Differential MicroRibonucleic Acid Expression in Cardioembolic Stroke

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Background: MicroRNAs (miRNA) are a class of small, endogenous (17-25 nucleotide) noncoding ribonucleic acids implicated in the transcriptional and post-transcriptional regulation of gene expression. This study examines stroke-specific miRNA expression in large vessel territory cardioembolic stroke. *Methods:* Peripheral blood was collected from controls and ischemic stroke patients 24 hours after stroke onset. Whole blood miRNA was isolated and analyzed for differential expression. A total of 16 patients with acute middle cerebral artery territory strokes of cardioembolic origin were included in this pilot study. MiRNA profiling was conducted by miRCURY LNA™ microRNA Array. *Results:* In patients with cardioembolic stroke, significant differential expression of 14 miRNAs was observed when compared to controls. Ten of these miRNA had not previously been associated with ischemic stroke (miR-664a-3p, -2116-5pp, -4531, -4765-5p, -647, -4709-3p, -4742-3p, -5584-3p, -4756-3p, and -5187-3p). Subanalysis of severe strokes (NIHSS > 10) identified an additional 5 differentially expressed miRNA. No significant effects of sex or tissue plasminogen activator treatment were seen on miRNA expression. *Conclusions:* Ischemic stroke patients show a differential miRNA expression profile as compared to controls. These new associations between circulating miRNAs and ischemic stroke may help to refine stroke subtype diagnosis and identify novel therapeutic miRNA targets for the treatment of ischemic stroke.

Key Words: Acute ischemic stroke—biomarkers—microRNA—translational research—clinical research—clinical stroke

Introduction

Stroke is a leading cause of disability and death worldwide.[1] Prior studies have identified numerous potential biomarkers for ischemic stroke, including ribonucleic acid (RNA)-based biomarkers.[1] As RNAs are continuously transcribed, translated, and turned over in response to physiologic and pathologic stimuli, the RNA profile of the cell serves as a useful reflection of its current functional state.[1-,3]

MicroRNAs (miRNA) are a class of endogenous, small (approximately 17-25 nucleotide), noncoding ribonucleic acids implicated in the transcriptional and post-transcriptional regulation of gene expression.[3] MiRNA can impact cellular function by suppressing or activating downstream mRNA targets, which in turn regulates protein expression.[2-3] Preclinical studies have demonstrated specific changes in miRNA expression profiles after ischemic stroke.[3-5] In addition, human studies have identified specific miRNA expression profiles associated with ischemic stroke, including those involved in thrombosis and leukocyte extravasation.[4-8] However, at this point, there are no established serum biomarkers in routine clinical practice that predict stroke risk, etiology, or outcome. In this study, we sought to discover novel stroke-associated miRNA by analyzing a highly homogenous subset

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of patients with large cerebral artery strokes of embolic origin.

Materials and Methods

Ischemic stroke patients admitted to a tertiary hospital between January 2011 and March 2014 were considered for this pilot study. Blood samples were collected at 24 ± 6 hours from symptom onset. Ethical approval for human studies and waiver of written informed consent was obtained from the Institutional Review Board at Hartford Hospital and the University of Connecticut Health Center. All procedures for specimen collection and analysis were conducted in accordance with institutional guidelines.

Inclusion/Exclusion Criteria

Patients above 18 years of age presenting with an acute ischemic stroke were considered for study inclusion. Stroke diagnosis was confirmed by clinical and radiologic evaluation. Among ischemic stroke patients, only those with cardioembolic strokes in the middle cerebral artery (MCA) territory were considered for inclusion (N = 16). Exclusion criteria included history of brain neoplasia, active peripheral malignancy, past-traumatic brain injury or brain hemorrhage, or strokes not secondary to a cardioembolic source. Blood collected from outpatients with no known acute/chronic neurological deficits and matched vascular risk factors served as controls (n = 8).

MicroRibonucleic Acid Isolation

Blood samples were collected in PAX-gene tubes. MiRNA profiling and analysis was performed by Exiqon. The quality of RNA was assessed using an Agilent 2100 bioanalyzer profile. Total RNA was labeled using the miRCURY LNA miRNA Hi-Power Labeling Kit, Hy3/Hy5 prior to analysis by miRCURY LNA miRNA Array 7th Gen (Exiqon) with capture probes targeting all miRNAs for human, mouse, or rat registered in the miRBASE 18.0. Hybridization was performed using a Tecan HS4800 hybridization station (Tecan). After hybridization, microarray slides were scanned, using the Agilent G2565BA Microarray Scanner System (Agilent Technologies, Santa Clara, CA) and image analysis was performed using ImaGeneR 9 (Biodiscovery, Santa Monica, CA).

Statistics

A total of 560 miRNAs were analyzed using the described microarray technique. Based on our literature review, 173 miRNAs were analyzed for differential expression between ischemic stroke cases and controls. For expression analysis, calculated *P* values were based on Wilcoxon rank sum test. The Benjamini and Hochberg multiple testing adjustment method was applied to control false discovery rate at .05. Statistical analysis was performed using SAS 9.4 (SAS Software, Cary, NC).

Results

The demographic characteristics of study patients are given in Table 1. Among all stroke cases, 50% patients had a left-MCA infarct, and 50% had a right-MCA stroke. The mean NIH stroke scale (NIHSS) on admission was 12, with the NIHSS ranging from 5-19. The stroke etiology was cardioembolic in 14 cases (87.5% patients) whereas 2 cases (12.5%) had a high likelihood of a cardioembolic etiology based on minimal intracranial or extracranial atherosclerosis. Among patients with confirmed cardioembolic strokes, 71.4% had atrial fibrillation and 28.6% cases were not related to atrial fibrillation (one patient had a mechanical aortic valve and had not been on anticoagulation, one had a low ejection fraction, and another was noted to have a left ventricular thrombus postmyocardial infarction).

Our analysis identified significant differential expression of 14 miRNAs (11 downregulated, 3 upregulated) in acute ischemic stroke patients as compared to controls. MiRNAs miR-1273e (log FC -.426, $P = .011$), miR-5187-3p (log FC -.426, $P = .011$) were found to be downregulated in stroke patients. Other miRNAs showing significant downregulation included let 7e-5p (log FC -.309, $P = .039$); miR-4709-3p (log FC -.372, $P = .023$), miR-4756-3p (log FC -.372, $P = .023$), miR-5584-3p (log FC -.312, $P = .024$), and miR-647 (log FC -.283, $P = .024$). MiRNAs miR-4742-3p (log FC -.289, $P = .038$), miR-4764-5p (log FC -.251, $P = .041$), miR-4531 (log FC -.194, $P = .042$), and miR-2116-5p (log FC -.179, $P = .043$) were also depressed in stroke patients compared to controls. MiRNAs miR-664a-3p (log FC .227, $P = .024$), miR-943 (log FC .234, $P = .043$), and miR-145-5p (log FC .399 $P = .039$) were significantly upregulated in patients with acute ischemic stroke (Table 2). Interestingly, 10 of our identified miRNAs have not been previously described in association with acute ischemic stroke.

Table 1. *Patient demographics*

Category	Total (N = 16)
Age, mean (range)	74.3 (56, 91)
Sex (% male)	8 (50%)
Stroke risk factors	
Hypertension	93.6%
Coronary artery disease	50.0%
Diabetes	31.2%
Smoking	18.7%
Hyperlipidemia	75.0%
Race	
Caucasian	62.5%
African American	12.5%
Hispanic	6.25%
Asian	6.25%
Other	12.5%
Stroke location	
Left MCA	50%
Right MCA	50%

MCA, middle cerebral artery.

As initial stroke severity is a strong predictor of stroke pathophysiology and outcome, we then conducted a subgroup analysis comparing acute ischemic stroke patients with more severe stroke deficits with an initial presenting NIHSS above 10 (n = 10) with controls (n = 8). Additional miRNAs, including miR-29a-5p ($P = .022$), miR-151a-3p ($P = .023$), miR-487b-3p ($P = .025$), and let-7b-3p ($P = .046$), were significantly upregulated in severe ischemic stroke cases (Table 3). MiRNAs miR-4531 ($P = .023$) and miR-15b-5p ($P = .048$) were significantly downregulated in these patients. Importantly, no significant sex differences or thrombolysis treatment effects on miRNA expression were observed, although this is likely due to the low number of patient samples.

Discussion

MiRNAs are key regulators of gene function and play a pivotal role in the modulation of the complex cascade of molecular signaling associated with neuronal injury. Specific miRNAs have been associated with ischemic stroke and related processes, including atherosclerosis and inflammation.[3,4] Current evidence indicates that miRNAs may act as stroke biomarkers as well as potential targets for therapy.[4-8] While the majority of miRNAs are intracellular, miRNA also exists extracellularly. The circulating miRNA identified in our study of whole blood may have been derived from plasma and/or peripheral blood cells.[8] Further investigation of these miRNA may reveal new mechanisms of regulation in response to cerebral injury.

Our study identified 10 miRNAs that have not previously been associated with ischemic stroke (Table 2).[3-8] MiRNAs miR-4531, miR-4756-3p, and miR-5584-3p were downregulated in patients with ischemic stroke. Prior studies have not identified these miRNAs in patients with stroke/cerebrovascular disease or other neurologic, or medical comorbidities. In addition, we identified differential regulation in miRNAs including miR-5187-3p, miR-4742-3p, miR-664a-3p, miR-647, miR-4764-5p, miR-2116-5p, and miR-943 which were not previously described in the context of cerebral ischemia.

Several of these miRNAs have been previously described in the context of other diseases. Transforming growth factor beta receptor II, a target of miR-664, plays a critical role in the TGF beta signaling pathway that is necessary for tissue repair.[9] The upregulation of miR-664 in stroke patients seen in our study could contribute to increased TGF beta signaling, resulting in cellular proliferation and angiogenesis that may impact poststroke recovery.

In our study, miR-4742 and miR-4709-3p were downregulated in stroke patients. Both of these miRNAs have previously identified roles in the phosphotidyl inositol-3-kinase (PI3K-Akt) signaling pathway.[10] The PI3K pathway regulates neural stem cell physiology, and is thus instrumental in cell survival or regeneration after cerebral infarction. Changes in the level of these miRNAs may drive the alterations in PI3K pathway signaling seen after ischemic stroke. Additionally, we found downregulation of miR-647 and miR-2116 in stroke patients. Targets of these miRNAs have been linked to the regulation of immune and metabolic pathways, but no studies to date have found an association between these miRNA and ischemic stroke.[11,12]

In addition to the novel ischemic stroke miRNA, our study confirmed differential expression of miRNAs miR-1273e, miR-15b-5p, and miR-145, which have all been previously associated with ischemic stroke and atherosclerosis.[3,4,13] We also noted differential expressions of hsa-let-7e-5p, hsa-let-7b, and miR-487b-3p which have been previously described in cases of ischemic stroke. Subsequent subset analysis found no significant effect of patient sex or

Table 2. *Differential miRNA expression between controls (n = 8) and all stroke cases (N = 16). Known disease associations are listed*

MiRNA	*P* value	Log FC	Fold change	Regulation	Previous associations
hsa-miR-145-5p	.0398	.399	1.319	Upregulated	Ischemic stroke[3-5,7,8]
hsa-miR-943	.0437	.234	1.176	Upregulated	Cancer, [2] ischemic stroke[5,7]
hsa-miR-664a-3p	.0247	.227	1.170	Upregulated	Cancer[9]
hsa-miR-2116-5p	.0437	−.179	.883	Downregulated	Cancer[12]
hsa-miR-4531	.0428	−.194	.874	Downregulated	None
hsa-miR-4764-5p	.0419	−.251	.840	Downregulated	Rheumatoid arthritis[14]
hsa-miR-647	.0247	−.283	.822	Downregulated	Cancer[11]
hsa-miR-4742-3p	.0383	−.289	.818	Downregulated	Autism[10]
hsa-let-7e-5p	.0398	−.309	.807	Downregulated	Ischemic stroke[3-5]
hsa-miR-5584-3p	.0247	−.312	.806	Downregulated	None
hsa-miR-4756-3p	.023	−.333	.794	Downregulated	None
hsa-miR-4709-3p	.023	−.372	.773	Downregulated	Autism[10]
hsa-miR-5187-3p	.0113	−.426	.744	Downregulated	Endometriosis[15]
hsa-miR-1273e	.0113	−.426	.744	Downregulated	Ischemic stroke[5]

MiRNA, micro ribonucleic acid.

Table 3. *Additional differentially expressed microRNA between severe (NIHSS > 10) stroke (n = 10) and control patients (n = 8). Known disease associations are listed*

miRNA	*P*-value	Log FC	Fold change	Regulation	Previous associations
hsa-miR-29a-5p	.022	.279	1.214	Upregulated	Atherosclerosis[13]
hsa- miR-151a-3p	.023	.674	1.596	Upregulated	Ischemic stroke[7]
hsa-miR-487b-3p	.025	.524	1.438	Upregulated	Ischemic stroke[6,7]
let-7b-3p	.046	.420	1.338	Upregulated	Ischemic stroke[5,7]
hsa-miR-4531	.023	−.221	.858	Downregulated	None
hsa-miR-15b-5p	.048	−.560	.678	Downregulated	Atherosclerosis,[13] ischemic stroke[7]

tissue plasminogen activator treatment on miRNA expression after ischemic stroke, likely due to the small sample size.

Summary

This study identified several new miRNAs in specifically selected patients with cardioembolic strokes of similar stroke location and infarct size. Our selection methods reduced variability but did lead to a small sample size. This study should serve as a pilot study to evaluate miRNA expression in patients with a MCA territory cardioembolic stroke. Our work has identified multiple novel miRNAs that have not been previously described in the context of ischemic stroke, due in part to our focus on this homogeneous subset of ischemic stroke patients (MCA strokes of cardioembolic origin). These newly identified miRNAs may provide a reliable signature for cryptogenic strokes of possible cardioembolic origin but this will need to be validated in larger cohorts. Furthermore, identification of these miRNAs may allow for the development of new molecular targets and therapeutic strategies for management of large hemispheric embolic strokes.

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