Published in final edited form as:

Mol Neurobiol. 2018 February ; 55(2): 1419–1429. doi:10.1007/s12035-016-0347-8.

Characterization of Dysregulated miRNA in Peripheral Blood Mononuclear Cells from Ischemic Stroke Patients

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Abstract

Epigenetic modification may play an important role in pathophysiology of ischemic stroke (IS) risk. Micro-RNAs (miRNAs), which constitute one of the modes of epigenetic regulation, have been shown to be associated with a number of clinical disorders including IS. The purpose of this study was to investigate the miRNA profile in the peripheral blood mononuclear cells (PBMCs) of IS patients and compare it with stroke-free controls. Blood samples were obtained from 19 healthy age-gender-race matched individuals who served as controls to 20 IS patients. miRNA microarray analysis with RNA from PBMCs was performed and significantly dysregulated miRNAs common among IS patients were identified. We identified 117 miRNAs with linear fold values of at least ± 1.5 , of which, 29 were significantly altered (p value < 0.05). Ingenuity Pathway Analysis (IPA) indicated a role for the dysregulated miRNAs in conditions relevant to IS (e.g., organismal injury and abnormalities, hematological disease and immunological disease). Pro-inflammatory genes like STAT3, interleukin (*IL*) 12A and *IL* 12B were some of the highly predicted targets for the dysregulated miRNAs. Notably, we further identified three common and significantly up-regulated miRNAs (hsa-miR-4656, -432, -503) and one downregulated miRNA (hsa-miR-874) amongst all IS patients. Molecular interactive network analysis revealed that the commonly dysregulated miRNAs share several targets with roles relevant to IS. Altogether, we report dysregulation of miRNAs in IS PBMCs and provide evidence for their involvement in the immune system alteration during IS pathophysiology.

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Conflict of interest

The authors declare that there is no conflict of interest.

Ethical approval

All procedures performed in studies involving human participants were approved by the Institutional Review Board of University of South Carolina and obtained written consent from all participants/subjects.

Keywords

Ischemic stroke; miRNA; PBMCs; Inflammation

Introduction

South Carolina, described as “the buckle of the Stroke Belt”, has one of the highest age-adjusted stroke mortalities in the United States [1,2]. The REasons for Geographic And Racial Differences in Stroke (REGARDS) study reported an association between inflammatory markers and stroke amongst those residing in the Stroke Belt states [1,3]. Inflammation plays an important role in atherosclerosis; a common mechanism that leads to IS and heart attacks [1,4]. Recently, data from The Health and Retirement Study (HRS) and REGARDS study demonstrated that exposure to environmental conditions in the Stroke Belt contribute to an increased IS risk [4]. Environmental conditions and physiological stress can alter epigenetic marks resulting in changes in gene expression and translation without altering the DNA sequence [5]. This phenomenon may explain the mechanism by which exposure to environmental conditions in the Stroke Belt could contribute to an increased stroke risk later in life [4].

Well-recognized epigenetic mechanisms involve the pathways regulating the synthesis and action of miRNA. These miRNAs are small, ~22 nucleotide, non-coding RNA [6,7] and, they play a key role in gene regulation at different stages of cellular differentiation and development [8,9]. Moreover, many types of cancers and other human diseases have been associated with the dysregulation of miRNA expression [10–13]. Because of their master role in gene control, and ease of isolation, miRNAs are emerging as new biomarkers of human disease [14–19]. Interestingly, there are approximately a dozen miRNAs isolated either from serum, cerebrospinal fluid (CSF) or whole blood from IS patients that are potential biomarker candidates [20–35]. However, to our knowledge, there are no reports on dysregulated miRNAs from the peripheral blood mononuclear cells (PBMCs) of stroke patients.

Peripheral blood mononuclear cells constitute a critical component of the immune system and previous research has shown that these cells display significant alterations in cytokine production and undergo apoptosis after IS [36]. Ischemic stroke is known to trigger a systemic inflammatory response [37]. Whether such changes are associated with altered miRNA expression in PBMCs has not been previously investigated. Consequently, we profiled miRNAs from acute IS PBMCs by microarray. We observed 117 dysregulated miRNAs and investigated their biological targets and processes using Ingenuity Pathway Analysis (IPA), (QIAGEN, Redwood City, CA, <http://www.ingenuity.com/>), Panther Pathways [38] and Cytoscape [39]. We observed that many of the target genes have roles in immune system functioning and biological processes and pathways relevant to stroke. For example, *IL12A* and *IL12B* are predicted targets of hsa-miRs-874-3p and -3064-5p, respectively. As another example, STAT3 is targeted by six downregulated miRNAs from our dataset. Furthermore, we found four miRNAs (hsa-miRs-4656, -432, -503 and -874-3p) which are commonly dysregulated in all IS patients analyzed as compared to

healthy controls. Therefore, we suggest that these four miRNAs are promising candidates for further study as biomarkers for IS.

Materials and Methods

Patients and cell preparation

Ischemic stroke patients admitted to the stroke center at University of South Carolina Palmetto Health Richland Hospital, Columbia, South Carolina, were recruited for this study after proper consent was obtained. Confirmation of the stroke subtype was done by a vascular neurologist. All blood samples were collected within 48h of stroke symptom onset. Controls consisted of age, gender and race matched healthy volunteers without any history of stroke in the individuals or in their family. Twenty mL of peripheral blood was collected in EDTA containing vials (Vacutainer, BD) and processed using Ficoll-Paque (GE Healthcare, Uppsala, Sweden) density centrifugation to isolate PBMCs. Trypan-blue exclusion staining was used to determine the viability of PBMCs. We included 19 controls and 20 IS patients for the present study.

RNA isolation and miRNA microarray

Total RNA, including miRNA and other small RNA molecules, was isolated using AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Valencia, CA) from fresh PBMCs from both control and IS patients. Next, total RNA samples (5 each controls and IS patients) were used for miRNA array hybridization assay employing GeneChip array (miRNA-3_0). Linear fold-change in miRNA was then calculated to compare the differences in expression of 1009 miRNAs between controls and IS patients.

Data analysis, selection of candidate miRNAs and functional enrichment

First, two-tailed Student's t-tests on the expression values of all miRNAs in all the individuals of the control and IS groups were performed. Then, the volcano plot, measuring statistical significance and magnitude of fold change, was generated based on the log₂ fold change (X axis) and $-\log_{10} p$ value from Student's t-tests (Y axis). All the dysregulated miRNAs were selected on the basis of linear fold-change cut off of at least ± 1.5 in IS compared to controls (a log₂ fold change of ~ 0.6 is ~ 1.5 linear fold change), and a p value < 0.05 was considered significant.

Only the miRNAs meeting the above two criteria, highlighted by colored dots in the volcano plot (orange and light blue for greater than ± 1.5 and less than ± 2 ; red and dark blue for beyond ± 2), were then used to create a Venn diagram with multiple patients ($n=5$) to identify the miRNA that are commonly dysregulated in all IS patients. Next, a miRNA-gene interaction network of the dysregulated miRNAs was obtained by employing the IPA tool. IPA is a widely used bioinformatics tool to analyze biological molecule interactions, including miRNA, mRNA and proteins. IPA was performed with only the miRNA that met the above selection criteria. Further down, the target genes of the dysregulated miRNA were extracted from IPA for functional analysis using Panther Pathways. Panther is a database maintained by the University of California, Santa Cruz that is used for functional enrichment of gene products. Finally, the commonly dysregulated miRNAs were selected for further

analysis, as they were most promising based on our set criteria for selection. The targets of these commonly dysregulated miRNAs were extracted from the following databases: www.mirwalk.org, www.mirdb.org and www.microrna.org. The genes that were known or predicted to be targeted by two or more miRNAs from the selected miRNAs (commonly dysregulated miRNAs) were then analyzed by Cytoscape to find their interactions and gene ontology.

Data validation by qRT-PCR analysis

Complementary DNA was prepared using the miScript II RT kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Real-time PCR was performed with 3 ng cDNA, for validating the miRNA expression, using miScript Primer Assays and miScript SYBR Green PCR Kit (Qiagen, Valencia, CA) following manufacturer's instructions. We included 7 miRNAs (hsa-miRs-503, -376c, -487b, -130a-3p, -432, -4656 and -874-3p) for validation.

Of the numerous target genes revealed based on the miRNA-gene interaction analysis, we selected 8 genes (*SMAD3*, *TGFB2*, *TGFB3*, *TGFBRI*, *MKL2*, *ETS1*, *IL12A* and *IL12B*) for expression analysis in PBMCs by qRT-PCR evaluation of their transcripts. Real-time PCR was performed using iQTM Universal SYBR[®] Green Supermix (Bio-Rad, USA) by using 25 ng of the cDNA as template. All the qRT-PCR was run in a ViiA7 Real-Time PCR System from Applied Biosystems. SNORD96A and 18S rRNA was used as internal controls for the miRNA and target genes, respectively. Expression level was presented after calculating the fold-change (2^{-C_T}). Micro-RNA and the target gene transcript levels were measured in PBMCs from each of the 19 controls and 20 IS patients.

Dual luciferase reporter assay

We obtained the sequence of only the hsa-miR-432-5p binding sites on the 3' UTRs of human *Tgfb3*, *Celsr2* and *Itih2* (as shown in Fig. 5a) as per information provided on www.microrna.org. After adding appropriate restriction sites, we procured the oligos from Integrated DNA Technology (IDT, Iowa). The double stranded oligos were cloned in pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, cat# E1330). Since the seed sequence of hsa-miR-432-5p was common for all the three genes, we used only one mutant UTR with completely different nucleotides in the seed sequence region. Overnight cultured THP1 cells were transfected simultaneously with the recombinant vectors and the miRNA mimic for hsa-miR-432-5p using lipofectamine 3000 from Invitrogen in separate experiments. After 48h, luciferase activity was detected using the Dual-Glo[®] Luciferase Assay System kit from Promega (cat# E2920) following the instructions by the provider.

Statistics

A two-tailed Student's t-test was used to calculate *p* values for miRNA expression level and qRT-PCR data. A *p* value <0.05 was considered significant. The *p* values indicated in the pathway and gene ontology analyses were obtained from the respective tools without any changes. To test the reliability of our *t* test used to identify significantly dysregulated miRNAs, we used power test to calculate the power of our tests (Supplemental Fig. III).

Results

miRNA expression in PBMCs from IS patients

A total of 39 participants were included in the analysis (20 IS patients and 19 healthy controls). The mean age for IS patients and controls was 61 years and 50 years, respectively. The median NIHSS for the IS group was 4 with a range of 0–23. By TOAST Classification of Subtypes of Ischemic Stroke, the most recurrent source of IS for the case group was cardioembolic (45%). Baseline characteristics for both groups can be found in Table 1 [40].

We performed miRNA microarray on total RNA from PBMCs from control and IS patients, on 5 subjects per group. A total of 1009 miRNA (Fig. 1a and 1b) were analyzed by microarray. The microarray data was used to identify differentially expressed miRNA after obtaining the linear fold-change of individual miRNA. Based on a ± 1.5 linear fold-change threshold, we observed 117 miRNAs that were either up-regulated ($n=76$) or downregulated ($n=41$) in IS PBMCs relative to controls (Supplemental Table I). The linear fold-change value for the most up-regulated miRNA (hsa-miR-409-3p) was 4.14 and for the most downregulated miRNA (hsa-miR-3188) was -2.40 , in IS when compared to controls. This analysis showed only 29 miRNAs (Fig. 1c) that were at least ± 1.5 fold up- or downregulated in IS patient PBMCs with a p value < 0.05 (referred to as ‘significantly dysregulated miRNAs’). Of the 29 significantly dysregulated miRNAs, eight miRNAs (hsa-miRs-31, $-342\text{-}5\text{p}$, $-3619\text{-}5\text{p}$, $-4281\text{-}5\text{p}$, $-4687\text{-}3\text{p}$, $4739\text{-}3\text{p}$, $-874\text{-}3\text{p}$, $-877\text{-}3\text{p}$) were significantly downregulated and hsa-miRs-181a*, $-199\text{a-}3\text{p}$, $-199\text{-b-}3\text{p}$, $-223\text{-}3\text{p}$, $-29\text{-}3\text{p}$, $-30\text{b-}3\text{p}$, $-30\text{e-}3\text{p}$, $-424\text{-}3\text{p}$, $-4656\text{-}3\text{p}$, $-4667\text{-}5\text{p}$, $-625\text{-}3\text{p}$, $-628\text{-}5\text{p}$, $-629\text{-}3\text{p}$, $-671\text{-}3\text{p}$, $-301\text{a-}3\text{p}$, $-376\text{c-}3\text{p}$, $-409\text{-}5\text{p}$, $-432\text{-}3\text{p}$, $-454\text{-}3\text{p}$, $-503\text{-}3\text{p}$, $-550\text{a-}3\text{p}$ were significantly up-regulated in the IS patients when compared to healthy controls.

Functional enrichment and biological pathway analysis

To identify the target genes of the dysregulated miRNAs, we employed IPA to analyze: 1) all the 117 dysregulated miRNAs (Supplemental Table I), and 2) only the 29 significantly dysregulated miRNAs shown in Fig. 1c. The top two networks obtained after IPA analysis with the first group of miRNAs were categorized under: (a) organismal injuries and abnormalities, reproductive system disease and cancer (Fig. 2a), and (b) cancer, hematological disease and immunological disease (Supplemental Fig. I). Under category (a), 112 miRNAs and in category (b), 71 miRNA were recorded. However, using IPA to investigate only the 29 significantly dysregulated miRNAs (Fig. 2b), the top network identified was categorized under: cancer, organismal injury and abnormalities, reproductive system diseases, with 24 of 29 miRNAs included in the network (Fig. 2c).

To identify the gene ontologies (GO) for the target genes of the dysregulated miRNAs, all target molecules were extracted from IPA and used for GO analysis using Panther Pathways. Panther analysis provided 12 biological processes and 14 pathways in which the genes were involved (Supplemental Fig. IIA and IIB). Within the biological process, the majority of the genes were included under ‘cellular, metabolic and developmental processes’. Among the various pathways, majority of the genes were involved in the Wnt signaling and P53 pathways. Protein class and molecular function classification of the genes indicated that

many of them are receptors and transporters (Supplemental Fig. IIC and IID). Many of the receptors are members of the GPCR (G-protein coupled receptors) family and a major proportion of the transporters are members of the solute carrier (SLC) family.

Identification of commonly dysregulated miRNAs in IS patients and functional enrichment of targets

Next, we identified miRNA that are commonly dysregulated in all 5 IS patient samples when compared to controls, assayed by the microarray. Consequently, we found only 6 dysregulated miRNAs (hsa-miRs-4656, -432, -487b, -503, -409-3p and -874-3p) (Fig. 3a and 3b, Table 2) that was found in all IS patient PBMCs. Of the 6 commonly dysregulated miRNAs, only hsa-miR-874-3p was downregulated and the rest were up-regulated in all five IS patients compared to controls (Table 2). Only the hsa-miRs-432-5p, -503, -4656 and -874-3p had significant *p*-value (<0.05). Thus, these 4 miRNAs were used for further analysis. The targets of the select miRNAs were obtained from three databases (www.mirwalk.org, www.mirdb.org and www.microrna.org) and analyzed in Cytoscape to generate the miRNA-gene interaction network. We found that many of the target genes were strongly predicted to be common targets of two or more of the select miRNAs (Fig. 3c). Functional enrichment of the common targets of the dysregulated miRNAs indicated that the top gene ontology is: cellular differentiation and developmental process (Supplemental Table II).

qRT-PCR validation of select miRNAs and target genes

To validate our miRNA microarray data, we performed qRT-PCR analysis for seven miRNAs (Fig. 4a, b) using cDNA samples prepared from total RNA of PBMCs from 19 healthy controls and 20 IS patients. The hsa-miRs-4656, -432, -503, -376c, -130a-3p and -487b were up-regulated and hsa-miR-874-3p was down-regulated as per our microarray analysis and similar results were observed after qRT-PCR. The hsa-miRs-4656, -432, -503 and -487b were among the commonly up-regulated in all IS patients included in the microarray relative to controls and in more than 90% when validated by qRT-PCR. To confirm that our microarray data was reliable, we included hsa-miR-320a for the qRT-PCR validation. This miRNA was shown by microarray to have similar expression levels in the controls and IS patients (1.01 linear fold-changes). Our qRT-PCR analysis also showed similar expression level for the miRNA in both controls and patients (Fig. 4b). Next, we selected 8 genes (*SMAD3*, *TGFB2*, *TGFB3*, *TGFBR1*, *MKL2*, *ETS1*, *IL12A* and *IL12B*) that were predicted to be targets of dysregulated miRNAs from our dataset and performed qRT-PCR to quantify their transcript level in the PBMCs. Our qRT-PCR analysis showed that *SMAD3*, *TGFB2*, *TGFB3*, *TGFBR1*, *MKL2* and *ETS1* were downregulated and *IL12A* and *IL12B* were up-regulated in the PBMCs of IS patients (Fig. 4c). As expected, expression of the target genes inversely correlated with the expression of the miRNAs predicted to target them.

Validation of miRNA and target gene UTR interaction

We selected three genes (human *TGFB3*, *CELSR2* and *ITM2C*) for the study of the miRNA and target gene interaction. These genes were selected as they were predicted to be targets (Fig. 5a) of the dysregulated miRNA, hsa-miR432-5p, as per analysis presented in Fig. 2c

and also targeted by more than one dysregulated miRNAs (hsa-miR-432-5p and miR-4656a), as per evidence provided in Fig. 4c. The hsa-miR-432-5p was selected to study further as it was one of the significantly dysregulated miRNAs present in all the IS patients (commonly dysregulated) included for the study. Dual luciferase reporter assay performed after 48h of transfection with the miRNA mimic along with the cloned UTR showed almost 50% significant reduction in the luciferase activity compared to the mutant sequence of UTRs (Fig. 5b) and control.

Discussion

The current study was initiated to identify dysregulated miRNAs in the PBMCs that could be used as epigenetic markers of ischemic stroke risk. Consequently, we first noted dysregulated miRNA based only on the level of their expression by setting a threshold of ± 1.5 linear fold-change and $p < 0.05$. Based on this, we found 117 miRNAs that were dysregulated in the PBMCs of IS. As expected, approximately a dozen of the miRNAs from our dataset were reported in studies of miRNAs isolated either from experimental mouse models of IS, human serum samples, CSF or whole blood cells from IS patients [20–35]. This observation indicated that irrespective of the cells or clinical material used for the analysis, these miRNAs are dysregulated in IS thereby further suggesting that the changes in microRNA seen in IS may be systemic and that PBMCs may represent a convenient target to study microRNA changes.

One of the main observations from our miRNA-gene interaction analyses is that various genes involved in the immune system pathways are targets of many of the dysregulated miRNAs. For example, *STAT3* is targeted by 6 downregulated miRNAs (hsa-miRs-3064-5p, -4651, -3935, -1207-5p, -4434 and -874-3p) from our dataset, implying that *STAT3* could be up-regulated during IS. *STAT3* is expressed in a diverse array of tissues and cells compared to other members of the *STAT* family. Depending on the type of signaling cytokine, activation of *STAT3* is shown to have a pleiotropic effect [41]. For example, in the presence of IL-6, pro-inflammatory activity of *STAT3* is rapidly induced in dendritic cells [42]. Systemic induction of pro-inflammatory cytokines within 24h of stroke incidence has been reported [37]. Thus, our data imply that pro-inflammatory cytokine induction can occur after IS through involvement of the *STAT3* signaling pathway. As another example, we observed that miRNAs targeting *IL12A* and *IL12B* (hsa-miR-874-3p and hsa-miR-3064-5p, respectively) are downregulated in the PBMCs of IS patients. We confirmed higher transcript levels for both the genes in the IS PBMCs. *IL12A* and *IL12B* are monomers of the functionally active *IL12* p40, which acts as a pro-inflammatory cytokine involved in the *JAK/STAT* signaling pathway to induce interferon (IFN). Serum *IL12* is elevated after IS incidence [43] but, there is no report correlating its elevated expression with dysregulated miRNAs in IS. Together, these observations imply a role for dysregulated miRNAs in the control of the expression of pro-inflammatory cytokines during IS.

Hematological and immunological disease was one of the top networks that we identified in IS using IPA. Correlating with this, increased neutrophil count in IS may contribute to pathogenesis [44,45]. We have also recorded a higher neutrophil count in IS patients (unpublished data). Analysis for GO and biological processes of the dysregulated miRNA

targets revealed ‘immune system process’ (GO:0002376) as one of the top biological processes. An altered immune response during acute IS onset has been reported previously [36,37,46,47]. GO analysis indicated ‘Toll receptor signaling pathway’ (P00054) as one of the top ontologies. Toll like receptors (TLRs) influence the body’s response to numerous forms of injury. Furthermore, the TLR signaling pathway has been associated with IS as neuroprotective if stimulation of this pathway is achieved before ischemia [48].

Ischemic stroke involves injury and damage to brain cells and tissues. Functional enrichment by IPA of the dysregulated miRNAs revealed ‘organismal injury and abnormality’ as the top network. Inhibition of miR-329, miR-487b, miR-494 and miR-495 improves neovascularization after ischemia in animal studies [49]. These miRNAs were up-regulated in IS patients in our study. Interestingly, hsa-miR-376c is in the same genomic cluster as hsa-miR-487b (www.mirbase.org). Our microarray and qRT-PCR analysis both showed up-regulation of this miRNA in IS patients. Altogether, these observations suggest that the expression of miRNA which are negatively involved in the vascularization process are up-regulated in IS and may exacerbate the disease outcome.

Studies have reported dysregulated miRNAs in the serum, whole blood cells, or cerebrospinal fluids during IS [20–35]. Therefore, we were interested in finding novel miRNAs in the IS PBMCs that could serve as biomarkers for IS. Consequently, for the first time, we identified four differentially expressed miRNAs (hsa-miRs-4656, -432 -503 and has-miR-874-3p, referred as select miRNAs) in the PBMCs from IS patients. Even after employing specific selection criteria (at least ± 1.5 linear fold-change, p value 0.05 and commonly dysregulated in all IS patients tested), these miRNAs were found to be significantly dysregulated in all IS patient samples analyzed by microarray and in >90% of the samples (n=19) after qRT-PCR validation, indicating that these miRNAs could serve as reliable epigenetic markers. Molecular interactive network analysis of the select miRNAs showed that more than one miRNA targets the same gene(s) (Fig. 3c). The major biological processes included cellular development, nervous system development, generation of neurons, etc., which are relevant to the pathophysiology of IS. This information clearly indicates roles for the select miRNAs in IS and thus further studies are warranted. Furthermore, South Carolina is one of the several states considered as having a particularly high rate of stroke nationally by United States Centre for Disease Control and Prevention (CDC) [50]. Our study samples were all collected from South Carolina and there is no previous report on miRNA expression profiles in PBMCs from the Stroke Belt region. A deeper study of the miRNAs in this report may enable identification of markers unique to those at risk for stroke.

To prove that the miRNAs commonly dysregulated in all the IS patients indeed interact with the predicted genes as per our analysis, we chose to study the interaction of hsa-miR-432-5p with the UTRs of human Tgfb3, Celsr2 and Itm2c. We found that luciferase activity was reduced to almost 50% in the UTR inserted recombinant constructs indicating that the hsa-miR-432-5p directly interacts with the UTR of the said genes. Thus, these data suggest that dysregulation of hsa-miR-432-5p could have implications on IS pathology involving the above mentioned genes. In this context, there are reports showing that Tgfb3 is involved in the regulation of the immune system in that they can lead to induction of FOXP3+ CD4+ T

cells [51]. As far as *Celsr2* is concerned, it is known that this gene is linked to cardiovascular diseases and evidence suggests that people with cardiovascular disease have higher risk of developing stroke [52]. *Itm2c* is also linked to stroke. For example, it was reported that siRNA mediated knockdown of *ITM2C* led to increase in apoptosis and decreased survival in neuronal cells [53].

In summary, the present work identified several dysregulated miRNAs in PBMCs from IS patients which are predicted to regulate expression of genes involved in the immune system and vascularization processes. Most importantly, we have identified four novel miRNAs that are dysregulated in IS. These miRNAs stand to be very good candidates for further analysis as epigenetic markers for IS. Further studies with larger sample sizes are required to examine the precise roles of these miRNAs and for inclusion as reliable epigenetic markers for prediction and risk stratification of IS. Also of interest is whether these miRNAs are involved in the induction of IS and could be useful in prediction of IS, or are an outcome of the IS incident. Finally, the unique miRNAs signatures of IS patients could reveal more about the pathological mechanisms underlying stroke and warrant further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study is supported in part by SC Smart State Funds, National Institutes of Health grants P01AT003961, R01AT006888, R01ES019313, R01MH094755, AI29788, and P20GM103641 to PN and MN.

References

1. Feigin VL, Krishnamurthi R. Public health strategies could reduce the global stroke epidemic. *Lancet Neurol.* 2010; 9(9):847–848. S1474-4422(10)70190-3 [pii]. DOI: 10.1016/S1474-4422(10)70190-3 [PubMed: 20723839]
2. NINDS Brain Basics: Preventing Stroke. National Institutes of Neurological Disorders and Stroke. http://www.ninds.nih.gov/disorders/stroke/preventing_stroke.htm
3. Faber JE, Zhang H, Lassance-Soares RM, Prabhakar P, Najafi AH, Burnett MS, Epstein SE. Aging causes collateral rarefaction and increased severity of ischemic injury in multiple tissues. *Arterioscler Thromb Vasc Biol.* 2011; 31(8):1748–1756. ATVB AHA.111.227314 [pii]. DOI: 10.1161/ATVB AHA.111.227314 [PubMed: 21617137]
4. D'Agostino RB, Wolf PA, Belanger AJ, Kannel WB. Stroke risk profile: adjustment for antihypertensive medication. The Framingham Study. *Stroke.* 1994; 25(1):40–43. [PubMed: 8266381]
5. Krupinski J, Slevin M. Emerging molecular targets for brain repair after stroke. *Stroke Res Treat.* 2013; 2013:473416.doi: 10.1155/2013/473416 [PubMed: 23365789]
6. Ambros V. The functions of animal microRNAs. *Nature.* 2004; 431(7006):350–355. nature02871 [pii]. DOI: 10.1038/nature02871 [PubMed: 15372042]
7. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell.* 2004; 116(2):281–297. S0092867404000455 [pii]. [PubMed: 14744438]
8. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell.* 2009; 136(2):215–233. S0092-8674(09)00008-7 [pii]. DOI: 10.1016/j.cell.2009.01.002 [PubMed: 19167326]
9. Fabian MR, Sonenberg N, Filipowicz W. Regulation of mRNA translation and stability by microRNAs. *Annu Rev Biochem.* 2010; 79:351–379. DOI: 10.1146/annurev-biochem-060308-103103 [PubMed: 20533884]

10. Mraz M, Pospisilova S. MicroRNAs in chronic lymphocytic leukemia: from causality to associations and back. *Expert Rev Hematol*. 2012; 5(6):579–581. DOI: 10.1586/ehm.12.54 [PubMed: 23216588]
11. He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, Powers S, Cordon-Cardo C, Lowe SW, Hannon GJ, Hammond SM. A microRNA polycistron as a potential human oncogene. *Nature*. 2005; 435(7043):828–833. nature03552 [pii]. DOI: 10.1038/nature03552 [PubMed: 15944707]
12. Mraz M, Pospisilova S, Malinova K, Slapak I, Mayer J. MicroRNAs in chronic lymphocytic leukemia pathogenesis and disease subtypes. *Leuk Lymphoma*. 2009; 50(3):506–509. 910218367 [pii]. DOI: 10.1080/10428190902763517 [PubMed: 19347736]
13. Cui JW, Li YJ, Sarkar A, Brown J, Tan YH, Premyslova M, Michaud C, Iscove N, Wang GJ, Ben-David Y. Retroviral insertional activation of the Fli-3 locus in erythroleukemias encoding a cluster of microRNAs that convert Epo-induced differentiation to proliferation. *Blood*. 2007; 110(7):2631–2640. blood-2006-10-053850 [pii]. DOI: 10.1182/blood-2006-10-053850 [PubMed: 17586726]
14. Zhang HD, Jiang LH, Sun DW, Li J, Tang JH. MiR-139-5p: promising biomarker for cancer. *Tumour Biol*. 2015; 36(3):1355–1365. DOI: 10.1007/s13277-015-3199-3 [PubMed: 25691250]
15. Khoury S, Tran N. Circulating microRNAs: potential biomarkers for common malignancies. *Biomark Med*. 2015; 9(2):131–151. DOI: 10.2217/bmm.14.102 [PubMed: 25689901]
16. Guess MG, Barthel KK, Harrison BC, Leinwand LA. miR-30 family microRNAs regulate myogenic differentiation and provide negative feedback on the microRNA pathway. *PLoS One*. 2015; 10(2):e0118229. PONE-D-14-35015 [pii]. doi: 10.1371/journal.pone.0118229 [PubMed: 25689854]
17. Zhuang LP, Meng ZQ. Serum miR-224 reflects stage of hepatocellular carcinoma and predicts survival. *Biomed Res Int*. 2015; 2015:731781.doi: 10.1155/2015/731781 [PubMed: 25688365]
18. Ravo M, Cordella A, Rinaldi A, Bruno G, Alexandrova E, Saggese P, Nassa G, Giurato G, Tarallo R, Marchese G, Rizzo F, Stellato C, Biancardi R, Troisi J, Di Spiezio Sardo A, Zullo F, Weisz A, Guida M. Small non-coding RNA deregulation in endometrial carcinogenesis. *Oncotarget*. 2015; 6(7):4677–4691. 2911 [pii]. DOI: 10.18632/oncotarget.2911 [PubMed: 25686835]
19. Li M, Wang Y, Song Y, Bu R, Yin B, Fei X, Guo Q, Wu B. MicroRNAs in renal cell carcinoma: a systematic review of clinical implications (Review). *Oncol Rep*. 2015; 33(4):1571–1578. DOI: 10.3892/or.2015.3799 [PubMed: 25682771]
20. Li P, Teng F, Gao F, Zhang M, Wu J, Zhang C. Identification of circulating microRNAs as potential biomarkers for detecting acute ischemic stroke. *Cell Mol Neurobiol*. 2015; 35(3):433–447. DOI: 10.1007/s10571-014-0139-5 [PubMed: 25410304]
21. Wang W, Sun G, Zhang L, Shi L, Zeng Y. Circulating microRNAs as novel potential biomarkers for early diagnosis of acute stroke in humans. *J Stroke Cerebrovasc Dis*. 2014; 23(10):2607–2613. S1052-3057(14)00281-X [pii]. DOI: 10.1016/j.jstrokecerebrovasdis.2014.06.002 [PubMed: 25287657]
22. Sorensen SS, Nygaard AB, Nielsen MY, Jensen K, Christensen T. miRNA expression profiles in cerebrospinal fluid and blood of patients with acute ischemic stroke. *Transl Stroke Res*. 2014; 5(6):711–718. DOI: 10.1007/s12975-014-0364-8 [PubMed: 25127724]
23. Zhou J, Zhang J. Identification of miRNA-21 and miRNA-24 in plasma as potential early stage markers of acute cerebral infarction. *Mol Med Rep*. 2014; 10(2):971–976. DOI: 10.3892/mmr.2014.2245 [PubMed: 24841240]
24. Leung LY, Chan CP, Leung YK, Jiang HL, Abrigo JM, Wang de F, Chung JS, Rainer TH, Graham CA. Comparison of miR-124-3p and miR-16 for early diagnosis of hemorrhagic and ischemic stroke. *Clin Chim Acta*. 2014; 433:139–144. S0009-8981(14)00115-6 [pii]. DOI: 10.1016/j.cca.2014.03.007 [PubMed: 24650689]
25. Septhamianam S, Tan JR, Tan KS, DeSilva DA, Tavintharan S, Woon FP, Wang CW, Yong FL, Karolina DS, Kaur P, Liu FJ, Lim KY, Armugam A, Jeyaseelan K. Circulating microRNAs as biomarkers of acute stroke. *Int J Mol Sci*. 2014; 15(1):1418–1432. ijms15011418 [pii]. DOI: 10.3390/ijms15011418 [PubMed: 24447930]

26. Long G, Wang F, Li H, Yin Z, Sandip C, Lou Y, Wang Y, Chen C, Wang DW. Circulating miR-30a, miR-126 and let-7b as biomarker for ischemic stroke in humans. *BMC Neurol.* 2013; 13:178. 1471-2377-13-178 [pii]. doi: 10.1186/1471-2377-13-178 [PubMed: 24237608]
27. Li WY, Jin J, Chen J, Guo Y, Tang J, Tan S. Circulating microRNAs as potential non-invasive biomarkers for the early detection of hypertension-related stroke. *J Hum Hypertens.* 2014; 28(5): 288–291. jhh201394 [pii]. DOI: 10.1038/jhh.2013.94 [PubMed: 24132136]
28. Tsai PC, Liao YC, Wang YS, Lin HF, Lin RT, Juo SH. Serum microRNA-21 and microRNA-221 as potential biomarkers for cerebrovascular disease. *J Vasc Res.* 2013; 50(4):346–354. 000351767 [pii]. DOI: 10.1159/000351767 [PubMed: 23860376]
29. Jickling GC, Ander BP, Zhan X, Noblett D, Stamova B, Liu D. microRNA expression in peripheral blood cells following acute ischemic stroke and their predicted gene targets. *PLoS One.* 2014; 9(6):e99283. PONE-D-13-46893 [pii]. doi: 10.1371/journal.pone.0099283 [PubMed: 24911610]
30. Wang Y, Zhang Y, Huang J, Chen X, Gu X, Zeng L, Yang GY. Increase of circulating miR-223 and insulin-like growth factor-1 is associated with the pathogenesis of acute ischemic stroke in patients. *BMC Neurol.* 2014; 14:77. 1471-2377-14-77 [pii]. doi: 10.1186/1471-2377-14-77 [PubMed: 24708646]
31. Moon JM, Xu L, Giffard RG. Inhibition of microRNA-181 reduces forebrain ischemia-induced neuronal loss. *J Cereb Blood Flow Metab.* 2013; 33(12):1976–1982. jcbfm2013157 [pii]. DOI: 10.1038/jcbfm.2013.157 [PubMed: 24002437]
32. Yang Q, Yang K, Li A. microRNA-21 protects against ischemia-reperfusion and hypoxia-reperfusion-induced cardiocyte apoptosis via the phosphatase and tensin homolog/Akt-dependent mechanism. *Mol Med Rep.* 2014; 9(6):2213–2220. DOI: 10.3892/mmr.2014.2068 [PubMed: 24676391]
33. Selvamani A, Williams MH, Miranda RC, Sohrabji F. Circulating miRNA profiles provide a biomarker for severity of stroke outcomes associated with age and sex in a rat model. *Clin Sci (Lond).* 2014; 127(2):77–89. CS20130565 [pii]. DOI: 10.1042/CS20130565 [PubMed: 24428837]
34. Lee ST, Chu K, Jung KH, Yoon HJ, Jeon D, Kang KM, Park KH, Bae EK, Kim M, Lee SK, Roh JK. MicroRNAs induced during ischemic preconditioning. *Stroke.* 2010; 41(8):1646–1651. STROKEAHA.110.579649 [pii]. DOI: 10.1161/STROKEAHA.110.579649 [PubMed: 20576953]
35. Zeng L, Liu J, Wang Y, Wang L, Weng S, Tang Y, Zheng C, Cheng Q, Chen S, Yang GY. MicroRNA-210 as a novel blood biomarker in acute cerebral ischemia. *Front Biosci (Elite Ed).* 2011; 3:1265–1272. 330 [pii]. [PubMed: 21622133]
36. Kassner SS, Kollmar R, Bonaterra GA, Hildebrandt W, Schwab S, Kinscherf R. The early immunological response to acute ischemic stroke: differential gene expression in subpopulations of mononuclear cells. *Neuroscience.* 2009; 160(2):394–401. S0306-4522(09)00263-2 [pii]. DOI: 10.1016/j.neuroscience.2009.02.050 [PubMed: 19258025]
37. Grond-Ginsbach C, Hummel M, Wiest T, Horstmann S, Pflieger K, Hergenahn M, Hollstein M, Mansmann U, Grau AJ, Wagner S. Gene expression in human peripheral blood mononuclear cells upon acute ischemic stroke. *J Neurol.* 2008; 255(5):723–731. DOI: 10.1007/s00415-008-0784-z [PubMed: 18465111]
38. Mi H, Thomas P. PANTHER pathway: an ontology-based pathway database coupled with data analysis tools. *Methods Mol Biol.* 2009; 563:123–140. DOI: 10.1007/978-1-60761-175-2_7 [PubMed: 19597783]
39. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* 2003; 13(11):2498–2504. [pii]. DOI: 10.1101/gr.123930313/11/2498 [PubMed: 14597658]
40. Adams HP Jr, Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, Marsh EE 3rd. Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment. *Stroke.* 1993; 24(1):35–41. [PubMed: 7678184]
41. Hutchins AP, Diez D, Miranda-Saavedra D. Genomic and computational approaches to dissect the mechanisms of STAT3's universal and cell type-specific functions. *JAKSTAT.* 2013; 2(4):e25097. [pii]. doi: 10.4161/jkst.250972013JAKS0150R [PubMed: 24416643]

42. Braun DA, Fribourg M, Sealfon SC. Cytokine response is determined by duration of receptor and signal transducers and activators of transcription 3 (STAT3) activation. *J Biol Chem.* 2013; 288(5): 2986–2993. M112.386573 [pii]. DOI: 10.1074/jbc.M112.386573 [PubMed: 23166328]
43. Zaremba J, Losy J. Interleukin-12 in acute ischemic stroke patients. *Folia Neuropathol.* 2006; 44(1):59–66. 5689 [pii]. [PubMed: 16565932]
44. Kochanek PM, Hallenbeck JM. Polymorphonuclear leukocytes and monocytes/macrophages in the pathogenesis of cerebral ischemia and stroke. *Stroke.* 1992; 23(9):1367–1379. [PubMed: 1519296]
45. Muir KW, Tyrrell P, Sattar N, Warburton E. Inflammation and ischaemic stroke. *Curr Opin Neurol.* 2007; 20(3):334–342. [pii]. DOI: 10.1097/WCO.0b013e32813ba15100019052-200706000-00016 [PubMed: 17495630]
46. Kawabori M, Yenari MA. Inflammatory responses in brain ischemia. *Curr Med Chem.* 2015; 22(10):1258–1277. CMC-EPUB-65093 [pii]. [PubMed: 25666795]
47. Pei J, You X, Fu Q. Inflammation in the pathogenesis of ischemic stroke. *Front Biosci (Landmark Ed).* 2015; 20:772–783. 4336 [pii]. [PubMed: 25553478]
48. Gesuete R, Kohama SG, Stenzel-Poore MP. Toll-like receptors and ischemic brain injury. *J Neuropathol Exp Neurol.* 2014; 73(5):378–386. DOI: 10.1097/NEN.0000000000000068 [PubMed: 24709682]
49. Welten SM, Bastiaansen AJ, de Jong RC, de Vries MR, Peters EA, Boonstra MC, Sheikh SP, La Monica N, Kandimalla ER, Quax PH, Nossent AY. Inhibition of 14q32 MicroRNAs miR-329, miR-487b, miR-494, and miR-495 increases neovascularization and blood flow recovery after ischemia. *Circ Res.* 2014; 115(8):696–708. CIRCRESAHA.114.304747 [pii]. DOI: 10.1161/CIRCRESAHA.114.304747 [PubMed: 25085941]
50. CDC. Centers for Disease Control and Prevention: State-specific mortality from stroke and distribution of place of death--United States, 1999. *MMWR Morb Mortal Wkly Rep.* 2002; 51:429–433. [PubMed: 12056498]
51. Chikuma S, Suita N, Okazaki IM, Shibayama S, Honjo T. TRIM28 prevents autoinflammatory T cell development in vivo. *Nat Immunol.* 2012; 13(6):596–603. DOI: 10.1038/ni.2293 [PubMed: 22544392]
52. How Cardiovascular & Stroke Risks Relate. http://www.strokeassociation.org/STROKEORG/LifeAfterStroke/HealthyLivingAfterStroke/UnderstandingRiskyConditions/How-Cardiovascular-Stroke-Risks-Relate_UCM_310369_Article.jsp#
53. Yang L, Xiong Y, Hu XF, Du YH. MicroRNA-323 regulates ischemia/reperfusion injury-induced neuronal cell death by targeting BRI3. *Int J Clin Exp Pathol.* 2015; 8(9):10725–33. eCollection 2015. [PubMed: 26617783]

Fig. 1. miRNAs are dysregulated in IS PBMCs. **a** Heat map showing the expression level of all 1009 miRNAs (number of probes used to detect miRNAs during the array) in the controls (n=5) and IS samples (n=5) used for the microarray analysis with RNA isolated from PBMCs. **b** Scatter plot of the linear fold-change values of all 1009 miRNAs analyzed in the microarray. **c** Volcano plot showing the expression levels of the miRNAs with fold change above the cutoff value (± 1.5) and $p < 0.05$ (X-axis: Log2 fold-change; Y-axis: $-\log_{10}$ of p -values). On the X-axis, 0.585 is ~ 1.5 linear fold-change and 1.0 is ~ 2 linear fold-change. On the Y-axis, the line denotes the p -value 0.05).

Fig. 2. Identification of molecular networks using IPA. **a** The most significant network by IPA: ‘Organismal injury and abnormalities, Reproductive system disease, Cancer’. To enhance the reliability of the results, only the miRNAs with a p -value < 0.05 were selected thereby obtaining 29 miRNAs. **b** Heat map showing the expression levels of only the 29 significantly

dysregulated miRNAs. (C: control, P: IS patients). **c** Top network obtained from IPA, with only the 29 significantly dysregulated miRNAs, under the category: ‘Cancer, organismal injury and abnormalities’ and ‘reproductive system diseases’. In the network, the following genes were manually added based on information for miRNA-gene interaction available on www.microrna.org (*SMAD3*, *TGFB2*, *TGFB3*, *MKL2*, *ETS1*, *TGFBR1*, *IL12A*, *IL12B*).

Fig. 3.

Identification of the dysregulated miRNAs common in all the IS samples included for the miRNA microarray. Linear fold-change of all the miRNAs was calculated for individual IS patients. A threshold of ± 1.5 was set and the miRNAs with expression level beyond the threshold was analyzed by Venn to get the common miRNAs seen in IS patients. **a** Venn diagram showing miRNAs (5) that are commonly up-regulated (hsa-miRs-4656, -432, -487b, -503, -409-3p) in the 5 IS patients. **b** Venn diagram showing miRNAs that are commonly downregulated (hsa-miR-874-3p) in the 5 IS patients compared to controls. The targets of four miRNAs (hsa-miRs-4656, -432, -503 referred to as select miRNAs and -487b) were analyzed by Cytoscape to generate the molecular interaction network. **c** Molecular interaction network of the select miRNAs, hsa-miR487b and their target genes.

Fig. 4.

Validation of select miRNAs and target genes by qRT-PCR. **a** Linear fold-change values of the commonly dysregulated miRNAs after microarray. **b** Relative abundance (RA) after qRT-PCR validation of representative dysregulated miRNAs. We randomly selected the miRNAs for validation from dysregulated miRNAs of which miRNAs, hsa-miRs-4656, -432 and -503, were among the commonly up-regulated miRNAs in all the IS patients. Greater than 90% of the IS patients had higher expression for hsa-miRs-4656, -432 and -503 upon validation by qRT-PCR. The hsa-miR-320a was included here to further confirm the reliability of our microarray result. **c** Result from qRT-PCR validation of 8 target genes of significantly dysregulated miRNAs. The values represent relative abundance of the transcripts in IS PBMCs (Control n=19, IS n=20). 18S rRNA was used as an internal control.

A

3' gguGGGUUACUGGAUGAGGUUCu 5' hsa-miR-432 	mirSVR score: -0.1098 PhastCons score: 0.5080
835:5' uccCCCACUCCCC--CUCCAAGa 3' TGFB3	
3' gguGGGUUACUGGAU---GAGGUUCu 5' hsa-miR-432 : :	mirSVR score: -1.0134 PhastCons score: 0.7396
224:5' gacCCCUGGGGCCAACAUCUCCAAGa 3' CELSR2	
3' ggUGGGUUACUGGAUGAGGUUCu 5' hsa-miR-432 : : :	mirSVR score: -0.4704 PhastCons score: 0.4705
814:5' gaGCUCAUUG-CUUUCUCCAAGc 3' ITM2C	

B **Fig. 5.**

MiR-432-5p directly interacts with the UTRs of human TGFB3, CELSR2 and ITM2C. **a** Nucleotide sequence of hsa-miR-432-5p and the UTR of the genes predicted to interact with miR-432-5p. The sequence details, miRSVR and PhastCons scores were obtained from www.microrna.org. **b** The UTRs were cloned into pmiRGLO vector and co-transfected with the hsa-miR-432-5p mimics into THP1 cells for luciferase activity measurement. The bar graph shows the relative luciferase activity 48h after transfection with the different recombinant vectors compared to control and mutant. Because the seed sequence complementary bases was same for all the three UTRs, we used only one mutant which had all 7 nucleotides of the seed sequence changed.

Table 1

Baseline characteristics of participants (N=39).

Characteristic	Control (N=19)	Case (N=20)
Demographics		
Age (yr), mean $\pm$ SD	50 $\pm$ 8.1	61 $\pm$ 10.1
Male sex – no. (%)	9 (47%)	11 (55%)
Race – no. (%)		
White	9 (47%)	10 (50%)
Black	10 (53%)	10 (50%)
Clinical history – no. (%)		
Stroke at entry	N/A	20 (100%)
History of prior stroke	0	4 (20%)
History of hypertension	8 (42%)	18 (90%)
Dyslipidemia	2 (11%)	11 (55%)
Diabetes	1 (5%)	7 (35%)
History of coronary artery disease	0	6 (30%)
Obstructive Sleep Apnea	3 (16%)	2 (10%)
Atrial Fibrillation	0	7 (35%)
Family history of stroke	8 (42%)	6 (30%)
Physical Examination		
NIHSS, median (range)	N/A	4 (0–23)
TOAST classification		
Large artery atherosclerosis	N/A	5 (25%)
Small vessel/Lacunar	N/A	4 (20%)
Cardioembolic	N/A	9 (45%)
Others/undetermined	N/A	2 (10%)

Table 2

Linear fold-change (LFC) of the common miRNAs in individual IS and control samples. Linear fold-change of miRNA in every IS individual was calculated against the microarray mean expression values of all the controls and vice-versa. The two-tailed Student's t-test p-values and linear fold-change values (column 13) were calculated by averaging all the controls and IS patients' microarray expression level values. The bottom two rows indicate age and gender of the individuals. C: control, S: ischemic stroke, LFC: linear fold-change, m: male, f: female.

miRNAs	C1	C2	C3	C4	C5	S1	S2	S3	S4	S5	p-value	LFC
hsa-miR-409-3p	1.00	-1.48	-27.70	-16.05	-1.87	2.45	7.10	2.78	3.66	6.92	0.082	4.14
hsa-miR-487b	-1.88	-2.48	-2.95	-22.06	-1.52	2.28	5.94	1.82	3.11	6.03	0.052	3.41
hsa-miR-432	-1.43	-2.66	-6.92	-10.12	-1.37	1.85	6.76	1.85	2.61	6.66	0.047	3.25
hsa-miR-503	-3.49	-1.62	-5.44	-3.01	-1.66	2.88	3.19	2.59	2.36	2.75	0.002	2.74
hsa-miR-4656	-3.74	-2.70	-1.25	-1.28	-1.57	1.84	1.71	1.68	2.39	2.01	0.021	1.91
hsa-miR-874-3p	1.06	1.40	3.05	1.99	1.70	-1.58	-2.65	-1.51	-1.55	-1.57	0.006	-1.73
Age (y)	51	48	41	53	36	72	63	54	71	68		
Gender	m	m	f	f	m	m	m	m	m	f		