRNAs that are involved in hypoxic conditions have also been found in their miRNA profiles<sup>[47]</sup>. Circulatory microRNA-145 expression was significantly higher in ischemic stroke patients than in control subjects raising the possibility of being a biomarker of ischemic stroke<sup>[48]</sup>. The microRNA profile of low or no risk ischemic stroke patients could differ substantially from those with pre-existing risk factors, which may be of great clinical importance<sup>[49]</sup>.

Another study showed that circulating miR-30a and miR-126 levels were markedly down-regulated in all patients with ischemic stroke until 24 weeks. However, circulating let-7b was lower in patients with large-vessel atherosclerosis than healthy volunteers, whereas circulating let-7b had higher level in patients with other kinds of ischemic stroke until 24 weeks. Among all patients, circulating miRNAs levels returned to normal 48 weeks after symptom onset. These data suggest that miR-30a, miR-126 and let-7b might be useful biomarkers for ischemic stroke (and for stroke subtype) in humans<sup>[50]</sup>. Furthermore, miR-223 may indicate worse clinical outcome in acute stroke patients<sup>[51]</sup>. In another study in patients with acute ischemic stroke, miR-122, miR-148a, let-7i, miR-19a, miR-320d, miR-4429 were decreased and miR-363, miR-487b were increased compared to vascular risk factor controls. These miRNA are predicted to regulate several genes in pathways previously identified by gene expression analyses, including toll-like receptor signaling, NF-kb signaling, leukocyte extravasation signaling, and the prothrombin activation pathway raising the possibility of their use as clinical markers<sup>[52]</sup>.

Recently, it has been proposed that the presence of genetic variants in miRNA genes could affect the processing and subsequent maturation of miRNAs, and collectively affect the risk and/or prognosis of diseases<sup>[53]</sup>. Current literature is controversial, further studies are needed to clarify their clinical importance<sup>[54-56]</sup>.

## SUMMARY

Stroke is one of the leading causes of death and disability worldwide. Enormous efforts have been made to improve stroke services worldwide. In past decades, researchers have studied the physiopathology and biochemistry of stroke, but knowledge of the molecular mechanisms underlying this disease remains at an early stage.

MicroRNAs have been identified as negative regulators of gene expression in a post-transcriptional manner. Accumulating studies have demonstrated that miRNAs are essential determinants of vascular endothelial cell biology/angiogenesis as well as contributors to stroke pathogenesis based on both animal and human studies. MiRNAs likely represent both new biomarkers and new therapeutic targets for many human diseases.

The discovery of miRNAs that mediate post-transcriptional silencing of specific target genes has shed light on how non-coding RNAs can play critical roles in the pathogenesis of ischemic stroke. Based on animal studies clusters of miRNA could be supposed in the background of ischemic injury and some of them possibly control the expression of different genes<sup>[10,11]</sup>. The microRNA pattern seem to be associated with stroke subtype (ischemic vs. hemorrhagic), but based on preliminary data miR-298 was the only miRNA that was upregulated in both brain and blood after almost all experimental conditions<sup>[12]</sup>. Stroke-induced cell death seem to be associated with different signaling pathways in male and female rats raising the

possibility of genetic differences in stroke-related apoptosis<sup>[16]</sup>.

Ischemic preconditioning was also shown to alter the abundance of miRNA expression, including miR-132, the miR-200 family, the miR-182 family, and others that may promote ischemic tolerance and trigger neuroprotective signaling pathways<sup>[14,57]</sup>.

MicroRNAs are also involved in specific pathways which are associated with stroke related injury or seem to be associated with neuroprotection. It has been demonstrated that stroke alters miRNA expression in SVZ neural progenitor cells and miR-124a mediated stroke-induced neurogenesis by targeting the JAG-Notch signaling pathway; the knockdown of endogenous miR-181 provides protection against ischemia/reperfusion-induced brain cell death by targeting GRP78, a well-studied and important molecular chaperone and cytoprotective protein<sup>[17,18]</sup>. They are also involved in glutamate excitotoxicity (miR-223), neural cell death caused by arachidonic acid insult (miR29b), key regulators of p75NTR (miR592) and overexpression of miR-132 in the hippocampal CA1 of living rats affords robust protection against ischemia-induced neuronal death<sup>[21,22,25,27]</sup>. Different microRNA patterns could be detected in ischemic core and penumbra raising the possibility of identifying different targets in neuroprotection<sup>[24]</sup>. They are also involved in acute stroke phase and recovery<sup>[19,23,26]</sup>.

Antagomirs are RNA-like oligonucleotides that harbor various modifications for RNase protection and pharmacologic properties such as enhanced tissue and cellular uptake. The efficient use of oligonucleotides to target miRNAs for functional studies and therapeutic use will be dependent on characterizations based on miRNA targets in vivo<sup>[30]</sup>. For example antagomirs to two miRNAs, miR1 and Let7f confer their role in neuroprotection in female rats, miR-181a antagomir reduced neuronal injury in vitro and hippocampal neuronal loss in vivo after forebrain ischemia, microRNA-103-1 selectively downregulates brain NCX1 and its inhibition by anti-miRNA ameliorates stroke damage and neurological deficits<sup>[31-33]</sup>. Direct miR therapy can be beneficial in experimental stroke, treatment with miR-424 decreased infarct volume and inhibited neuronal apoptosis after ischemia/reperfusion, reduced reactive oxygen species and malondialdehyde levels in the cortex, and increased the expression and activation of MnSOD as well as the expression of extracellular SOD and the redox-sensitive transcription factor nuclear factor erythroid 2-related factor suggesting that MiR-424 protects against transient cerebral ischemia/reperfusion injury by inhibiting oxidative stress<sup>[44]</sup>.

Circulatory microRNA expression can be biomarker of ischemic stroke (miR-145) and 30a, miR-126 and let-7b might be useful biomarkers for ischemic stroke (and for stroke subtype) in humans<sup>[49,50]</sup>. They also can be used as predictors of outcome in ischemic stroke patients<sup>[52]</sup>. The role of polymorphisms involving human microRNA expression are controversial with regards to future cardiovascular events.

Human phase I and II studies are in progress to clarify the efficacy of antagomir treatment in different human conditions<sup>[45,46]</sup>.

On the other hand, the small size of miRNAs poses a unique challenge in their detection, requiring a highly sensitive and discriminatory methods<sup>[58,59]</sup>. Most commercially available technologies for global miRNA expression profiling are based on DNA microarrays spotted onto a glass surface spotted onto a glass surface, which are not optimal for miRNA detection. Using this technique discrimination between single-nucleotide differences is not always possible due to cross-hybridization and lack of specificity<sup>[59,60]</sup>.

Even though genomic technologies are advanced, scientists continue to build upon them to make these even better. Two cutting-

edge technologies in modern biology, microarrays and Real Time PCR (Polymerase Chain Reaction) are in the lead to revolutionize biology and medicine. RNAi, a powerful new approach for achieving targeted gene silencing using siRNA/miRNA as the triggering agent, offers remarkable specificity, scalability, potency, and reproducibility. MicroRNAs can be a challenge to study because of their small size<sup>[60]</sup>. They require specialized and dedicated tools for analysis. For qRT-PCR and microarray applications, the following are the requirements: (1) Effective method of miRNA isolation from samples; (2) RT-PCR and Microarray reagents optimized for miRNA detection; (3) Assays specific to the miRNAs of interest; (4) Real-time and Microarray analytical instruments and reagents validated for miRNA detection protocol.

Although miRNAs represent a potentially powerful treatment paradigm, much work remains to be done in describing individual specific miRNA-mRNA interactions, understanding the regional and cell type specific distribution of miRNAs within the nervous system, and in developing organ-targeted delivery systems to optimize the therapeutic potential of miRNAs.

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