Molecular Neurobiology (2019) 56:6976–6985
https://doi.org/10.1007/s12035-019-1562-x

Novel miRNA PC-5P-12969 in Ischemic Stroke

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Received: 4 January 2019 / Accepted: 13 March 2019 / Published online: 5 April 2019

Abstract

Circulating microRNAs (miRNAs) have been used effectively as peripheral biomarkers and mechanistic targets for human diseases such as stroke, Alzheimer's, and cancer. The purpose of our study is to determine noninvasive, blood-based early detectable biomarkers for ischemic stroke (IS). Based on our previous global miRNA sequencing study, four miRNAs were previously unreported (novel) in IS condition. Among these, miRNA PC-5P-12969 was exclusively expressed in the IS condition; otherwise, it was not expressed in normal condition, and therefore, we focused on miRNA PC-5P-12969 for further studies. In the present study, we investigated novel miRNA PC-5P-12969 for its expression levels using quantitative real-time PCR assay (qRT-PCR) in an in vitro, oxygen, and glucose deprivation/reoxygenation (OGD/R)-treated mouse primary hippocampal neuronal cells (HT22) and in an in vivo using a photothrombotic stroke model. In an in vitro study of stroke-induced HT22 cells, we found a two fold increase of PC-5P-12969 expression levels, in agreement with our original global miRNA study. In the cerebral cortex of photothrombotic stroke mice, we found significantly upregulated levels of PC-5P-12969 in 4 hours and 1 day post-stroke relative to the control mice. However, we did not find any change in the expression of PC-5P-12969 in the cerebellum (unaffected in IS) of both stroke and control mice. Based on findings from this study, together with our earlier original global microRNA study results, we conclude that PC-5P-12969 is a potential candidate of the peripheral marker and also a drug target for IS. This is the first study validating that the miRNA PC-5P-12969, might be a potential biomarker for IS.

Keywords Ischemic stroke · Novel miRNA · Biomarker · HT22 · Infarct volume · Photothrombotic stroke model

Introduction

Stroke is a multifactorial disease caused by a sudden interruption of blood flow to a specific brain region and is usually accompanied by atherosclerosis, hypertension, and diabetes mellitus [1]. The traditional definition of stroke offered by the World Health Organization is, "rapidly developing clinical signs of focal or global disturbance of cerebral function, with symptoms lasting 24 hours or longer, or leading to death with no apparent cause other than vascular origin" [2]. Stroke is the fifth leading cause of death in the USA, with more than 140,000 Americans dying each year from stroke. Each year, approximately 795,000 people are affected by stroke worldwide. About

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600,000 of these are first attacks [3]. The absolute number of patients experiencing their first stroke continues to increase annually because of the aging population in rapidly economically growing countries [4].

Stroke is broadly classified into two major clinical types: hemorrhagic (bleedings) and ischemic stroke (IS). To diagnose stroke, modern neuroimaging approaches (e.g., computed tomography, magnetic resonance imaging) are used, which however are costly and require advanced training. A relatively low-cost alternative for diagnosis of stroke could be through stroke-specific biomarkers. However, such biomarkers have not been identified yet.

MicroRNAs are 18–25 nucleotides in length, noncoding RNA molecules in different tissues and fluids in mammals, including humans. Peripheral miRNAs are stable, once released to fluids from tissues. Many miRNAs were found to be highly conserved, and these miRNAs act as an antisense regulator of other RNAs [5]. The miRNAs are endogenous, short, and small RNAs that regulate the expressions of ~ 60% of human protein-coding genes [6]. MicroRNAs modulate diverse biological processes, including cell proliferation, cell differentiation, metabolism, cell cycle, apoptosis, and cellular stress response [7]. Recent research has focused on peripheral biomarkers as having a potential role in stroke [8–11].

MicroRNAs are the main regulators of homeostasis in neurons, and their dysregulation consequences in pathological settings in the brain [12]. Several recent miRNA studies provided compelling evidence indicating the functional relevance of miRNAs in neuroprotection and neurodegeneration [13–15]. MiRNAs play an important role in the normal function of cell regulation and dysregulated conditions are associated with different disease pathologies including diabetes, cancer, Alzheimer's disease, and other neurodegenerative disorders [16–19].

In our previous study, we used miRNA-Illumina deep sequencing method and identified differentially expressed miRNAs in serum samples from IS patients relative to healthy controls [8]. Further, we validated the trend of selected miRNAs using postmortem IS brains, lymphoblastoid IS cell lines, oxygen and glucose deprivation/reoxygenation (OGD/R)-treated human (SH-SY5Y) and mouse neuroblastoma (N2a) cells and hypoxia and ischemia (HI)-induced stroke mouse model.

Out of 16 differentially expressed miRNAs, 4 were previously unreported in the IS field. Interestingly, miRNA PC-5P-12969 was exclusively expressed in the IS condition; otherwise, it was not expressed in normal condition, and therefore, we focused on miRNA PC-5P-12969 for further studies. In the present study, we sought to evaluate miRNA PC-5P-12969 expression levels which are predominantly expressed (potential biomarker) in ischemic conditions. We focused on the novel miRNA PC-5P-12969 and determined its expression level in OGD/R-treated mouse hippocampal neuronal cells (HT22) and in affected and unaffected regions of a brain in a mouse model of photothrombotic stroke at 4 time points: 4 hours and days 1, 3, and 7 post-stroke in the IS group.

Materials and Methods

Study Design and Mouse Model

Twenty, 10- to 12-week-old, male CD-1 mice (Charles River Laboratories, Wilmington, MA, USA) were maintained over a period of 14 days in a 12-h light/dark cycle. During this time, they were fed ad libitum. This study was approved by the IACUC at Texas Tech University Health Sciences Center. Two investigators individually handled the mice for about 2 min at a time, once or twice daily for 4–5 days. After handling the mice for this time period, the investigators assessed the baseline performance of mice in grid-walking and cylinder tests [9]. Following the baseline evaluation, the mice were randomly divided into a sham and four different stroke groups (4 h, 1, 3, and 7 days post-stroke; $n = 4$ per group). The mice in IS group underwent photothrombosis on the third day as described by Alamri et al. [9]. In brief, under isoflurane anesthesia at 36.9 ± 0.5 °C, the skull was exposed and cleared of connective tissue. The brains of the mice in the stroke group underwent illumination at 1.5 mm lateral from bregma 0 (right hemisphere, 2-mm diameter, irradiation for 15 min). Illumination was through a fiber optic halogen lamp, 5 min after intraperitoneal administration of Rose Bengal solution (8 mg/ml, 10 ml/kg volume). Sham animals underwent the same procedure except for the light illumination. The mice were housed individually after stroke. During the course of the study, no mouse died, and no mouse was eliminated from the study for any reason.

Behavioral Assessment

To determine motor functional impairment, mice in both groups were tested on the grid-walking and cylinder tasks as described by Alamri et al. [9]. Evaluations were carried out by personnel who were blind to the experimental groups.

Briefly, in the grid-walking test, mice walked on an elevated wire grid (12-mm square wire mesh, 33 cm × 20 cm total area) for 5 min while being video-recorded. Footfaults for each forelimb and the total normal steps were counted to calculate the footfault index as [(no. affected forelimb footfaults − no. unaffected forelimb footfaults) / (no. affected forelimb footfaults + no. unaffected forelimb footfaults + no. normal steps)].

For the cylinder test, mice were placed in a clear acrylic cylinder (17 cm height and 10 cm diameter) for 5 min to video-record and determine forelimb symmetry during exploratory rearing. During each rear, the use of the affected, unaffected, or both forelimbs was counted, followed by

calculation of forelimb use symmetry index as [(no. affected forelimb use – no. unaffected forelimb use) / (no. affected forelimb use + no. unaffected forelimb use + no. use of both forelimbs)].

Tissue Collection

Following the behavioral assessment, the mice were deeply anesthetized by isoflurane and decapitated. The whole brain was quickly removed and was placed on a chilled glass on ice.

Cerebral infarction (on all four-time points after stroke) and edema (on days 1 and 3 post-stroke) were verified visually. The frontoparietal cortex (~bregma 1.5 to − 1.5) and the cerebellum were dissected from the infarcted and contralateral hemispheres and frozen at − 80°C for future processing and analysis [10]. The brains of sham mice were handled in the same way, except that the brain parts from both hemispheres were collected and considered as control.

Cresyl Violet Staining

A separate cohort of mice was deeply anesthetized with isoflurane on days 1, 3, and 7 post-stroke and cardially perfused with 4% paraformaldehyde, followed by additional overnight paraformaldehyde perfusion, sucrose cryopreservation, and cryosectioning in the coronal plane (40 μm thickness). Cresyl violet staining and histologic assessment of infarct size followed methods described in Alamri et al. [9].

RNA Extraction from Brain Tissues and cDNA Synthesis

Total RNA was isolated from the 80 mg of tissues, using the TRIzol RT reagent (Ambion, City, State, USA) per the manufacturer's instructions. The value of absorbance of each RNA sample (A_{260}/A_{280}) was 1.8 to 2.0. RNAs were extracted and cDNA was synthesized, following Vijayan et al. [8]. Briefly, 1 μg of total RNA polyadenylated with a miRNA First-Strand cDNA synthesis kit (Agilent Technologies Inc., CA, USA), following manufacturer's instructions. PolyA reaction was prepared by mixing RNA with 4 μl of 5X poly A polymerase buffer, 1 μl of rATP (10 mM), and 1 μl of *Escherichia coli* poly A polymerase, producing a final volume of 20 μl with RNase-free water. The tube with these components was incubated at 37 °C for 30 min, followed another incubation at 95 °C for 5 min to terminate the adenylation reaction [11]. Then, 10 μl of polyadenylated miRNAs was processed for cDNA synthesis with the miRNA First-Strand cDNA synthesis kit (Agilent Technologies Inc.). The following reaction components were combined in a tube: 2 μl of 10X AffinityScript RT buffer, 0.8 μl of dNTP mix (100 mM), 1 μl of RT adaptor primer (10 μM), 1.0 μl of AffinityScript RT/RNase Block enzyme, and polyadenylated RNA. The combination resulted in a reaction volume of 20 μl RNase-free water. This reaction mixture was incubated at 55 °C for 5 min, then at 25 °C for 15 min, followed by an incubation at 42 °C for 30 min, and a final incubation at 95 °C for 5 min in a Veriti 96-well Thermal Cycler (Applied Biosystems, USA). Resulting cDNAs were diluted with 20 μl of RNase-free water and stored at − 80 °C for further analysis.

Validation of PC-5P-12969 Using Quantitative Real-Time RT-PCR

PC-5P-12969 was the focus of this investigation because it has been found to be a potential biomarker in our recent microRNA study [8]. Oligonucleotide primers for PC-5P-12969 (forward: 5′-GCAGGAGCCGGGACTGGCTTC-3′) and SnoR-202 (5′-AGTACTTTTGAACCCTTTTCCA-3′) were synthesized commercially (Integrated DNA Technologies, Inc., Iowa, USA). SnoR-202 was used as the endogenous, internal control for real-time RT-PCR. The qRT-PCR reaction was performed by preparing a reaction mixture containing 1 μl of PC-5P-12969-specific forward primer (10 μM), 1 μl of a universal reverse primer (3.125 μM) (Agilent Technologies Inc., CA, USA), 10 μl of 2X SYBRVR Green PCR master mix (Applied Biosystems, NY, USA), and 1 μl of cDNA. To this mixture, RNase-free water was added up to a 20-μl final volume. The reaction mixture of each sample was prepared in triplicates. The reaction was set in the 7900HT Fast Real-Time PCR System (Applied Biosystems, USA) using following reaction conditions: initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 10 s, annealing at 60 °C for 15 s, and extension at 72 °C for 25 s. The detailed protocol and fold change of each miRNAs were calculated as described previously by Vijayan et al. [8].

Cell Cultures

To verify the differentially expressed novel miRNA PC-5P-12969 in vitro, we used HT22 cells (immortalized mouse hippocampal neuronal cells), a kind gift from Dr. David Schubert.

They were fed DMEM (no. 11995, Gibco) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were cultured at 50–55% relative humidity, in 10% CO_2 at 37°C.

Oxygen and Glucose Deprivation/Reoxygenation Protocol

HT22 cells (OGD/R) deprived and reoxygenated cells were cultured following Vijayan et al. [8]. Briefly, the complete culture medium was replaced with deoxygenated DMEM without glucose. To represent OGD/R conditions, the HT22 cells were then incubated in a hypoxia chamber (Biospherix, ProOx model 110, Parish, New York, USA) with 95% N_2 and

5% CO_2 for 4 h at 37 °C. After OGD, the HT22 cells were washed in PBS. The medium was replaced with the complete culture medium and then placed in a humidified incubator at 37 °C for 20–24 h to represent reoxygenation.

Statistical Analysis

For the behavioral analysis, data were presented as mean ± SD and analyzed using two-way repeated measures ANOVA, followed by Dunnett's or Sidak's post hoc test, as appropriate, for multiple comparisons. The threshold cycle (CT) values and the fold change of each miRNA were calculated, as described in Vijayan et al. [8]. *P* value was calculated, based on the paired *t* tests for analyzing two groups (the stroke and sham groups). For both groups of mice, the miRNA levels between day 1 and day 3 post-stroke and between day 3 and day 7 post-stroke were analyzed using a one-way repeated measures ANOVA. A comparative analysis was carried out using the Holm-Sidak's multiple comparison tests. Sensitivity and specificity of measured variables for biomarkers were examined using a receiver operating characteristic (ROC) curve analysis under a nonparametric approach. A *P* value of < 0.05 was considered to be statistically significant. All analyses were performed by GraphPad Prism (version 7.02; GraphPad Software, La Jolla, CA, USA).

Results

Oxygen and Glucose Deprivation/Reoxygenation-Treated HT22 Cells

To investigate the potential role of novel miRNA-PC-5P-12969 in OGD/R-induced stroke condition, we first evaluated its expression pattern in HT22 cells to build an OGD/R model in vitro to mimic ischemia and reperfusion. Results of qRT-PCR showed that miRNA-PC-5P-12969 expression level was significantly upregulated ~ 2-fold, when the re-oxygenation continued for 24 h following 4 h of OGD (Fig. 1), indicating that miRNA PC-5P-12969 might be involved in regulating OGD/R-induced stroke condition.

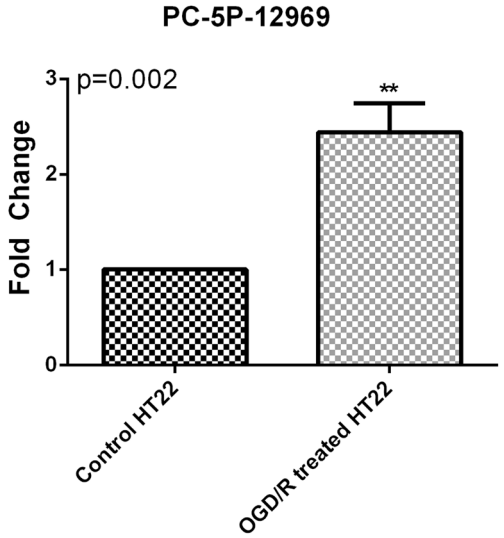

Fig. 1 MiRNA PC-5P-12969 expression in the OGD/R treated mouse hippocampal neuronal cell line (HT22). Using qRT-PCR, we validated miRNA PC-5P-12969 in OGD/R treated HT22 cells. Data are presented as the mean±SD of 3 independent experiments

Photothrombotic Mouse Model for Ischemic Stroke

Infarct Volume

Representative images of cresyl violet-stained brain sections are presented in Fig. 2.

As shown in Fig. 2, lesions were not found in the brains of the mice from the sham group, whereas the estimated stroke infarct volume for 1, 3, and 7-day post-stroke brains were 2.66, 2.92, and 1.1 mm^3, respectively.

Behavioral Assessment

Focal cerebral stroke caused a significant deficit in the affected forelimb function of the IS mice, determined by the grid-walking and cylinder tests (Fig. 3) (group × day interaction, *F*

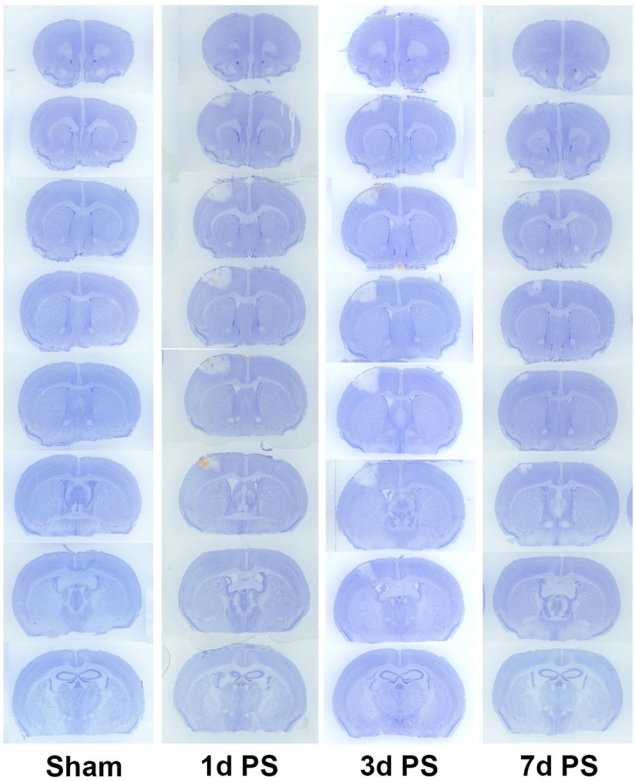

Fig. 2 Representative cresyl violet-stained mouse brain sections (d PS, day post stroke). The estimated stroke volume is 0, 2.66, 2.92, and 1.1mm3 in sham, days 1, 3, and 7 post stroke brains, respectively

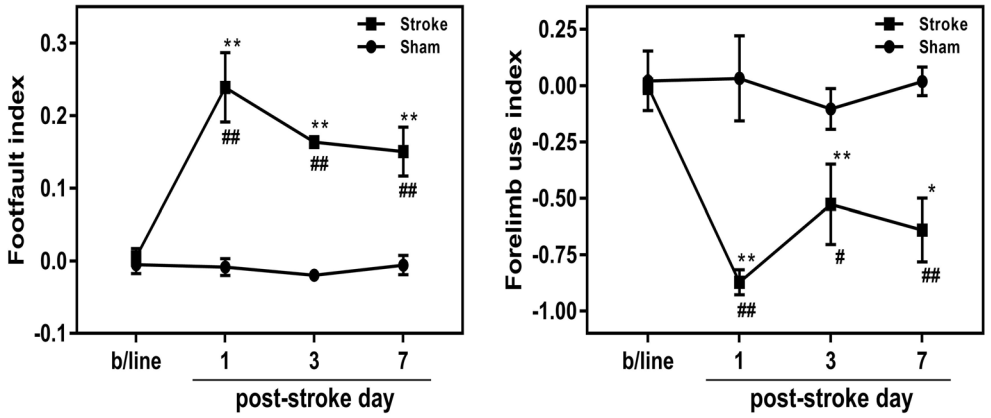

Fig. 3 Behavioral analysis of photothrombotic mouse model ischemic stroke. Following photothrombosis, mice exhibited functional deficits of the affected forelimb in grid walking (left panel, increased number of foot faults) and cylinder (right panel, decreased use the affected forelimb upon rears) tests. The observed deficit was absent in the sham mice ($n = 4$ for each group; $^{*}p = 0.05$, $^{**}p \leq 0.01$ compared to the baseline of the stroke group; $^{\#}p < 0.05$, $^{\#\#}p \leq 0.01$ compared to the baseline of the sham group)

(2, 4) = 36.81, $p = 0.0027$ and F (2, 4) = 9.647, $p = 0.0295$, respectively). Post hoc analyses with Dunnett's correction revealed significant differences in forelimb function between baseline and all three post-stroke evaluation days in the mice from the IS group ($p < 0.05$) but not in the mice from the sham group ($p > 0.05$). Within-day comparisons of sham and stroke groups (post hoc analyses with Sidak's correction) showed statistically significant differences in the forelimb function of the IS and the sham groups on days 1, 3, and 7 post-stroke ($p < 0.05$) but not at baseline ($p > 0.05$).

Novel miRNA PC-5P-12969 Expression in Cerebral Cortex

At 4 hours and days 1, 3, and 7 post-stroke, we measured PC-5P-12969 miRNA expression in the stroke-induced brain region (cerebral cortex) and in the brain tissues from the sham group. There was a significant upregulation of PC-5P-12969 at 4 h and 1-day post-stroke in the IS group compared to the sham group ($p = 0.0001$) (Fig. 4a). However, we did not find a significant difference between the IS group and the sham group for miRNA levels of PC-5P-12969 at day 3 and day 7 post-stroke. Interestingly, when we analyzed the data for 4 h and 1-day post-stroke group vs days 3 and 7 post-stroke groups, we found a decreased level of PC-5P-12969 in the IS group in days 3 and 7. When we analyzed the data between 4 h and 1-day post-stroke group, we did not find a significant difference.

We also measured the expression level in the cerebral cortex (contralateral) which is considered as a healthy side. Interestingly, there was no change observed in any of the post-stroke group compared to the sham control group (Fig. 4b). We compared the PC-5P-12969 expression levels in the ipsilateral vs the contralateral areas of the cerebral cortex at 4 h and days 1, 3, and 7 post-stroke in the IS group. The

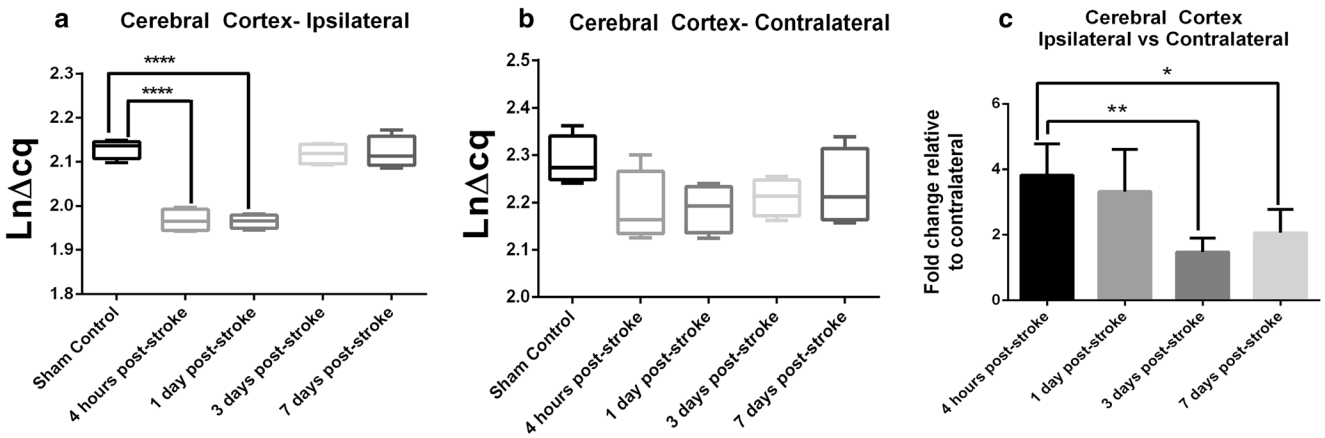

Fig. 4 Quantitative RT-PCR analysis of novel miRNA PC-5P-12969. **a** Ipsilateral region of cerebral cortex of photothrombotic stroke model, **b** Contralateral region of cerebral cortex and **c** Ipsilateral vs contralateral region of cerebral cortex. Fold change was calculated by 2- ΔΔCT method. Significant difference among groups were calculated by one way anova with Holm-Sidak's multiple comparison test, $P < 0.05$ is considered significant

levels of PC-5P-12969 were significantly upregulated in all post-stroke mice relative to control sham mice (Fig. 4c). These results indicated that the expression levels of miRNA-PC-5P-12969 are tissue-specific and time-dependent.

Cerebellum

The cerebellum is not affected by stroke in this model and cerebellar tissue was used as a negative control in this study. We quantified miRNA PC-5P-12969 expression levels in the cerebellum, which is unaffected in the IS mice. There was no change in the miRNA PC-5P-12969 expression level on 4 hours and days 1, 3, and 7 post-stroke in the stroke group compared to the sham group (Fig. 5a). We also compared the miRNA PC-5P-12969 expression levels in the ipsilateral areas of the cerebral cortex and the cerebellum. Interestingly, the miRNA PC-5P-12969 expression levels were upregulated in the ipsilateral area of the cerebral cortex compared to the ipsilateral area of the cerebellum in all post-stroke groups (Fig. 5b). These observations strongly suggested that stroke is confined to the cerebral cortex that is affected in the photothrombotic stroke mouse model.

Receiver Operating Characteristic Curve Analysis

The miRNA PC-5P-12969 expression level was consistently upregulated in the stroke mice and in the HT22 in vitro cell line model. Therefore, we evaluated the diagnostic value of the miRNA PC-5P-12969 by plotting a ROC curve in the IS mice and in HT22 cells. The curves were plotted, based on the ΔCt value of candidate miRNA expression in the IS mice and HT22 cells. Upon analysis, PC-5P-12969 (AUROC = 1.0; $P = 0.02$) showed the significant area under the ROC at 4 h and 1-day post-stroke compared to the area at this same time point in the sham mice (Fig. 6a). This same trend was observed in the HT22 cells (Fig. 6b). Thus, ROC analysis confirmed that the profile of the miRNA PC-5P-12969 expression level possibly could be used as a specific, noninvasive molecular biomarker for IS.

Discussion

The objective of our current study was to identify early detectable peripheral biomarkers for IS. The present study demonstrated the upregulation of miRNA PC-5P-12969 expression levels in the OGD/R-treated HT22 cells and in brain tissues from photothrombotic stroke mice. Based on our previous global miRNA study in serum samples from IS patients and control subjects, we investigated the expression levels of few differentially expressed miRNAs in IS serum samples, postmortem human IS brains, lymphoblastoid IS cell lines, SH-SY5Y cells, mouse neuroblastoma (N2a) cells, and hypoxia and ischemia mouse model [8]. From these miRNAs, we selected a novel and previously unreported miRNA PC-5P-12969 for this study. Our extensive analysis revealed that miRNA PC-5P-12969 is exclusively expressed in the IS condition; otherwise, it was not expressed in the normal condition. The selected possible molecular targets for the novel miRNA PC-5P-12969 and gene ontology enrichment for target genes were reported in Table 1.

MiRNAs have been observed to be involved in the post-transcriptional regulation and they have been identified as potential circulating biomarkers in several diseases, such as cancer, Alzheimer's, and ischemic stroke [20–25]. Increasing evidence indicates that various miRNAs might play critical roles in ischemic stroke pathogenesis [26–30]. Several studies showed that specific miRNA expression and function could regulate post-ischemic neural death by altering the expression

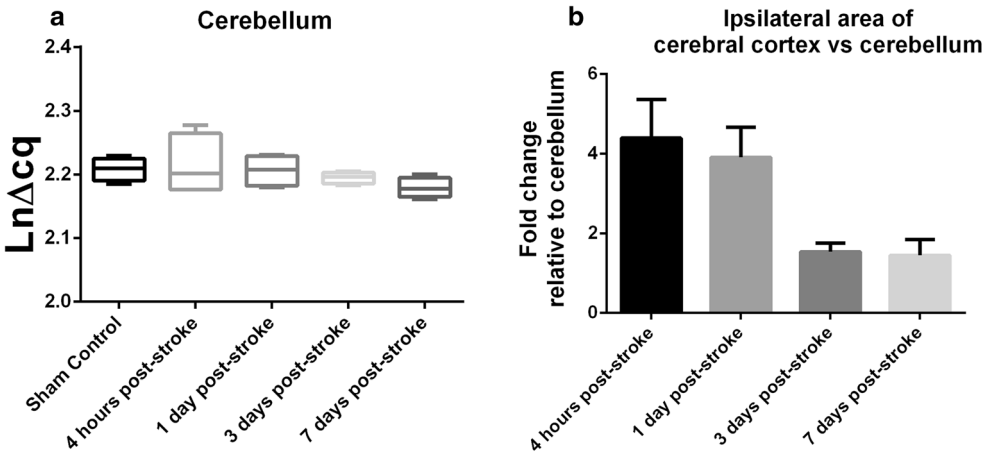

Fig. 5 Novel miRNA PC-5P-12969 expression. **a** Cerebellum of photothrombotic stroke model and **b** Ipsilateral region of cerebral cortex vs cerebellum. Fold change was calculated by 2-ΔΔCT method. Significant difference among groups were calculated by one way anova with Holm-Sidak's multiple comparison test, $P < 0.05$ is considered significant

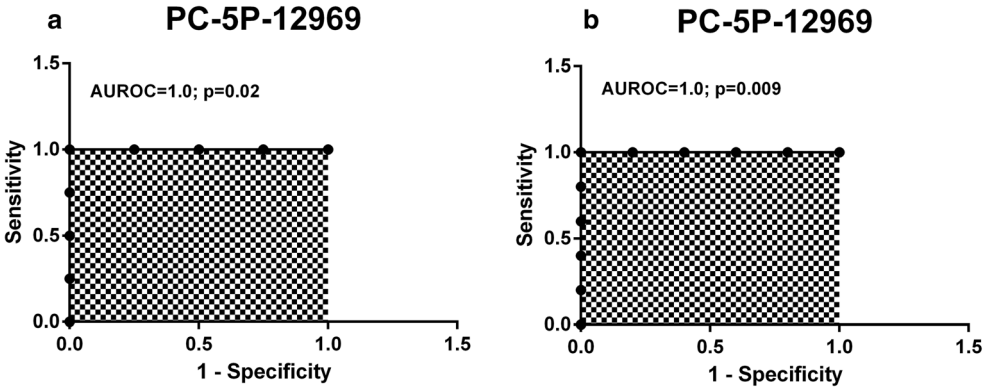

Fig. 6 Receiver operating characteristics curve analysis of novel miRNA PC-5P-12969. **a** Photothrombotic stroke mouse model (cerebral cortex) and **b** OGD/R-treated HT22 cell lines

of the target genes [31]. A broad analysis of miRNA expression amid IS patients and healthy controls would help us to better understand the roles of miRNAs in the progress of the disease as well as to find novel biomarkers for IS diagnosis and prognosis for personalized therapy.

Currently, the clinical diagnosis of stroke depends on a patient's medical history, clinical examination, and results from various neuro/radiological imaging techniques. However, this clinical diagnosis method has limited availability and is expensive. There are no reliable circulating biomarkers for the diagnosis of IS [30, 32]. Therefore, simple and convenient biomarkers are urgently needed to help diagnose IS at an early stage. Findings from our current study together with our previous study [8] strongly indicate miRNA PC-5P-12969 is a promising early detectable peripheral biomarker for IS.

The understanding of the molecular mechanisms underlying ischemia/reperfusion-induced neuronal death and neurological dysfunction might provide therapeutic targets for ischemic stroke [33]. Hypoxia induces a time-dependent alteration of miRNAs expression levels, suggesting their involvement in the cellular response to ischemic injury [34]. In the present study, we set out to reveal the expression of novel miRNA following ischemia in vitro. To this end, we performed OGD/R on HT22 cells to mimic ischemia in vitro. The expression level of PC-5P-12969 was upregulated in the OGD/R-treated HT22 cells. In recent times, there were several other novel roles of miRNAs reported in brain diseases. The miRNA-346 upregulates amyloid-β precursor protein (APP) via targeting of APP mRNA 5′-untranslated region.

The expression level of a few miRNAs could be differently modulated in both in vivo and in vitro experimental models [36, 37]. The photothrombotic stroke

Table 1 The selected possible molecular targets involved in miRNA PC-5P-12969 and gene ontology enrichment for target genes

miRNA	Genes	Abbreviations	Biological process	Cellular component	Molecular function
PC-5P-12969	ADA	Adenosine deaminase 2	Purine-containing compound salvage	Cytosol	Zinc ion binding
	BMX	BMX non-receptor tyrosine kinase	–	Cytosol	–
	CCR2	C-C motif chemokine receptor 2	Positive regulation of T cell activation	Cytosol	–
	DISC1	Disrupted in schizophrenia 1	Protein localization to centrosome	–	Sequence-specific DNA binding
	EIF4B	Eukaryotic translation initiation factor 4B	–	Cytosol	–
	GSK3A	Glycogen synthase kinase 3 alpha	Regulation of mitophagy	Cytosol	–
	PARK2	Parkin RBR E3 ubiquitin protein ligase	Regulation of autophagy, positive regulation of tumor necrosis factor-mediated signaling pathway, proteasomal protein catabolic process, vesicle-mediated transport, cellular protein catabolic process	Cytosol	Zinc ion binding, transcription factor activity, sequence-specific DNA binding
	HTRA2	HtrA serine peptidase 2	Regulation of mitophagy, cellular protein catabolic process	Cytosol	–
	PDGFR Beta	Platelet-derived growth factor receptor beta	Phosphatidylinositol phosphorylation	–	–

model used in this study is based on intravascular photo-oxidation, which leads to well-defined ischemic lesions in the cortex and modifications in the striatum [38]. We measured the function of miRNA using a photothrombotic stroke induced in 10–12-week-old, male CD-1 mice. The cerebral cortex region of the stroke-induced mouse brain showed the most consistent differential expression of miRNA compared to the cerebellum.

From our previous miRNA sequencing study, we found this novel miRNA PC-5P-12969 which has a unique expression only in the ischemic condition. We did not observe the expression level in the normal condition in both in vitro and in vivo. We used the hypoxia ischemic stroke model and OGD/R-induced neuroblastoma (human and mouse) cell lines in our previous study [8]. In the present study, we used a photothrombotic stroke model and IS-induced HT22 cell line and investigated expression levels of PC-5P-12969 (Fig. 7) in order to strengthen the novelty of PC-5P-12969 for IS.

In summary, the findings from this study showed that PC-5P-12969 upregulation in early ischemic conditions (4 h and 1-day post-stroke) in an in vivo and in vitro suggesting that PC-5P-12969 is a potential biomarker for the ischemic stroke. Further studies from our lab will focus on

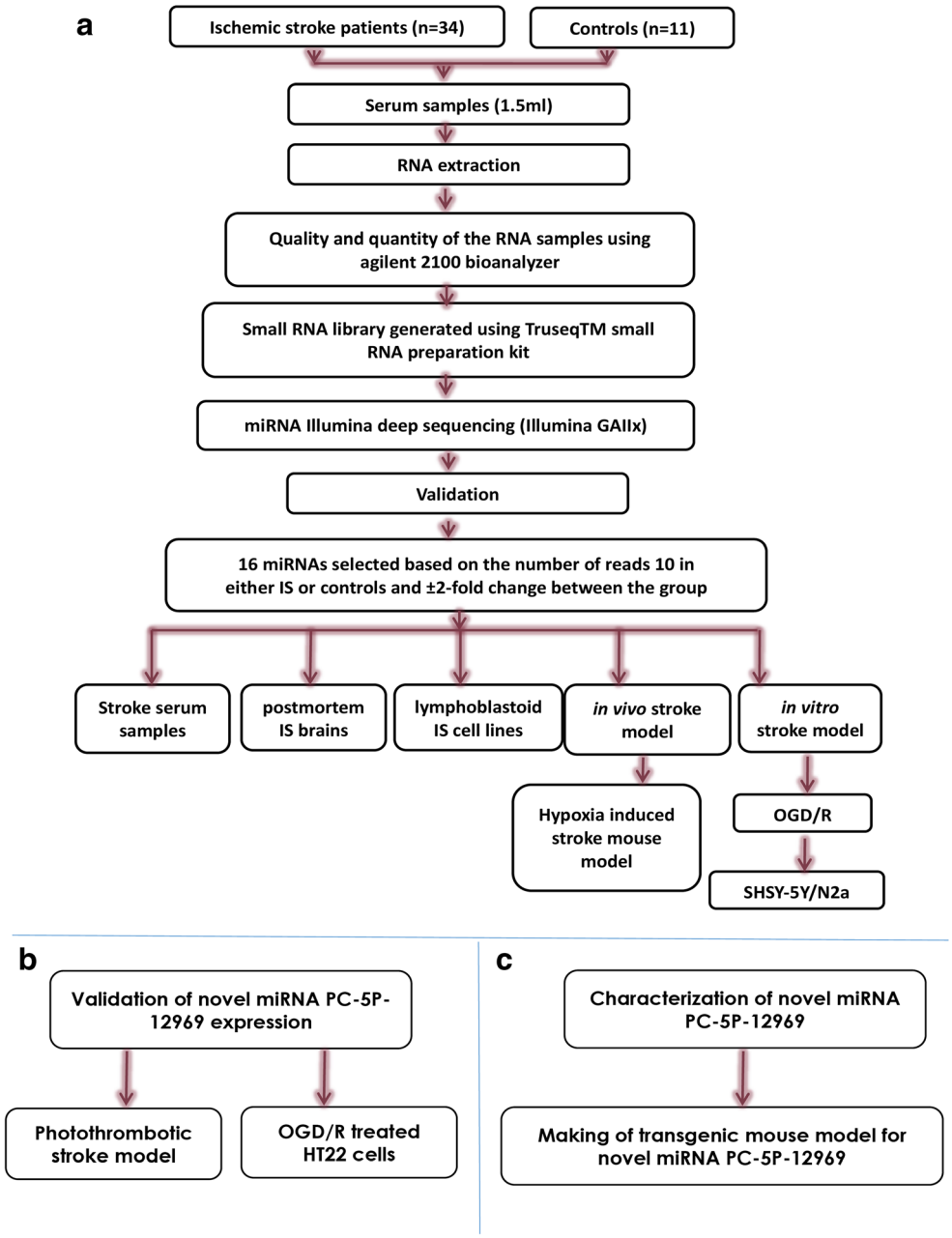

Fig. 7 Schematic representation of past, current, and future work flowchart of novel miRNA PC-5P-12969. **a** miRNA sequencing and validation of selected 16 miRNAs using IS serum samples, postmortem IS brains, lymphoblastoid IS cell lines, OGD/R-treated human and mouse neuroblastoma cells, and mouse models of hypoxia and ischemia (HI)-induced stroke. **b** Validation of novel miRNA PC-5P-12969 expression in photothrombotic stroke mouse model and OGD/R-treated HT22 cell lines. **c** Characterization and making of transgenic mouse model for novel miRNA PC-5P-12969

making and characterizing a transgenic mouse model for miRNA PC-5P-12969 and is expected to provide mechanistic insights for ischemic stroke. To our knowledge, this is the first study reporting that the miRNA PC-5P-12969 is a potential early detectable biomarker for IS.

Acknowledgements We thank Dr. David Schubert (Salk Institute for Biological Studies) for providing the HT22 cells for this study as a gift.

Funding The research presented in this article was supported by the National Institute of Health grants AG042178, AG47812, and NS105473 and grants from the Garrison Family Foundation and the CH Foundation (PHR).

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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