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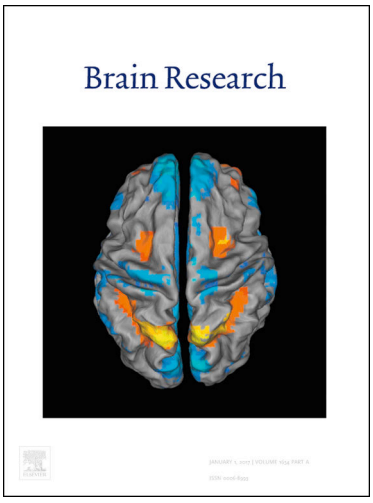

Registered Report Stage II

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Differentially expressed miRNA profiles of serum derived extracellular vesicles from patients with acute ischemic stroke

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ABSTRACT

Background: MicroRNAs (miRNAs) participate in diverse cellular changes following acute ischemic stroke (AIS). Circulating miRNAs, stabilized and delivered to target cells via extracellular vesicles (EVs), are potential biomarkers to facilitate diagnosis, prognosis, and therapeutic modulation. We aimed to identify distinctive expression patterns of circulating EV-miRNAs in AIS patients.

Methods: miRNA profiles from EVs, isolated from plasma samples collected within 24 hours following AIS diagnosis, were examined between a dataset of 10 age-, gender- and existing comorbidities-matched subjects (5 AIS and 5 healthy controls, HC). We measured 2578 miRNAs and identified differentially expressed miRNAs between AIS and HC. An enrichment analysis was conducted to delineate the networks and biological pathways implicated by differentially expressed microRNAs. An enrichment analysis was conducted to delineate the networks and biological pathways implicated by differentially expressed microRNAs.

Results: Five miRNAs were differentially expressed between stroke (AIS) versus control (HC). hsa-let-7b-5p, hsa-miR-16-5p, and hsa-miR-320c were upregulated, whereas hsa-miR-548a-3p

and hsa-miR-6808-3p, with no previously reported changes in stroke were downregulated. The target genes of these miRNAs affect various cellular pathways including, RNA transport, autophagy, cell cycle progression, cellular senescence, and signaling pathways like mTOR, PI3K-Akt, and p53. Key hub genes within these networks include TP53, BCL2, Akt, CCND1, and NF-κB. These pathways are crucial for cellular function and stress response, and their dysregulation can have significant implications for the disease processes.

Conclusion: Our findings reveal distinct circulating EV-miRNA expression patterns in AIS patients from Qatar, highlighting potential biomarkers that could aid in stroke diagnosis and therapeutic strategies. The identified miRNAs are involved in critical cellular pathways, offering novel insights into the molecular mechanisms underlying stroke pathology. Circulating EV-miRNAs differentially expressed in AIS may have a pathophysiological role and may guide further research to elucidate their precise mechanisms.

Keywords: Brain, Ischemia, Stroke, miRNAs, Extracellular vesicles

1 INTRODUCTION

Stroke accounted for 6.55 million deaths in 2019 and contributed to 143 million disability-adjusted life years (DALYs), positioning it as the third leading cause of death and disability combined(Feigin et al., 2021). The stroke council of the American Stroke Association (ASA)/ American Heart Association (AHA) defines ischemic stroke as a central nervous system infarction with overt symptoms confirmed by neuropathological, neuroimaging, or clinical evidence of ischemic cell death (Sacco et al., 2013). Preventive strategies are crucial as most ischemic strokes are preventable, and the development of predictive biomarkers is essential for identifying high-risk individuals (Powers, 2020). Diagnosing acute ischemic stroke (AIS) can be challenging due to limitations in traditional neuroimaging techniques (Parody et al., 2015; Wardlaw et al., 2007). Circulating biomarkers can enhance early detection and diagnosis of AIS, providing a more efficient method. Likewise, prognostic biomarkers offer insights into the likelihood of stroke recurrence and unfavorable outcomes, aiding in the management of patients at risk of hemorrhagic transformation (van Kranendonk et al., 2019). A comprehensive approach to stroke management includes predictive and prognostic biomarkers, as well as preventative, therapeutic, and rehabilitation measures.

MicroRNAs (miRNAs) are small, non-coding RNA molecules that regulate gene expression by affecting mRNA stability or translation efficiency (Tétreault and De Guire, 2013). They are involved in various mechanisms of stroke pathophysiology and recovery, including apoptosis, inflammation, angiogenesis, oxidative stress, excitotoxicity, ischemia, and blood-brain barrier dysfunction (Kumari et al., 2022). Due to their presence in circulation, miRNAs are extensively studied as biomarkers for various pathophysiological conditions including stroke (Eyileten et al., 2018; Kadir et al., 2022; Li et al., 2015). miRNA profiling can predict stroke subtypes (Modak et al., 2019; Toor et al., 2022a; Zhou et al., 2022). Also, distinct miRNA signatures have been reported in stroke patients with comorbidities like diabetes, revealing new regulatory molecular pathways impacting stroke outcomes (Burlacu et al., 2022; Toor et al., 2022b). miRNAs are stabilized in circulation and delivered to target cells via extracellular vehicles (EVs) (Montecalvo et al., 2012), nanoscale membrane-bound vesicles known to carry cell-specific nucleic acids, proteins, and lipids to target cells, reprogramming the recipients (Zhang et al., 2019)). Indeed, in-vitro and ex-vivo studies have shown that circulating vesicles can target the endothelium, inducing vascular dysfunction (Agouni et al., 2011, 2008). Concerning stroke, an increase in endothelial-derived circulating EVs has been reported in AIS patients (Agouni et al., 2019). Despite the anticipated potential of circulating vesicles carrying miRNAs as significant biomarkers and therapeutic agents for various diseases, studies on EV-miRNA expression in AIS patients remain limited (Xu et al., 2022).

In this study, we compared circulating EV-miRNA profiles between a small cohort of stroke patients and a control group matched for age and existing comorbidities. Using microarray, we identified a set of 5 miRNAs that were differentially regulated between the two groups. We then explored the biological and molecular pathways associated with the differentially expressed miRNAs to identify the experimentally validated gene targets of these miRNAs, allowing us to identify the pathophysiological pathways that may be impacted by their dysregulation in AIS. Further functional studies on these pathways could identify potential therapeutic targets and facilitate the clinical translation of our findings.

2 MATERIALS AND METHODS

2.1 Patients and samples

The study adhered to the guidelines set by the Declaration of Helsinki and received approval from the Hamad Medical Corporation IRB (MRC-03-19-020). Participants or their authorized representatives provided written informed consent after being fully informed about the study. The study included 10 individuals consisting of 05 clinically diagnosed AIS patients admitted to Hamad General Hospital in Doha, Qatar and 05 controls matched by age, gender, and comorbidities (other than AIS) with the experimental group. Eligible participants were those over 18 years of age, within 24 hours of AIS onset, and willing to provide informed consent. Exclusion criteria included computed tomography/Magnetic Resonance Imaging (CT/MRI) based diagnosis other than stroke, stroke etiology related to ICH, ruptured aneurysm or ruptured AV malformation, active unstable coronary artery disease, inability to undergo MRI imaging, pregnancy, participation in a concurrent trial related to stroke or vascular disease and concurrent conditions such as end-stage renal or hepatic dysfunction, impaired cognition (with Montreal Cognitive Assessment (MoCA) Scale), or severe systemic illness that would prevent the patient from completing at least one year in the trial. Blood samples obtained from patients and controls through venipuncture and collected in K2 EDTA tubes (Vacutainer; Becton Dickinson) were processed within 2 h of the blood draw. Cell-free plasma was collected by centrifuging the blood at 1500 *g* for 15 min at 4 °C and stored immediately at -80°C until further downstream processing.

2.2 Purification of EVs

Size exclusion chromatography-based Izon qEVoriginal 35 nm Smart columns (Izon Science, Christchurch, New Zealand) were used to isolate and purify EVs from the cell free plasma, and the whole procedure was carried out at RT as per the manufacturer's instructions. Briefly, plasma samples were thawed at RT before EV isolation, and centrifuged at 1500 *g* for 10 minutes, followed by another round of centrifugation at 10,000 *g* for 10 minutes at room temperature, to remove cells and large particles. qEV columns and buffer (PBS) were equilibrated to RT, and columns were flushed with one column volume (10ml) of PBS. The prepared centrifuged plasma samples were loaded on top of the qEV columns, and after collecting the void volume (≈3 ml in total), four EV-rich fractions (0.5 ml each) were collected and pooled together. The pooled EV samples were concentrated using Izon Concentration Kit (Izon Science, Christchurch, New Zealand) to a final volume of 200 µl in PBS.

2.3 Validation of the purified EVs

Total protein content of the isolated concentrated EV-rich samples was determined by Pierce BCA Protein Assay Kit (Thermo Scientific). Samples were subjected to qualitative assessment for markers known to be present or enriched in EVs CD63, CD81, ALIX, FLOT1, ICAM1, EpCam, ANXA5, GM130, and TSG101 (REF: PMID: 25536934), using Exo-Check antibody array from System Biosciences (#EXORAY200B-4, #EXORAY210B-8, 2438 Embarcadero Way Palo Alto, CA). Briefly, 10X lysis buffer was added to 50 µg of EV-rich sample to a final 1X concentration and vortexed for 30 sec. Next, 1 µl of Labelling reagent was added to the mixture and incubated at room temperature for 30 minutes. The labeled EV lysate was then eluted using equilibrated column filters by centrifuging at 800 *g* for 2 minutes to remove the excess labeling reagent and

mixed with 5 ml Blocking Buffer. The resulting labeled EV lysate/blocking buffer mixture was applied on the prewetted membrane and incubated overnight at a 4°C shaker. Next day, the membrane was washed with 1X Wash Buffer and incubated in 5 ml Detection Buffer for 30 minutes at room temperature on a shaker. Finally, after washing with 1X Wash Buffer, the membrane was developed using Clarity™ Western ECL Substrate (#170-5061, Hercules, California, United States) and imaged using BioRad blot scanner (ChemiDoc MP System, Hercules, CA, USA).

2.4 RNA isolation

Isolation of total RNA (including miRNA) from the concentrated pooled EV-rich samples was achieved using miRNeasy Serum/Plasma Kit from Qiagen (# 1071073, Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. Briefly, 200 µl of the concentrated pooled EV-rich samples were lysed with QIAzol, and chloroform was added to the resulting QIAzol eluate. The aqueous phase was recovered and mixed with ethanol. The total RNA, including miRNA, was then bound to a spin column, and washed three times before elution. RNA yield and purity were evaluated spectrophotometrically (Nanodrop ND-1000, Thermo, Wilmington, DE, USA).

2.5 Microarray profiling

Isolated RNA samples were labeled using the FlashTag® Biotin HSR RNA Labeling Kit for Affymetrix® GeneChip® miRNA 4.0 Arrays to analyze the miRNA expression profiles in AIS patients and controls as per the recommended instructions by the manufacturer. Briefly, 200 ng of total RNA adjusted to 8 µl reaction volume with Nuclease-Free Water was mixed with 2 µl of RNA Spike Control Oligos and 5 µl of Poly(A) Tailing Master Mix, and then incubated at 37°C for 15 minutes. Next, 4 µl of 5 × FlashTag Biotin HSR Ligation Mix was added to the Poly(A) Tailing Mix followed by 2 µl of T4 DNA Ligase and incubated at 25°C for 30 minutes. The reaction was halted by adding 2.5 µl of Stop Solution. The resulting biotin-labeled RNA was then hybridized on GeneChip™ miRNA Arrays. The chips were then stained, scanned using an Affymetrix Gene Array scanner 3000 7G, and analyzed according to the Affymetrix technical manual (Affymetrix, Santa Clara, CA, USA). The hybridization process was carried out at 48°C at 60 rpm for 16 hours in Affymetrix GeneChip® Hybridization Oven 640 (Central Expressway Santa Clara, CA). The chips were subsequently washed and stained using Affymetrix GeneChip® Fluidics Station 450 (Central Expressway Santa Clara, CA), and images were acquired. The Hybridization Controls were added to the hybridization cocktail for monitoring the array of hybridization, washing, and staining. The spike-in control 2, 23, and 29 were added to confirm poly(A) tailing and ligation. Oligo 31 was spiked-in to confirm ligation while oligo 36 to confirm ligation and lack of RNAse in the RNA sample.

2.6 Data Analysis and Differential Gene Expression Analysis

The CEL files were imported into Transcriptome Analysis Console (TAC) for quality control, normalization, and annotation using the miRNA 4.0 library files. Quality control measures were implemented through the examination of signals generated by hybridization and spike-in controls. The signals were then normalized using the robust multi-array average (RMA) method. To identify present or absent genes, the (Detection Above Background) DABG method with a default

threshold of 0.05 was applied. The RMA method normalizes signals across multiple arrays, while the DABG method with a 0.05 threshold distinguishes between genes that are actively expressed (present) and those that are not (absent) based on background noise levels. Over-representation analysis (ORA) was performed using the miRNA Enrichment Analysis and Annotation (miEAA) tool to identify significantly enriched biological pathways, functions, and tissues associated with the differentially expressed miRNAs. A reference list comprising all miRNA probes on the microarray was used as the background set to ensure unbiased results. This approach allowed us to determine whether the identified miRNAs were statistically overrepresented in specific biological contexts compared to what would be expected by chance. miRNA family classification was performed using the miRViz web tool (http://mirviz.prabi.fr/) based on the seed sequences of the identified miRNAs.

To analyze differential gene expression, R programming language in R studio (Version 2022.12.0) and packages from the Bioconductor project were utilized. An empirical Bayes moderated t-test, implemented through the limma package, was applied to detect differences in gene expression between samples (Ritchie et al., 2015). P-values were adjusted for multiple testing using the method of Benjamini and Hochberg, and a statistical significance level of FDR adjusted p-value < 0.05 and log2FC of 1 were employed (Benjamini and Hochberg, 1995). The Bioconductor package EnhancedVolcano was utilized to create volcano plots (Blighe et al., 2023). Additionally, the 'heatmaps' package (26) was employed for hierarchical cluster analysis of the microarray data using complete linkage and the generation of heatmaps (Perry, 2023). GraphPad Prism (Version 19.0) was used for visualizing the log2FC values of the identified signal in AIS patients and controls.

2.7 Expression of the identified signature in GEO datasets

Two publicly available Gene Expression Omnibus (GEO) datasets, GSE169353 and GSE199942 were utilized, to validate the expression of five miRNAs previously identified as differentially expressed AIS patients. One dataset (GSE270958) containing the transcriptomic data of the mouse ischemic cortex was included. GSE169353 included serum exosomal miRNA profiles from 3 AIS patients and 3 healthy controls, while GSE199942 comprised miRNA expression data from serum micro vesicles of 5 AIS patients and 5 controls, with both studies employing the GPL16791 Illumina HiSeq 2500 platform for sequencing. The raw read counts were extracted and normalized, ensuring consistency and comparability. The expression levels of the selected miRNAs were then statistically compared between AIS patients and healthy controls using a t-test, allowing for the assessment of their differential expression. GSE270958 dataset contains the transcriptomic profiles from cortex of 3 control mice and 3 mice with ischemic brain injury induced by the middle cerebral artery occlusion (MCAO). The differential expression of the genes was downloaded from the GEO database. The mouse gene IDs were converted into ortholog human gene IDs using ensemble database and the upregulated and the direction of expression was compared with the target genes of the miRNAs in our data.

2.8 Target identification and functional enrichment analysis

In this study, we employed a multi-step methodology for functional enrichment analysis. Initially, we discerned the targets of differentially expressed miRNAs utilizing miRTargetLink 2.0 (Kern et al., 2021), with a focus on experimentally validated miRNA-gene interactions. To ensure the

robustness of our results, we applied filters based on the strength of experimental evidence supporting these interactions. Subsequently, we conducted a comprehensive functional enrichment analysis using both the enrichR and the Ingenuity Pathway Analysis software. This analysis aimed to unveil the top diseases and functions associated with the target genes of the differentially expressed miRNAs. To assess the statistical significance of these associations, we compared the gene lists derived from miRNA targets against annotated gene sets representing prior biological knowledge to determine over-representation of functional categories in the input list. This rigorous approach allowed us to gain valuable insights into the biological implications of the differentially expressed miRNAs in our study.

3 RESULTS

3.1 Patients and samples

Five (05) subjects with AIS and 05 age, gender and comorbidity matched controls were included in this study. The diagnosis of AIS was made by CT and the severity assessment was done using the National Institute of Health Stroke Scale (NIHSS). The demographic information for both the AIS patients and the control participants is provided in Table 1. The study population contained 04 male and 01 female patients each in AIS and control group. No statistically significant ($p < 0.05$) age difference was found between the groups and the average age of the AIS group was 57 ± 3.39 years vs 62.2 ± 7.82 of the control group. All patients and controls had a history of hypertension and diabetes (known risk factors of AIS) and were receiving treatment before admission to the hospital. The average systolic blood pressure of the AIS group was significantly higher than the systolic blood pressure of the control group. However, the difference between diastolic blood pressure was non-significant between the two groups.

EVs from cell-free plasma were isolated using size exclusion chromatography-based qEV columns and their endosomal origin characterized by Exo-Check antibody array. A positive expression of known exosomal markers CD63, CD81, ALIX, FLOT1, ICAM1, EpCam, ANXA5, and TSG101 indicated that our EV preparation is highly enriched in exosomes (Figure 1A).

3.2 Microarray profiling and Differential gene expression analysis

Microarray analysis of the samples revealed the expression of 383 miRNAs after accounting for background noise. Of these, 245 miRNAs were expressed in both groups, while 90 and 48 miRNAs were exclusively expressed in control and AIS samples, respectively (Supplementary Table S1). The most abundant miRNAs in the stroke group were categorized into families based on their seed sequences, predominantly belonging to the let-7b, miR-106, miR-16, and miR-320 families (Supplementary Figure 1).

miRNAs highly expressed in AIS samples only (average log2 expression > 2) were overrepresented in tissues such as adipocytes, arachnoid mater, arteries, brain, and dura mater. Gene Ontology (GO) analysis revealed an overrepresentation of terms including extracellular exosome, extracellular vesicle, negative regulation of BMP secretion, negative regulation of BMP signaling pathway, negative regulation of cysteine-type endopeptidase activity involved in apoptotic processes, and outflow tract morphogenesis (Supplementary Table S2). Circulating EV-miRNA profiles of patients were compared with healthy individuals and 5 miRNAs were found to

be significantly differentially expressed between the two groups (FDR P < 0.05, log2FC > 1) (Figure 1B and Figure1C). hsa-let-7b-5p, hsa-miR-16-5p, hsa-mir-320c were upregulated while hsa-mir-548a-3p, hsa-mir-6808-3p were downregulated in the AIS group as compared to control group (Figure 2). The log2FC values and the FDR P-values are given in table 2 along with the sequences of the differentially expressed miRNAs. The log2 signal intensities of the individual samples in the group are shown in Figure 2.

3.3 Expression of the identified signature in GEO datasets

In this study, we successfully leveraged two comprehensive GEO datasets, GSE169353 and GSE199942, to validate the differential expression of key miRNAs in AIS patients. One dataset (GSE270958) representing the ischemic brain injury from a mouse model was utilized to confirm the effect of dysregulated miRNAs on the target genes. Our analysis revealed significant alterations in the levels of hsa-let-7b-5p and hsa-mir-548a-3p in the GSE169353 dataset, and an upregulation of hsa-mir-16-5p in the GSE199942 dataset, underscoring their potential roles in AIS pathophysiology. However, the analysis was limited by the low expression of hsa-mir-6808-3p and hsa-mir-548a-3p in GSE169353 and GSE199942, respectively, leading to their exclusion (Figure 3A). The expression data from the mouse model confirmed that 65 (1.9%) target genes of the upregulated miRNAs in our dataset were significantly downregulated in the ischemic mouse brain. Conversely, only 17(0.8%) target genes of the downregulated miRNAs were upregulated in the ischemic cortex of mouse (Figure 3B).

3.4 Target prediction of miRNA and functional enrichment analysis

Using the miRTargetLink 2.0 database, we identified 2735 unique target genes along with experimental evidence for each miRNA as indicated in Supplementary Table S3. In order to visualize those genes that overlap as well as those that are unique among these miRNAs, a Venn diagram was constructed (Figure 4). Remarkably, hsa-miR-16-5p and hsa-let-7b-5p were responsible for the largest number of gene targets with both of them targeting concurrently 229 genes.

The up-regulated and down-regulated miRNA target genes were separately enriched using enrichR. Consequently, the enrichment analysis gave significant KEGG pathways such as signaling pathways regulating the pluripotency of stem cells and TGF-beta signaling pathways upregulated in AIS (Figure 5). The GO biological process enrichment highlighted regulation of translation and negative regulation of transcription. GO molecular function showed a clear and significant increase in the mRNA-UTR binding in stroke indicating a regulatory role of miRNAs the regulation of gene expression. Go cellular component enrichment showed downregulation of the focal adhesion in AIS and upregulation in cytoplasmic stress granule related genes (Figure 5).

Target genes of the dysregulated miRNAs wer clustered into eight distinct networks. The radial network diagrams illustrated TP53-, BCL2-, Akt-, CCND1- and NF-kB-centered top five networks (Figure 6). Consequently, these central genes play a critical role in shaping their respective network structures and functioning

4 DISCUSSION

The prognosis of AIS patients depends on accurate diagnosis and timely management. Current imaging tools can examine infarct volume and location but are ineffective for early detection and identifying the underlying cause. Biomarkers can indicate physiological and pathological conditions (Makris et al., 2018), and show distinct expression levels allowing their profiling to predict stroke subtypes and severity (Kadir et al., 2022; Parray et al., 2022; Tan et al., 2009; Toor et al., 2022a, 2022b). However, the lack of direct brain tissue access complicates linking peripheral biomarkers to neuronal expression. Identifying peripheral biomarkers of neurological origin or biomarkers directly linked to neuronal changes can improve our understanding and prognosis of acute stroke(Osier et al., 2018).

Recent studies suggest EVs play a role in ischemic stroke pathophysiology. Injured brain cells release EVs, initiating various responses. Some EVs have neuroprotective effects; for example, exosomes secreted by astrocytes and oligodendrocytes offer neuroprotection, while those derived from mesenchymal stem cells promote neural repair and improve neurological deficits (Fröhlich et al., 2014; Wang et al., 2011). Conversely, some EVs, like endothelium-derived exosomes, are associated with neurodegeneration and can damage the blood-brain barrier (BBB) (Pan et al., 2016). EVs can cross the BBB and circulate in blood and cerebrospinal fluid, making them ideal non-invasive biomarkers for AIS diagnosis and prognosis (Xu et al., 2022). However, most identified miRNAs from peripheral blood lack brain specificity, possibly reflecting systemic effects of brain damage. In this study, we isolated EVs from AIS patients' blood within 24 hours of onset and examined miRNA expression. Compared to controls, five EV-miRNAs showed significantly varied expression levels in AIS patients: upregulation of hsa-let-7b-5p, hsa-miR-16-5p, and hsa-miR-320c, and downregulation of hsa-miR-548a-3p and hsa-miR-6808-3p. The upregulated EV-miRNAs in our study (miR-16-5p, and miR-320c) were also upregulated in human stroke brain tissue resected during decompressive craniectomy, suggesting neuronal origin(Carlson et al., 2021). However, novel techniques are needed to efficiently confirm the identity of EVs derived from specific cells or brain tissues and establish their relationship with neurological disorders (Eitan et al., 2023; Guix et al., 2018).

Increased expression of hsa-let-7b-5p (human miRNA-7b-5p) in EVs from the blood of stroke patients in our study, in alignment with a previous study where its higher levels were also associated with worse stroke outcomes (Chi et al., 2020), is consistent with the reports that miRNAs remain stable and are retained in circulation months after stroke onset (Chen et al., 2008; Tan et al., 2009), implying thereby its potential as a prognostic biomarker in AIS. Recently, the therapeutic role of hsa-mir-7b-5p was investigated by Aday et al., hsa-let-7b levels were differentially present in the angiogenic EVs and its delivery to animal models of stroke reduced the brain injury and improved the functional recovery through enhanced angiogenesis (Aday et al., 2021). Among the gene targets of hsa-let-7b-5p, TGFBR1 is linked to Loeys-Dietz syndrome (LDS) which is characterized by severe cerebrovascular events with a potential to develop into stroke (Laterza et al., 2019; Zhou et al., 2021), while TLR4 has been identified to promote platelet-dependent thrombosis in SARS-CoV-2 (Carnevale et al., 2023). Further research is necessary to fully understand the role of hsa-let-7b-5p in AIS and to develop effective diagnostic and therapeutic strategies based on this miRNA.

Another miRNA, hsa-miR-16-5p that was upregulated in AIS patients, saw its circulating levels as detected by RT-qPCR increase acutely in stroke patient plasma (Rainer et al., 2016) , and in

serum (Wu et al., 2015). These findings corroborate with a recent bioinformatic analysis that identified miR-16-5p as a specific biomarker in AIS (Jiang et al., 2022). Additionally, plasma concentrations of hsa-miR-16 were also elevated in ischemic stroke patients compared to hemorrhagic stroke patients (Leung et al., 2014),), indicating its specificity for ischemic stroke... Several studies have associated miR-16-5p with rheumatoid arthritis (RA) (Dunaeva et al., 2018; Pauley et al., 2008). VEGFA, a validated gene target of miR-16-5p, has been linked to poor stroke outcomes, with elevated levels seen in ischemic stroke patients who died or had moderate to severe disability (Bhasin et al., 2019; Escudero et al., 2021). Other validated target genes of miR-16-5p, such as APP and BACE1, are known risk factors for Alzheimer's disease (AD) and their potential implications in AD have been investigated. miR-16 showed neuroprotective effects in several AD models via modulating amyloid precursor protein (APP) expression and its cleavage by β-site amyloid precursor protein-cleaving enzyme 1 (BACE1) (Liu et al., 2012; Zhang et al., 2015; Zhong et al., 2018). Whether increased miR-16 levels observed in stroke patients in this study represent a compensatory mechanism to prevent neuronal damage, like the case in AD models, remains to be determined.

We also report a significant higher circulating hsa-miR-320c levels in EVs in AIS patients, consistent with previous studies showing elevated hsa-miR-320 levels in both blood and brain samples of rodent stroke models (Dharap et al., 2009; Jeyaseelan et al., 2008) and in human stroke patient blood samples (Tan et al., 2009). Dysregulated hsa-miR-320 has been implicated in angiogenesis, as shown in type 2 diabetic Goto-Kakizaki rats where elevated miR-320 levels correlated with impaired angiogenesis in myocardial microvascular endothelial cells (MMVEC), and inhibition of miR-320 improved cell proliferation and migration(Wang et al., 2009). Similarly, increased hsa-miR-320c levels were observed after pro-inflammatory lipopolysaccharide treatment (Matamala et al., 2021). In contrast, traumatic brain injury reduced hsa-miR-320 levels in a rodent model (Redell et al., 2009) , and its downregulation restored normal cell functions through regulation of the apoptotic suppressor Mcl-1(Chen et al., 2009). Likewise, stroke patients with a good outcome (mRS<2) showed slight downregulation of hsa-miR-320c levels (Tan et al., 2009), hinting at a potential predictive role for favorable outcomes likely via activated angiogenesis. Therefore, more studies are warranted for further elucidation of its dysregulation in stroke pathology. PRDM1 (also known as B lymphocyte-induced maturation protein-1, BLIMP-1), crucial for B lymphocyte development, plasma cell differentiation (Shaffer et al., 2002; Shapiro-Shelef et al., 2003), and T cell hyporesponsiveness which prevents autoimmune pathology and transplantation-induced damage (Guo et al., 2022), is a key target of miR-320c.

Two miRNAs, hsa-miR-548a-3p and hsa-miR-6808-3p, have not been previously implicated in stroke and to our knowledge, we the first to report their downregulation AIS patients. Downregulation of miR-548a-3p levels was observed in serum exosomes and PBMCs of rheumatoid arthritis (RA) patients, correlating with higher levels of C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and rheumatoid factor (RF) (Wang et al., 2019). Since, a downregulation of TLR4 mRNA and protein levels was observed after exogenous miR-548a-3p overexpression in pTHP-1cells (Wang et al., 2019), it is likely that miR-548a-3p regulates TLR4-mediated inflammatory responses involved in RA. ECHS1, the only gene target of miR-548a-3p was reported to be one of the causes of Leigh disease (Peters et al., 2014). In the context of stroke, however, the probable mechanism needs to be elucidated.

miRNAs exert their effects by targeting several pathological processes involved in ischemic stroke progression, like autophagy, BBB disruption, excitotoxicity, oxidative stress, inflammation, and caspase-mediated cell death signaling (Khoshnam et al., 2017). Our pathway analysis indicates that the five differentially regulated miRNAs identified in this study are broadly involved in regulating cellular organization, proliferation, growth, development, survival, and death, as well as nervous system development and function. IPA analysis revealed that the target genes of these miRNAs are interconnected into five critical networks with TP53, BCL2, Akt, CCND1, and NFKB as hub genes. The TP53 pathway responds to various intrinsic and extrinsic stresses, leading to cell cycle arrest, cellular senescence, or apoptosis (Harris and Levine, 2005). The apoptotic effects of TP53 may involve the interaction with BCL2 family of proteins (Yan et al., 2021). Both TP53 and BCL2 are experimentally validated targets of the differentially expressed hsa-miR-16-5p. The PI3K/Akt pathway, which regulates cell survival, growth, and metabolism, is known to be dysregulated in AIS (X. Liu et al., 2022).). It also mediates blood vessel formation, crucial for stroke prevention and recovery (Karar and Maity, 2011). Akt is an experimentally validated target of both hsa-let-7b-5p and hsa-mir-16-5p, and their downregulation is known to protect the cells by activating the Akt pathway (Calderon-Dominguez et al., 2021; Li et al., 2020). The role of CCND1 beyond cellular proliferation is evidently increasing and a pathological increase of CCND1 in focal neuronal ischemia is reported (Y. Liu et al., 2022; Osuga et al., 2000). Thus, the observed overexpression of miR-16-5p, which targets CCND1 and related cyclin-dependent kinase family proteins, might be protective. In a cell model of irritable bowel disease, miR-16-5p decreases the activation of NFκB to limit inflammation (Xi et al., 2022). While several studies have implicated the pathological role of NFκB in ischemic stroke, its role in the context of miR-16-5p requires further evaluation.

5 Conclusion

In conclusion, our data show that acute ischemic stroke (AIS) patients have a distinctive miRNA expression profile in circulating EVs compared to healthy controls. Five differentially expressed miRNAs—hsa-let-7b-5p, hsa-miR-16-5p, hsa-miR-320c, hsa-miR-548a-3p, and hsa-miR-6808-3p identified here can regulate multiple genes and pathways relevant to stroke. However, the cause and consequence merits further studies. The upregulated miRNAs hsa-let-7b-5p and hsa-miR-16-5p, also elevated in human stroke brain tissue, are implicated in crucial biological pathways involving TP53, BCL2, Akt, CCND1, and NFKB. To our knowledge, this is the first study to report the dysregulation of two miRNAs between AIS and healthy individuals. While our findings provide valuable insights into the miRNA expression profile in circulating EVs of AIS patients, we acknowledge several limitations of our study. The small sample size limits the statistical power and generalizability of our results, necessitating validation in larger cohorts. Our cross-sectional design prevents us from establishing a clear cause-and-effect relationship between the identified miRNAs and stroke pathology, and we cannot account for potential changes in miRNA expression over time. Furthermore, our study focused on a specific set of miRNAs in acute ischemic stroke, which may not capture the full complexity of miRNA dysregulation in different stroke types or chronic conditions. We also recognize the need for functional validation of the identified miRNA-target interactions and their potential therapeutic implications in stroke models. The experimentally validated target genes provide a more reliable insight into the molecular mechanisms potentially contributing to the pathology of stroke and may offer targets for therapeutic intervention. Future studies should address these limitations, consider potential

confounding factors, and investigate the correlation between miRNA expression and clinical outcomes to further elucidate the role of these molecules in stroke pathophysiology and their potential as biomarkers or therapeutic targets.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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DATA AVAILABILITY STATEMENT

The raw datasets used in this study, which underpin the findings and conclusions presented in the manuscript, are securely stored, and maintained by the corresponding author. We are committed to promoting scientific rigor and open access to our data. We welcome inquiries and requests for access to the raw datasets. Interested parties who wish to obtain access to the data for further analysis or validation are encouraged to reach out to the corresponding author.

ETHICS APPROVAL AND INFORMED CONSENT

The study adhered to the guidelines set by the Declaration of Helsinki and received approval from the Institutional Review Board of Hamad Medical Corporation, Qatar (MRC-03-19-020). Participants or their authorized representatives provided written informed consent after being fully informed about the study.

ABRREVIATIONS

DALYs - Disability-adjusted life years

ASA - American Stroke Association

AHA - American Heart Association

AIS - Acute ischemic stroke

miRNAs - MicroRNAs

EVs - Extracellular vehicles

CT - Computed tomography

MRI - Magnetic Resonance Imaging

ICH - Intracranial hemorrhage

AV - Arteriovenous

MoCA - Montreal Cognitive Assessment

PBS - Phosphate-buffered saline

RT - Room temperature

RMA - Robust multi-array average

DABG - Detection Above Background

ORA - Over-representation analysis

miEAA - miRNA Enrichment Analysis and Annotation

FDR - False discovery rate

GEO - Gene Expression Omnibus

MCAO - Middle cerebral artery occlusion

NIHSS - National Institute of Health Stroke Scale

GO - Gene Ontology

BBB - Blood-brain barrier

RA - Rheumatoid arthritis

AD - Alzheimer's disease

APP - Amyloid precursor protein

BACE1 - β-site amyloid precursor protein-cleaving enzyme 1

MMVEC - Myocardial microvascular endothelial cells

mRS - Modified Rankin Scale

BLIMP-1 - B lymphocyte-induced maturation protein-1

PBMCs - Peripheral blood mononuclear cells

CRP - C-reactive protein

ESR - Erythrocyte sedimentation rate

RF - Rheumatoid factor

IPA - Ingenuity Pathway Analysis

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FIGURES

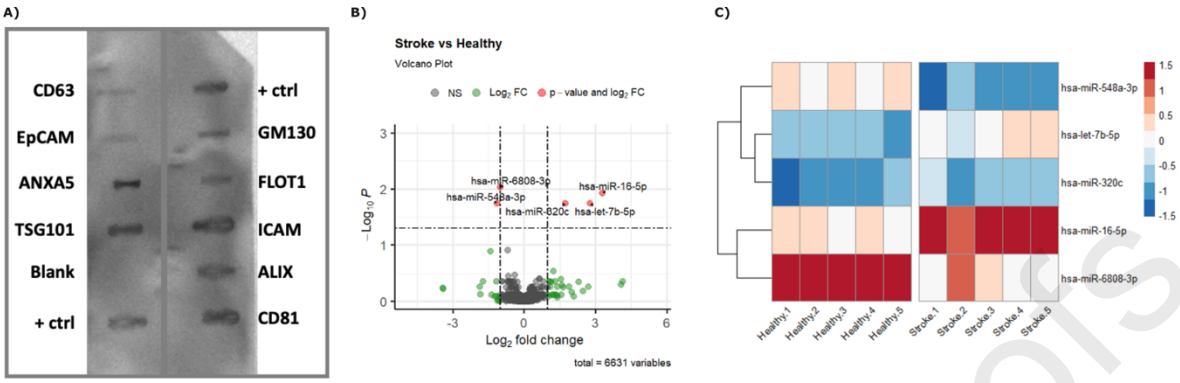


Figure 1. (A) Representative Exo-array blot from the Exo-array of known EV markers (CD63 and CD81: Tetraspanins, EpCAM: epithelial cell adhesion molecule, ANXA5: annexin A5, TSG101: tumor susceptibility gene 101, FLOT1: flotillin-1, ICAM: intercellular adhesion molecule 1, GM130: cis-golgi matric protein and ALIX: programmed cell death 6 interacting protein), and three controls including two positive controls (+ Ctrl), and a blank control (Blank). **(B)** Volcano plot showing the results of differential expression analysis. The x-axis represents the log2 fold change between the two groups, and the y-axis represents the negative log10 of the FDR p-value. Red dots indicate significantly differentially expressed genes at the thresholds ($p < 0.05$, log2FC >1). The horizontal line indicates the threshold for statistical significance and vertical line indicates the threshold for log2Fc. **(C)** A heatmap with clustering of miRNA expression showing both the relative expression levels of differentially expressed miRNAs and the relationships between the miRNAs. The rows of the heatmap correspond to individual miRNAs, while the columns correspond to the samples. The color scale in the heatmap represents the normalized range of expression levels over the rows.

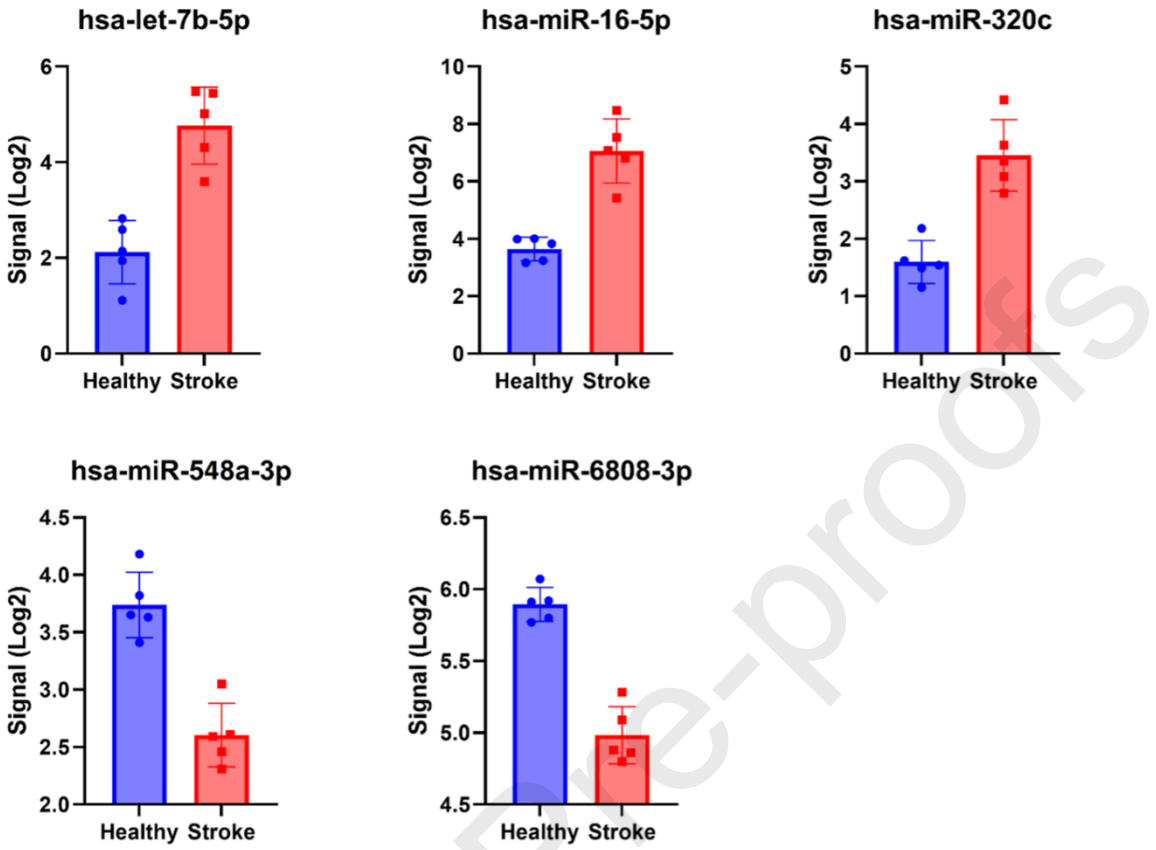


Figure 2. Bar plots showing log2 signal intensities of five miRNAs in AIS and control groups. hsa-let-7b-5p, hsa-miR-16-5p, hsa-mir-320c were upregulated while hsa-mir-548a-3p, hsa-mir-6808-3p were downregulated in the AIS group as compared to the control group.

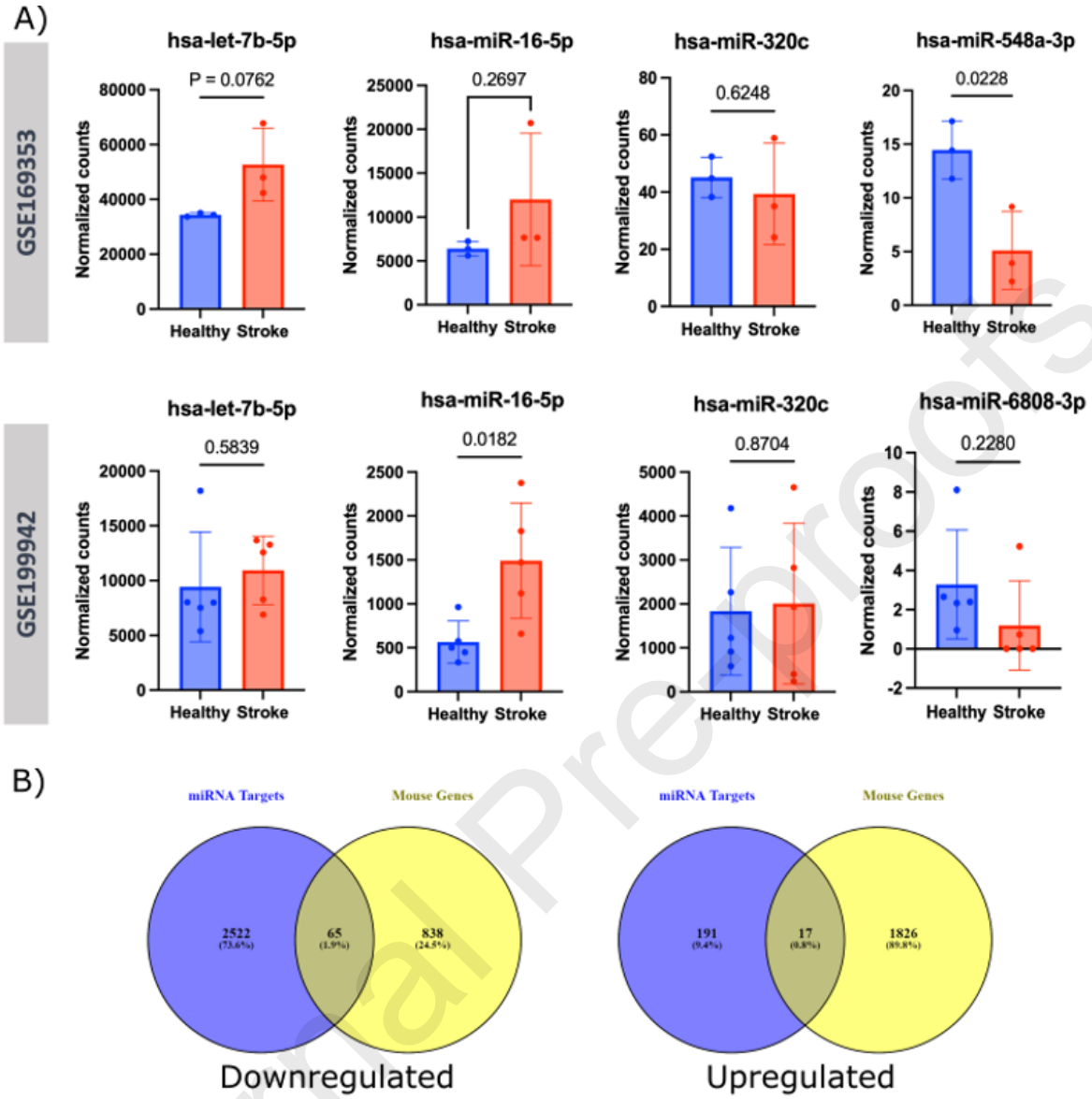
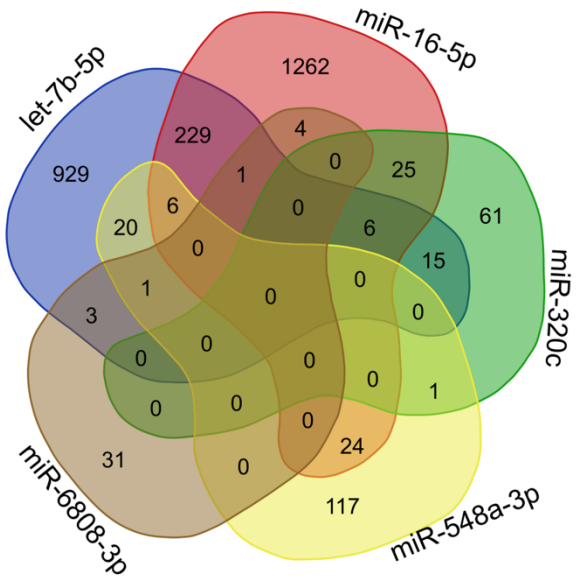


Figure 3. Comparative expression analysis of miRNAs in control and AIS patients in GEO datasets. (A) A statistically significant upregulation of hsa-let-7b-5p and downregulation of hsa-mir-548a-3p can be seen in the GSE169353 dataset, while an upregulation of hsa-mir-16-5p in the GSE199942 dataset. (B) Venn diagram showing the overlap of target genes with the dysregulated genes in the GSE270958 dataset.



miRNA	Number of targets	Number of unique targets
hsa-let-7b-5p	1508	1210
hsa-miR-16-5p	2279	1557
hsa-miR-320c	125	108
hsa-miR-548a-3p	221	169
hsa-miR-6808-3p	52	40
Overall number of unique targets		**2735**

Figure 4. Venn diagram showing the number of identified targets for each miRNA and the number of overlapping targets for combination of miRNAs.

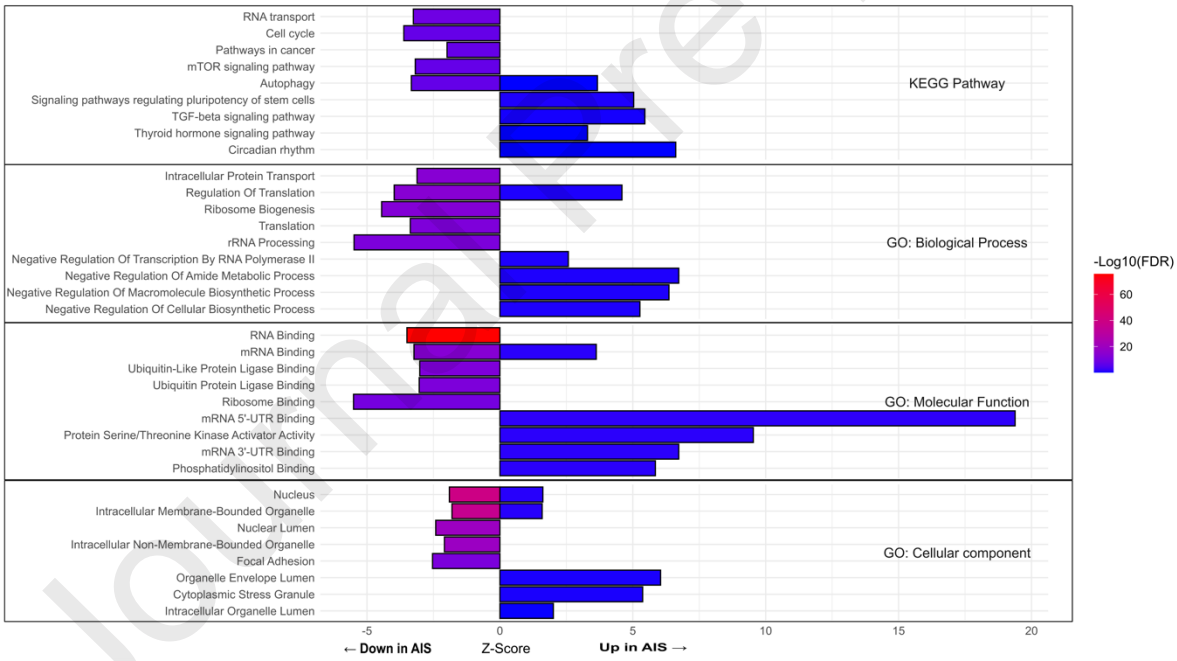


Figure 5. Functional enrichment analysis of miRNA target genes in Acute Ischemic Stroke (AIS). The figure displays enriched KEGG pathways, Gene Ontology (GO) Biological Processes, Molecular Functions, and Cellular Components associated with differentially expressed miRNA target genes in AIS. The x-axis represents the Z-score, with negative values indicating downregulation and positive values indicating upregulation in AIS. The color scale represents the -Log10(FDR) value, with red indicating higher statistical significance.

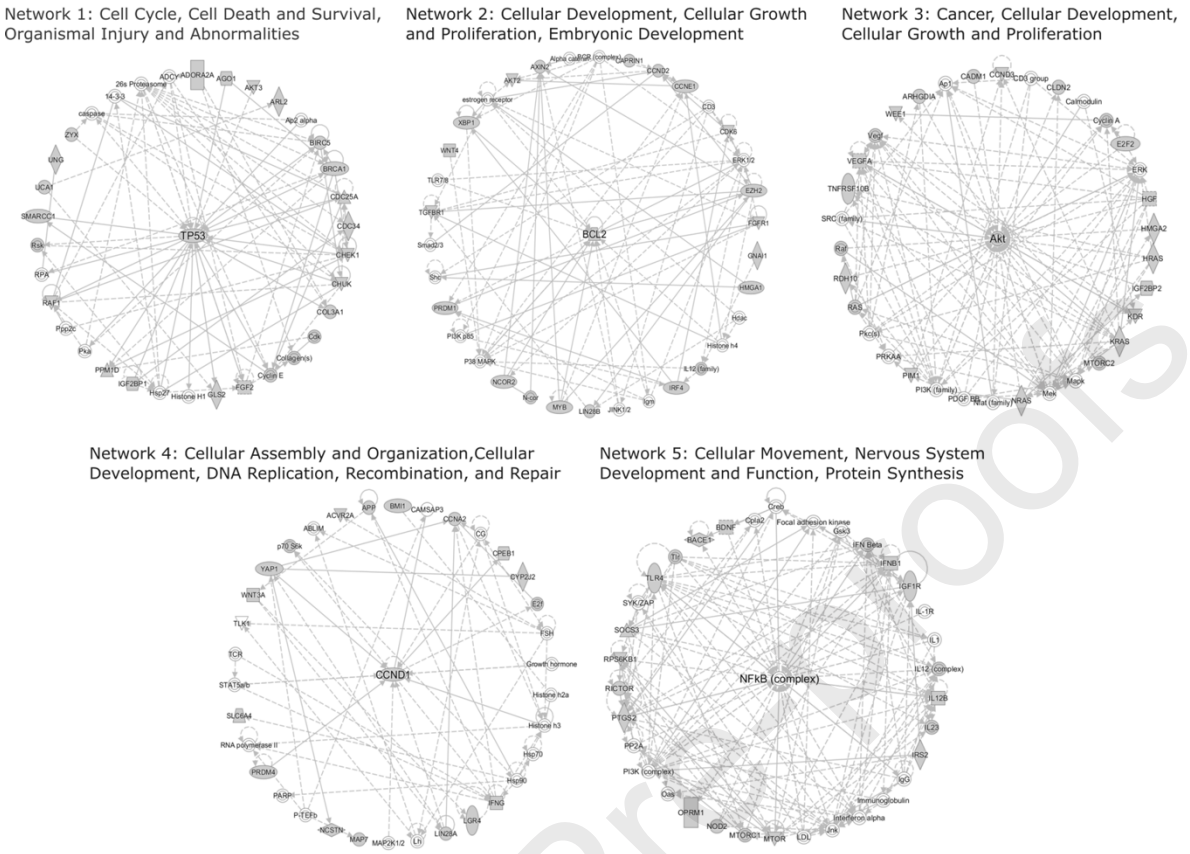


Figure 6. Ingenuity Pathway Analysis (IPA) network visualization of the target genes of differentially regulated miRNAs. The networks are centered around hub genes *TP53*, *BCL2*, *Akt*, *CCND1*, and *NF-κB*, and are enriched with various disease and functional pathways, providing insight into the potential roles of the miRNAs in different biological processes.

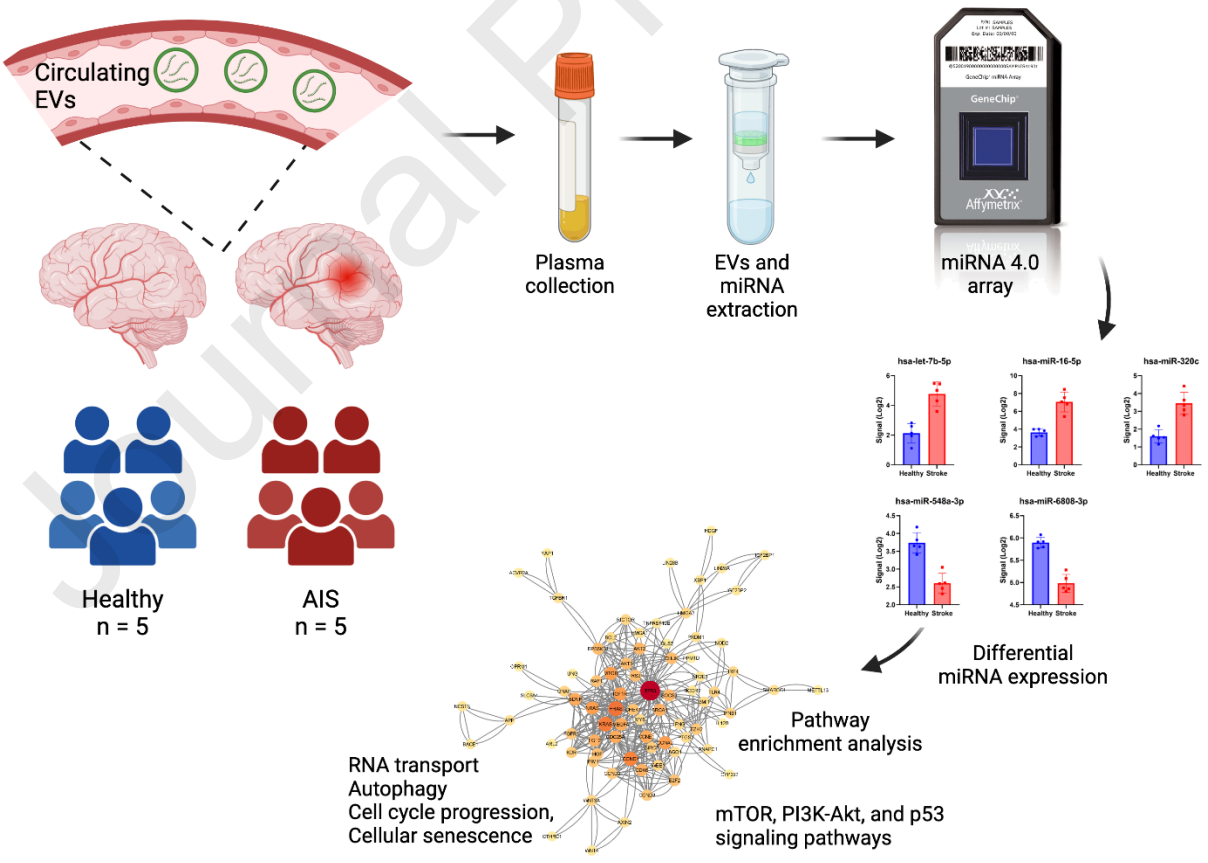
TABLES

Table 1. Demographic information and medication and disease history of the subjects in AIS and Control groups.

	CONTROL	STROKE
GENDER		
MALE	4 (80%)	4 (80%)
FEMALE	1 (20%)	1 (20%)
AGE	57 ± 3.39	62.2 ± 7.82
BLOOD PRESSURE		
SYSTOLIC	121.8 ± 5.58	151.6 ± 7.56
DIASTOLIC	71.6 ± 6.19	76.4 ± 12.42
MEDICATION HISTORY		
HYPERTENSION TX	5	5
DIABETES TX	5	5
DISEASE HISTORY		
HYPERTENSION	5	5
DIABETES	5	5
DYSLIPIDEMIA	4	4

Table 2. Differentially expressed miRNAs in the stroke group as compared to control group with their log2FC, FDR P-values and sequences.

miRNA ID	Log2FC	FDR P-value	Sequence (5′-3′)
hsa-let-7b-5p	2.77	0.0181	UGAGGUAGUAGGUUGUGUGGUU
hsa-miR-16-5p	3.29	0.0118	UAGCAGCACGUAAAUAUUGGCG
hsa-miR-320c	1.74	0.0181	AAAAGCUGGGUUGAGAGGGU
hsa-miR-548a-3p	-1.13	0.0181	CAAAACUGGCAAUUACUUUUGC
hsa-miR-6808-3p	-1	0.0094	GUGUGACCACCGUUCCUGCAG



Highlights

- Five circulating EV-miRNAs were differentially expressed in acute ischemic stroke.
- Novel downregulation of hsa-miR-548a-3p and hsa-miR-6808-3p was observed in stroke.
- EV-miRNAs implicate key pathways including RNA transport, autophagy, and cell cycle.
- Findings reveal potential biomarkers for stroke diagnosis and therapeutic strategies.
- Study provides insights into molecular mechanisms of stroke pathology.