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Alteration of *microRNA 340-5p* and Arginase-1 Expression in Peripheral Blood Cells during Acute Ischemic Stroke

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Abstract

Acute stroke alters the systemic immune response as can be observed in peripheral blood; however, the molecular mechanism by which microRNA (miRNA) regulates target gene expression in response to acute stroke is unknown. We performed a miRNA microarray on the peripheral blood of 10 patients with acute ischemic stroke and 11 control subjects. Selected miRNAs were quantified using a TaqMan assay. After searching for putative targets from the selected miRNAs using bioinformatic analysis, functional studies including binding capacity and protein expression of the targets of the selected miRNAs were performed. The results reveal a total of 30 miRNAs that were differentially expressed (16 miRNAs were upregulated and 14 miRNAs were downregulated) during the acute phase of stroke. Using prediction analysis, we found that *miR-340-5p* was predicted to bind to the 3′-untranslated region of the arginase-1 (*ARG1*) gene; a luciferase reporter assay confirmed the binding of miR-340-5p to *ARG1*. *miR-340-5p* was downregulated whereas *ARG1* mRNA was upregulated in peripheral blood in patients experiencing acute stroke. Overexpression of miR-340-5p in human neutrophil and mouse macrophage cell lines induced downregulation of the ARG1 protein. Transfection with *miR-340-5p* increased nitric oxide production after LPS treatment in a mouse macrophage cell line. Our results suggest that several miRNAs are dynamically altered in the peripheral blood during the acute phase of ischemic stroke, including miR-340-5p. Acute stroke induces the downregulation of *miR-340-5p*, which subsequently upregulates ARG1 protein expression.

Keywords Ischemic stroke · MicroRNA · Microarray · miR-340-5p · Arginase-1

Introduction

During cerebral ischemia, blood cells are known to participate in the pathophysiology of stroke [1]. Local injury in the central nervous system (CNS) induces activation of blood-borne inflammatory cells, which produce and release circulating pro-inflammatory cytokines and chemokines. Activated white blood cells infiltrate the ischemic brain and aggravate inflammation, eventually leading to neuronal death [2–4]. However, despite extensive research, the molecular mechanism by stroke alters the acute immune response in peripheral blood and contributes to brain damage is still unknown. Recent development of the microarray technique has allowed investigators to obtain a "molecular signature" of various diseases, including stroke. Several microarray studies have reported distinct genomic alterations in peripheral blood during stroke. Genomic profiling of peripheral blood helps to identify the diagnosis of stroke [5] and stroke subtype [6] and has enabled the discovery of novel molecular mechanisms of the pathophysiology of stroke [7].

MicroRNAs (miRNAs), small non-coding RNA molecules 18–25 nucleotides in length, are recognized as major endogenous regulators of gene expression. The miRNA molecule binds to the 3′-untranslated region (3′UTR) of target mRNAs and inhibits the expression of target genes by binding to and inducing the decay of mRNA molecules of target genes

Hanna Yoo and Jinkwon Kim contributed equally to this work.

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[8–10]. An estimated 1500 human miRNAs have been identified, and as many as 30% of mammalian genes may be regulated by miRNAs [11]. Many miRNAs play crucial roles in the regulation of the cell cycle, hematopoiesis, and cellular metabolism [12]. Several in vitro [13–15] and in vivo (human) [16, 17] studies have shown that miRNAs are temporally regulated during the progression of and especially during the reperfusion phase of a cerebral ischemic event, not only in the brain but also in peripheral blood. In human stroke, differentially expressed miRNAs were predicted to be related to inflammation, endothelial/vascular function, erythropoiesis, angiogenesis, neural function, and hypoxia [16–18]. These findings suggest a similar distinct miRNA signature should be present in peripheral blood cells as well; thus, this molecular pattern provides the molecular mechanism of stroke. However, few studies have investigated the expression pattern of blood miRNAs during the acute phase of human stroke. Therefore, further studies are needed to identify miRNA expression and their regulation of target gene expression during an acute ischemic attack.

In the present study, we utilized a miRNA microarray to create a profile of miRNA expression in the peripheral blood of patients experiencing stroke. We then sought to identify target genes of selected miRNAs identified in the microarray, including *miR-340-5p* and its target gene, arginase-1 (*ARG1*).

Methods

Study Subjects

A total of 21 peripheral blood samples from patients with a first-ever acute ischemic stroke who were admitted to our stroke center within 24 h after stroke onset ($n = 10$) along with and age- and sex-matched controls ($n = 11$) were obtained and used for the miRNA microarray experiments. In the stroke group, ischemic stroke was diagnosed based on acute neurological deficits relevant to stroke and confirmed by the presence of acute cerebral infarction on brain diffusion-weighted images as revealed by magnetic resonance imaging. Past history of vascular risk factors (e.g., hypertension, diabetes, hyperlipidemia, and smoking) were examined in all subjects by the same neurologist. The frequency of vascular risk factors was similar between stroke patients and controls (Table 1).

Table 1 Clinical characteristics of patients with ischemic stroke and controls for the miRNA microarray experiments

	Stroke ($n = 10$)	Control ($n = 11$)	*P*
Age (years)	73 (61–72)	73 (60–77)	0.34
Male (%)	4 (40.0)	5 (45.5)	0.84
Hypertension (%)	7 (70.0)	5 (45.5)	0.49
Diabetes mellitus (%)	1 (10.0)	0 (0.0)	0.96
Hyperlipidemia (%)	0 (0.0)	1 (9.1)	0.96
Smoking (%)	4 (40.0)	1 (9.0)	0.25
Etiology of stroke			
LAA (%)	5 (50.0)	–	–
CE (%)	5 (50.0)	–	–
Sampling time (h)	13.5 (10.0–18.0)	–	–

The continuous variables are presented as the median value and interquartile range (parenthesis). *P* value was calculated using a Mann-Whitney *U* test for continuous variables and using a chi-square test for categorical data

LAA large-artery atherosclerosis, *CE* cardioembolism

RNA Isolation

A total of 5 ml of whole blood was drawn via the antecubital vein within 24 h after symptom onset (median sampling time 13.5 h, interquartile range (IQR) 10.0–18.0 h after symptom onset in stroke patients). Blood samples were stored in PaxGene blood RNA tubes (Qiagen, CA, USA). Total RNA was extracted using TRIzol (Invitrogen Life Technologies, CA) and purified using RNeasy columns (Qiagen, CA). After processing with DNase digestion and associated cleanup procedures, the RNA samples were quantified, aliquoted, and stored at − 80 °C until use. RNA purity and integrity were evaluated by denaturing gel electrophoresis; the ratio of optical density at 260 and 280 nm ($OD_{260/280}$) was measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, CA). The $OD_{260/280}$ ratio of all of the samples was within a range of 1.8 to 2.2. Negative control, background, and noise signals were low (< 200) across all of the arrays, whereas the housekeeping (> 15,000) and biotin (> 30,000) signals were high.

MicroRNA Microarray

The miRNA microarray was performed using the Illumina Human MicroRNA Expression Profiling Assay V2 (Illumina, CA), which targets 1146 human miRNAs. Biotinylated cDNA was prepared from 0.2 to 1 μg total RNA using high-throughput gene expression profiling (cDNA-mediated annealing, selection, extension, and ligation assay: DASL) according to the manufacturer's protocol (Illumina, CA). For hybridization, fluorescently labeled cDNA polymerase chain reaction (PCR) products were hybridized to an Illumina Sentrix Beadchip U1536-16 according to the protocols provided by the manufacturer. The arrays were scanned on a BeadArray Reader and automatic image registration, and intensity extraction software was used to derive intensity data per the bead type that corresponded to each miRNA.

Data Processing of Microarray Test

The quality of hybridization and overall chip performance were monitored by visually inspecting both internal quality

control checks and raw scanned data. Raw data were extracted using the software provided by the manufacturer (Illumina BeadStudio v3.1.3; Gene Expression Module v3.3.8). Selected gene signal values were logarithmically transformed and normalized using the quantile method. Student's *t* tests were performed and fold differences (FD) were calculated. Statistical significance was adjusted using a false discovery rate (FDR) correction for multiple comparison hypotheses. Hierarchical cluster analysis was performed using complete linkage and Euclidean distance as a measure of similarity. All of the data analyses and visualization of differentially expressed genes were conducted using ArrayAssist (Stratagene, CA) and R statistical language v. 2.4.1.

TaqMan miRNA Assays

The expression of selected miRNAs obtained from the microarray data was quantified using TaqMan miRNA assays (Applied Biosystems, CA) for the validation of the microarray data. The primers used in this study were the following: *miR-186-5p* (5′-CAAAGAAUUCUCCUUUUGGGCU-3′), *miR-19a-3p* (5′-UGUGCAAAUCUAUGCAAAACUGA-3′), *miR-32-5p* (5′-UAUUGCACAUUACUAAGUUGCA-3′), *miR-340-5p* (5′-UUAUAAAGCAAUGAGACUGAUU-3′), *miR-579-3p* (5′-UUCAUUUGGUAUAAACCGCGAUU-3′), *let-7e* (5′-UGAGGUAGGAGGUUGUAUAGUU-3′), *miR-362-3p* (5′-AACACACCUAUUCAAGGAUUCA-3′), and *miR-1238-5p* (5′-GUGAGUGGGAGCCC CAGUGUGUG-3′). For miRNA expression analysis, 10 ng of total RNA was used along with the miRNA-specific primers supplied with the TaqMan miRNA assays. Customized reverse transcription primers were synthesized complementary to the sequences of the mature miRNAs. Complementary DNA templates were standardized to RNU6 and subjected to 40 PCR cycles according to the manufacturer's instructions. Data were generated using CFX Manager software (Bio-Rad, CA). The experiment was performed three times for each miRNA, and the mean value was chosen for the statistical analysis. The FD is represented as the 2^{-ddCt} value, which was calculated using the comparative threshold (C_T) cycle method.

Prediction of Target Genes of Selected miRNAs

For the identification of putative targets for 31 miRNAs (FD ≥ 2 and corrected $p < 0.05$) from the microarray data, we initially searched candidate targets whose binding region had at least a 7-mer-sequence-match using target prediction software such as miRBase (miRBase Release ver. 20., http://www.mirbase.org/), TargetScan (TargetScan Human ver. 6.2, http://www.targetscan.org/), or MicroCosm Target (MicroCosm Targets ver. 5., http://microrna.sanger.ac.uk/targets/v5). We searched for candidate genes that were plausibly associated with the pathophysiology of acute stroke in the literature [1, 4], specifically focusing on genes that were found to be differentially expressed in peripheral blood in previous microarray studies [5–7, 19]. After selection of miRNAs and their putative targets, we next performed functional studies to examine whether the selected miRNAs actually bound to the predicted targets.

Dual Luciferase Reporter Assay

XbaI-conjugated 3′UTR sequences of arginase-1 (*ARG1*) or interleukin-18 receptor 1 (*IL18R1*) messenger RNA (mRNA) were amplified using HiPi™ Plus Taq (ELPIS biotech, Korea) and cloned into the pmirGLO vector (Promega, WI). The miRNA mimics (mock, negative control [N.C.], miR-186-5p, miR-340-5p, and miR-19a-3p) were generated (Genolution, South Korea). Twenty-four hours before transfection, five million HeLa cells (ATCC, VA) were plated into 24-well culture dishes. Eighty nanograms of pmirGLO-3′ UTR vector for *ARG1* and *IL18R1* and 80 nM of corresponding miRNA mimics were transfected into cells using Lipofectamine 3000 (Invitrogen, CA). As *miR-340-5p* was predicted to have two potential binding sites to the 3′UTR of *ARG1*: (the 396th–402th nucleotide (nt) and 409th–416th nt of the 3′UTR of *ARG1* mRNA), we performed the luciferase activity of *ARG1* after transfection of the mutant constructs of *ARG1* 3′UTR. The pmirGLO-*ARG1* mutants were generated using a Muta-direct site-directed Mutagenesis kit (iNtRON Biotechnology, Korea) with mutation primers as follows: (1) ARG1_3′UTR_mut 1 covering the former region (forward: GTCATTCAAAAAATGTGATTTCCCGCGATAAA CTCTTTATAAC, reverse: GTTATAAAGAGTTTATCGCG GGAAATCACATTTTTTGAATGAC); (2) ARG1_3′ UTR_mut 2 covering the latter region (forward: TTTTTTAT AATAAACTGCCCGCGACAATCTAGAGTCGACCTGC, reverse: GCAGGTCGACTCTAGATTGTCGCGGG CAGTTTATTATAAAAAA); and (3) ARG1_3′UTR_mut 1&2 covering the both regions (forward: CCCGCGAT AAACTGCCCGCGACAATCTAGAGTCG, reverse: CGACTCTAGATTGTCGCGGGCAGTTTATCGCGGG). Twenty-four hours after transfection, firefly and Renilla luciferase activities were measured using a dual-luciferase assay system (Promega, WI); luminescence was measured on a VICTOR^3 analyzer (PerkinElmer, CA). Independent experiments were performed three times on different days.

Cell Culture

A human acute pro-myelocyte cell line (HL-60, Korea Cell Line Bank, Korea) was cultured at a density of 8×10^5 cells/ml in 24-well culture dishes. They were maintained in RPMI1640 medium containing 10% fetal bovine serum, 100 IU/ml penicillin, and 100 pg/ml streptomycin (Thermo

Fisher Scientific, Waltham). In order to differentiate into neutrophils, cell density was maintained at 1×10^6 cells/ml in the presence of 500 nM all-trans retinoic acid (ATRA, Sigma, MO) for 5 days. For determination of efficient neutrophil differentiation, real-time PCR of CD66b expression, a neutrophil-specific CD marker, was evaluated. In addition, an expression of neutrophil elastase (ELANE) protein was evaluated by immunocytochemistry using an anti-ELANE antibody (Abcam, Cambridge, UK).

Mouse macrophage cells (RAW 264.7, ATCC, VA) were cultured in DMEM high glucose medium (Welgene, Daegu, Korea) containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 pg/ml streptomycin (Thermo Fisher Scientific, Waltham, MA). The cells were seeded at a density of 1×10^6 cells/ml in 75-T culture flasks and incubated for 37 °C in a humidified atmosphere with 5% CO_2. After 2 days, the cells were harvested by cell scraping (BD Falcon, Becton, Dickinson).

Transfection of miR-340-5p Mimics

Chemically synthesized *miR-340-5p* and negative control miRNAs (NC) were purchased (Genolution, South Korea) and incubated in medium with 0.1% FBS. HL-60 cells or RAW264.7 cells were transfected with *miR-340-5p* mimics (100 μg), mock, or NC molecules using Lipofectamine 3000 reagent (Thermo Fisher Scientific, Waltham) following manufacturer's instructions. At 48 h after transfection, cell lysates were isolated for further experiments.

Real-Time PCR of ARG1 mRNA

Total RNA was reverse-transcribed to cDNA using SuperScript® II First-Strand Synthesis System (Invitrogen, CA). The expression of *ARG1* mRNA was quantified using a CFX™ real-time system (Bio-Rad, CA) and a Quantitect® SYBR Green PCR kit (Qiagen, CA). The RT primers for amplification of *ARG1* mRNA were as follows: TCTGTGGGAAAAGCAAGCGA (forward) and TTGCCAAACTGTGGTCTCCG (reverse). The data was normalized to *GAPDH* and calculated using the cycle threshold (2^{-ddCt}) method. Independent experiments were performed three times on different days.

Western Blot

Forty-eight hours after *miR-340-5p* mimic transfection, cells were lysed using a protein lysis buffer (PRO-PREP™, iNtRON Biotechnology, Korea) and subjected to immunoblot analysis according to the manufacturer's protocol. Whole proteins were separated on SDS-PAGE gels and immunoblotted using rabbit monoclonal antibodies to ARG1 (ab124917, Abcam, Cambridge, UK) and GAPDH as an internal control. Quantification of the bands was performed using the NIH ImageJ program. Independent experiments were performed three times on different days.

Nitric Oxide Assay

After transfection of mouse macrophage (raw 264.7 cells, 5×10^4/ml cells) with either the *miR-340-5p* mimic or an antisense *miR-340-5p* molecule (anti-miR340-5p) for 24 h, we conducted a nitric oxide (NO) assay. After transfection, cells were further treated using fresh medium containing lipopolysaccharide (LPS) 200 ng/ml and incubated for 0, 6, 24, or 48 h after LPS treatment, respectively; a total of 3 ml medium was then collected at each time point. The medium was used to measure the amount of NO present at each time point using a Griess assay kit (Promega, WI) according to the manufacturer's instructions. Briefly, 50 μl of the medium was transferred into a 96-well plate containing 50 μl Griess reagent I (sulfanilamide). The mixture was mixed and incubated for 10 min. After the first incubation, 50 μl of Griess reagent II (*N*-1-napthylethylenediamine dihydrochloride) was added to the mixture and the reaction was allowed to proceed at room temperature for 10 min. The absorbance of the mixture at 540 nm was read using a plate reader. A standard curve was constructed using twofold dilution of 0.1 M sodium nitrite.

Statistical Analysis

All data from quantitative real-time PCR and Western blots are expressed as the mean ± standard error of mean (SEM). The statistical significance between two groups was analyzed by Mann-Whitney *U* test. The statistical significance of multiple comparisons was analyzed using the Kruskal-Wallis test with a post hoc Conover's test for pairwise comparisons of subgroups. Statistical significance was considered at $p < 0.05$. Statistical analyses were conducted using MedCalc statistical software (MedCalc software, ver. 11.6., Mariakerke, Belgium). The statistical analyses used for miRNA microarray analyses are described above.

Results

Differentially Expressed miRNAs in Peripheral Blood After Acute Ischemic Stroke

Using an miRNA microarray, a total of 810 of 1146 miRNA molecules were found to be differentially expressed in peripheral blood after an acute ischemic stroke. Figure 1a shows the heat map of the miRNAs with an FD ≥ 2 and an uncorrected *p* value < 0.05 in their expression between the control and stroke groups (Fig. 1a). After FDR correction, a total of 30 miRNAs were significantly different in their expression in the

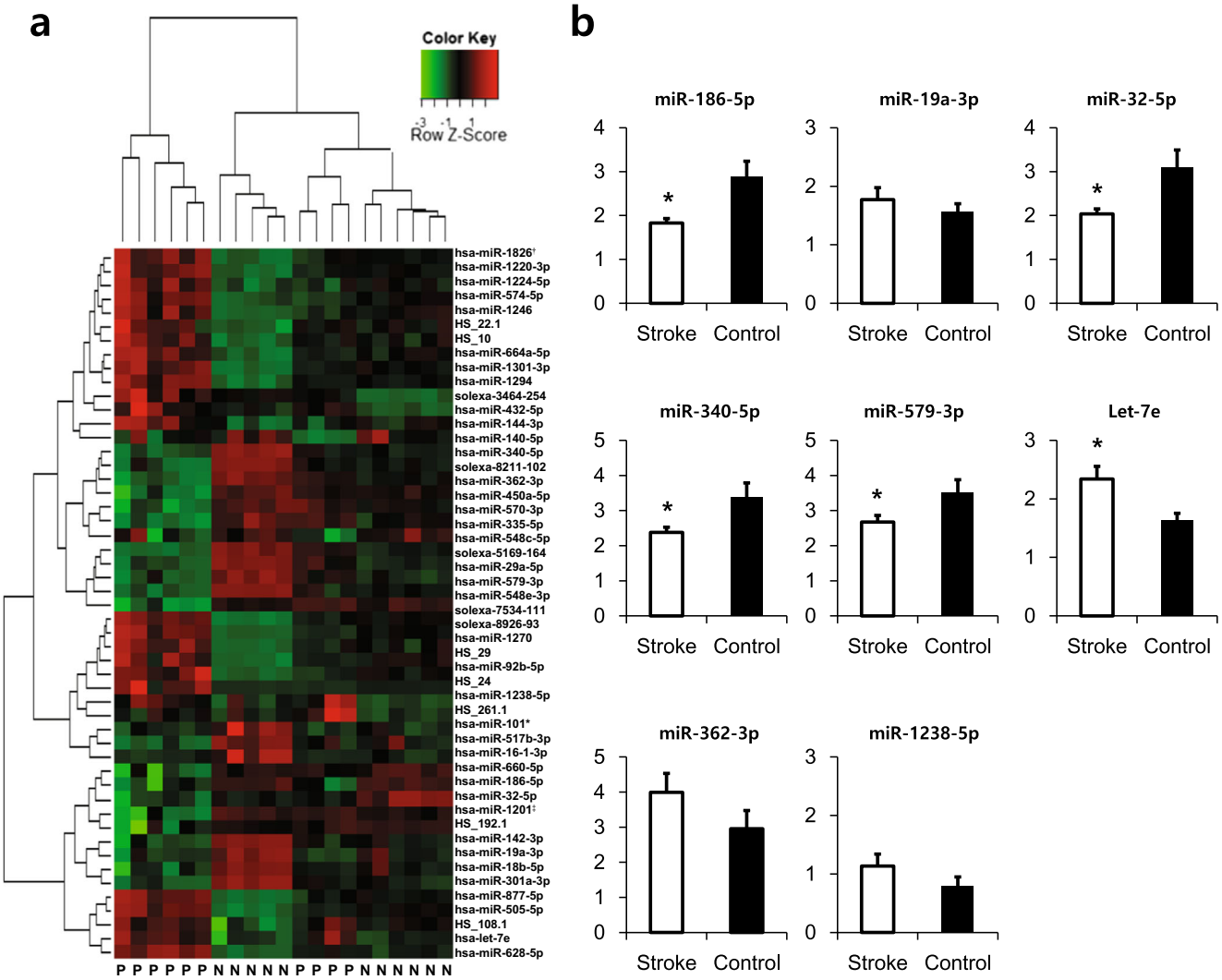

Fig. 1 miRNA microarray and TaqMan miRNA assay between stroke and control groups. **a** Heat map of miRNAs with fold difference ≥ 2 and raw $p < 0.05$ in their expression in stroke and control groups. On the heat map, red represents high expression and green represents low expression. P indicates stroke patient and N indicates normal control subject. hsa indicates *Homo sapiens*. **b** Expression of *miR-186-5p*, *miR-19a-3p*, *miR-32-5p*, *miR-340-5p*, *miR-579-3p*, *let-7e*, *miR-362-3p*, and *miR-1238-5p* in the TaqMan miRNA assay between control and stroke groups. Error bar indicates standard error of the mean (SEM). *$p < 0.05$ by Mann-Whitney *U* test. †Removed by miRBase database (http://www.mirbase.org/) because this annotated mature sequence is a fragment of 5.8S ribosomal RNA. *FD* fold difference. ‡Removed by miRBase database (http://www.mirbase.org/) because this microRNA overlaps an annotated snoRNA (SNORD126, Rfam 10.0, Infernal 1.0)

peripheral blood between patients with acute ischemic stroke as compared to controls (Table 2). Among these, 16 miRNAs (*miR-544a*, *solexa-3464-254*, *miR-1238-5p*, *HS_108.1*, *miR-505-5p*, *miR-877-5p*, *let-7e*, *miR-1229-3p*, *miR-1294*, *HS_10*, *HS_22.1*, *miR-628-5p*, *miR-1270*, *solexa-7534-111*, *miR-1301-3p*, and *miR-664a-5p*) exhibited high expression, whereas the remaining 14 miRNAs (*miR-579-3p*, *miR-140-5p*, *miR-186-5p*, *miR-301a-3p*, *miR-18b-5p*, *miR-19a-3p*, *miR-362-3p*, *miR-32-5p*, *miR-340-5p*, *miR-142-3p*, *miR-144-3p*, *miR-660-5p*, *miR-335-5p*, and *miR-517b-3p*) exhibited low expression in patients with stroke as compared to controls. To validate the expression pattern in the microarray data, we performed a TaqMan assay for eight miRNAs (*miR-186-5p*, *miR-19a-3p*, *miR-32-5p*, *miR-340-5p*, *miR-579-3p*, *let-7e*, *miR-362-3p*, and *miR-1238-5p*) (Fig. 1b). In the TaqMan assay, the expression of *miR-186-5p* (stroke group vs. control group 1.83 vs. 2.89, $p = 0.024$), *miR-32-5p* (stroke group vs. control group 2.04 vs. 3.10, $p = 0.024$), *miR-340-5p* (stroke group vs. control 2.38 vs. 3.39, $p = 0.049$), and *miR-579-3p* (stroke group vs. control 2.67 vs. 3.52, $p = 0.029$) were significantly lower in the stroke group than in the control group. Conversely, the expression of *let-7e* was higher in the stroke group than in the control group (stroke group vs. control group 2.34 vs. 1.63, $p = 0.012$). The expression of *miR-19a-3p*, *miR-362-3p*, and *miR-1238-5p* exhibited no difference in expression between patients with acute ischemic stroke and controls.

Table 2 List of significant miRNAs identified by microarray analysis in stroke patients and normal controls

miRNA	FD[a]	*p**
hsa-miR-579-3p	− 4.43	0.021
hsa-miR-140-5p	− 3.00	0.033
hsa-miR-186-5p	− 2.80	0.005
hsa-miR-301a-3p	− 2.57	0.042
hsa-miR-18b-5p	− 2.56	0.015
hsa-miR-19a-3p	− 2.49	0.012
hsa-miR-362-3p	− 2.43	0.026
hsa-miR-32-5p	− 2.43	0.014
hsa-miR-340-5p	− 2.27	0.036
hsa-miR-142-3p	− 2.24	0.026
hsa-miR-144-3p	− 2.24	0.047
hsa-miR-660-5p	− 2.10	0.033
hsa-miR-335-5p	− 2.08	0.026
hsa-miR-517b-3p	− 2.01	0.018
hsa-miR-664a-5p	2.04	0.045
hsa-miR-1301-3p	2.08	0.021
solexa-7534-111	2.09	0.025
hsa-miR-1270	2.11	0.026
hsa-miR-628-5p	2.11	0.009
HS_22.1	2.15	0.039
HS_10	2.20	0.017
hsa-miR-1294	2.25	0.017
hsa-miR-1229-3p	2.34	0.026
hsa-let-7e	2.44	0.004
hsa-miR-877-5p	2.51	0.021
hsa-miR-505-5p	2.54	0.034
HS_108.1	2.78	0.017
hsa-miR-1238-5p	3.08	0.016
solexa-3464-254	3.40	0.013
hsa-miR-544a	3.40	0.032

FD fold difference

[a] The control group is regarded as a reference

[b] Statistically significant after false discovery rate correction (FDR-$p < 0.05$)

Selection of miRNAs and Their Target Genes

After conducting the selection process for the identification of putative targets of significantly expressed miRNAs using target gene prediction programs (miRBase Release ver. 20., TargetScan, and MicroCosm Target), we selected four miRNAs and their putative target genes as follows: (1) *miR-340-5p* and *ARG1*, (2) *miR-186-5p* and *ARG1*, (3) *miR-19a-3p* and *IL18R1* (interleukin-18 receptor type 1), and (4) *miR-186-5p* and *IL18R1* (Supplementary Table 1). Prior studies in the literature have reported that *ARG1* and *IL18R1* are associated with pathophysiology of stroke [7, 19]. ARG1 is produced by macrophages and exerts anti-inflammatory actions in response to inflammation [20–22]. *ARG1* mRNA has been reported to be consistently upregulated in peripheral blood in mRNA microarray studies [7, 23]. IL18R1 is a receptor for interleukin-18 (IL-18), which is a member of the IL-1 superfamily. IL-18 is a pro-inflammatory cytokine that functions to induce inflammation and atherosclerosis [24, 25]. Serum IL-18 and expression of the *IL18R1* gene are both elevated in stroke patients [26, 27]. These findings suggest an important role for ARG1 and IL18R1 in the stroke-induced peripheral immune response.

In the bioinformatics analysis, genes were considered to be target genes if they were predicted to have at least a 7 nucleotide-seed match to the corresponding miRNA. As described above, there are two predicted binding sites for *miR-340-5p* in the 3′UTR of the *ARG1* mRNA molecule. The latter region (the most 3′ site) of *ARG1* was predicted to have a higher binding capacity for *miR-340-5p* than the former region. In addition, the latter *miR-340-5p* binding site in the *ARG1* is predicted to be evolutionally conserved across mammals (Supplementary Table 1).

ARG1 as a Target of miR-340-5p

To determine the real targets for selected miRNA molecules, we conducted a dual luciferase reporter assay for four putative pairs of miRNAs and their target genes (*ARG1* and *miR-340-5p, ARG1* and *miR-186-5p*, *IL18R1* and *miR-19-3p*, *ARG1* and *miR-186-5p*) (Fig. 2a). The luciferase activity of the 3′UTR of *ARG1* construct was significantly decreased following transfection with *miR-340-5p* mimic. By contrast, the luciferase activity of the *ARG1* and *IL18R1* constructs was not obviously altered after transfection with either the *miR-186-5p* or the *miR-19a-3p* mimic. As the 3′ UTR of *ARG1* mRNA was predicted to have two potential binding sites for *miR-340-5p*, we performed a luciferase activity for the *ARG1* construct after transfections with either a mutant construct of the *ARG1* 3′UTR at the former region (ARG1_3′UTR_mut 1), the latter region (ARG1_3′UTR_mut 2), and in both regions (ARG1_3′UTR_mut 1&2) (Fig. 2b). The luciferase reporter assay, after transfection of the ARG1_3′UTR mutant construct, indicated that luciferase activity was decreased only after transfection of an *ARG1* construct with a mutation in the former region (ARG1_3′ UTR_mut 1) (Fig. 2c). In contrast, there were no changes in luciferase activity after transfection with mutant construct of *ARG1* mutated in the latter putative binding site (ARG1_3′UTR_mut 2) or after transfection of a construct with a mutation in both putative binding sites (ARG1_3′UTR_mut 1&2). These results indicate that *miR-340-5p* binds to the latter region (409th–416th nt) of the 3′UTR of the *ARG1* gene.

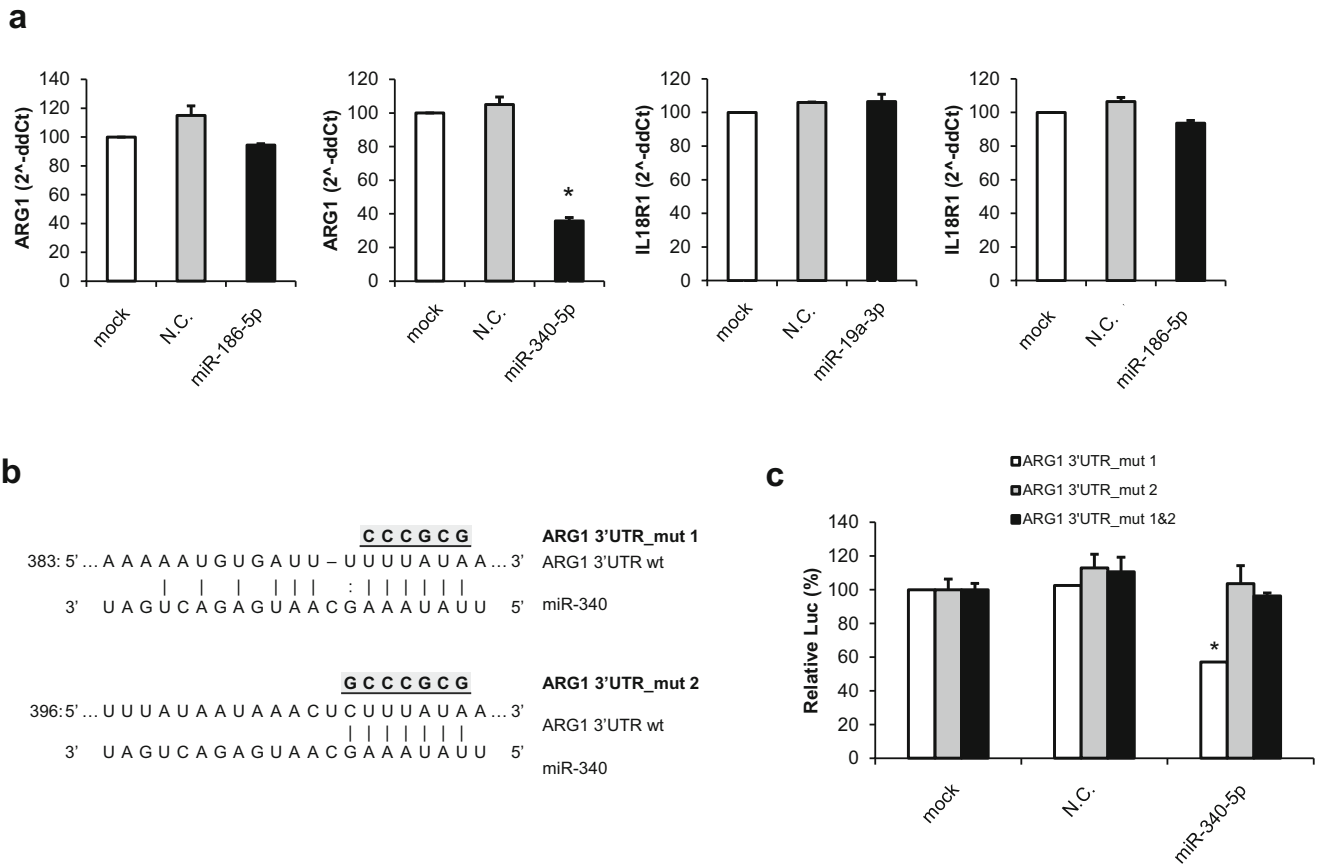

Fig. 2 Functional analysis of binding capacity of selected miRNA and their targets. **a** Dual-luciferase assay to confirm the predicted target genes. PmirGLO vectors containing the 3'UTR of *ARG1* or *IL18R1* were transfected with each miRNA mimics (mock, negative control (N.C.), *miR-186-5p*, *miR-340-5p*, and *miR-19a-3p*) to HeLa cells. Firefly luciferase activities were normalized to Renilla luciferase activities. Three independent experiments were performed. *$p < 0.05$ by Kruskal-Wallis test with a post hoc Conover's test. **b** The two predicted target sites in the *ARG1* 3'UTR with the *miR-340-5p* seed region are depicted. Mutations in the *ARG1* 3'UTR were generated with missense sequences that could not pair with *miR-340-5p* seed region. **c** Mutated *ARG1* 3'UTR vectors cotransfected with miRNA mimics. *ARG1* 3'UTR_mut 1&2 contains both 1 (former) and 2 (latter) mutation region. *$p < 0.05$ by Kruskal-Wallis test with a post hoc Conover's test. ARG1 arginase-1, IL18R1 interleukin-18 receptor 1

miR-340-5p Regulates Protein Expression of ARG1 in a Human Neutrophil Cell Line

We next examined the expression pattern of *miR-340-5p* and the *ARG1* gene in the blood of stroke patients. As described above, the expression of *miR-340-5p* was significantly downregulated in the peripheral blood of stroke patients. On the other hand, the expression of the *ARG1* gene was significantly upregulated in the peripheral blood of stroke patients (stroke group vs. non-stroke group 1.79 vs. 0.67, $p = 0.024$) (Fig. 3a), which is consistent with findings in previous studies [7, 23]. Based on the previous findings showing that blood *ARG1* expression in peripheral blood is highest in neutrophils as compared to other peripheral blood leukocytes in human [28], we hypothesized that *miR-340-5p* regulates ARG1 protein expression in neutrophils. Although we first tried to perform *miR-340-5p* transfection in human PMN cells from healthy blood donors, we found that the PMN cells are too short-lived to perform the transfection study (less than 12 h after blood collection using trypan blue staining). Therefore, we used HL-60 cell line-differentiated neutrophils using retinoic acid for *miR-340-5p* transfection. At 5 days after ATRA treatment, HL60 cells showed strong expression of ELANE using immunocytochemistry (Supplementary Fig. 1a). Furthermore, ATRA-treated HL60 cells showed strong expression of *CD66* mRNA, one of the neutrophil-specific markers, as compared to cells that did not receive ATRA (Supplementary Fig. 1b). Next, we transfected the *miR-340-5p* mimic into HL60 cells 5 days after ATRA treatment. At 48 h after *miR-340-5p* mimic transfection, the expression of *miR-340-5p* was approximately 1100-fold increased as compared to the negative control ($p < 0.05$) (Supplementary Fig. 1c), indicating that transfection was successful and efficient. Real-time PCR analysis at 24 h post-transfection with the *miR-340-5p* mimic indicated that *ARG1* mRNA expression was unchanged compared to controls

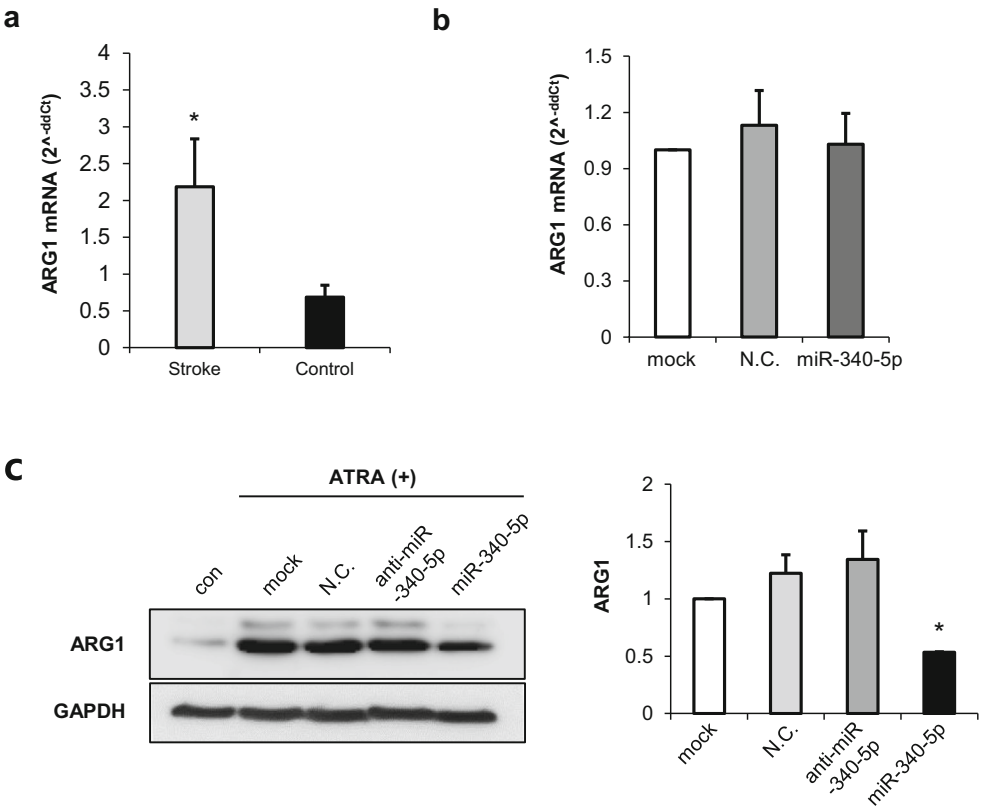

Fig. 3 Effect of *miR-340-5p* overexpression on protein expression of ARG1 and NO production in a human neutrophil cell line. **a** Expression of *ARG1* mRNA in blood of patients with stroke and controls. * $p < 0.05$ by Mann-Whitney *U* test. **b** Real-time PCR for *ARG1* mRNA after transfection with mock, scrambled (N.C.), and *miR-340-5p* mimic on ATRA-treated HL60 cells. **c** Western blot for ARG1 protein after transfection with mock, negative control (N.C.), and *miR-340-5p* mimic on ATRA-treated HL60 cells. Expression levels were normalized to *GAPDH*. NIH Image J was used for quantification of the Western blot. *$p < 0.05$ by Kruskal-Wallis test with a post hoc Conover's test. ARG1 arginase-1

(Fig. 3b). Western blotting revealed that ARG1 protein was detected in ATRA-treated HL60 cells as compared to cells without ATRA treatment (Fig. 3c). At 24 h post-transfection with the *miR-340-5p* mimic, ARG1 protein was significantly decreased in cells transfected with the *miR-340-5p* mimic as compared to cells transfected with either a mock transfection or the negative control (Fig. 3c). In cells transfected with antisense *miR-340-5p*, ARG1 protein levels did not differ from that of controls. These findings indicate that *miR-340-5p* regulates the *ARG1* expression at the post-transcriptional level in human neutrophils.

miR-340-5p Regulates Protein Expression of ARG1 and NO Production in a Mouse Macrophage Cell Line

In contrast to human *ARG1*, murine *ARG1* is expressed predominantly in macrophages, which regulates the immune response through nitric oxide (NO) production by competing with inducible nitric oxide synthase (iNOS) [20, 21, 29]. In a bioinformatics analysis (Targetscan software), murine *miR-340-5p* was predicted to bind the 3′UTR of *ARG1*. Furthermore, murine *miR-340-5p*, which is predicted to bind to a portion of the *ARG1* 3′UTR is fully homologous to human *miR-340-5p*. In order to examine whether *miR-340-5p* also regulates *ARG1* expression in mouse macrophages, we next evaluated the change in *ARG1* expression in RAW 264.7 cells treated with *miR-340-5p* mimics. In Western blot analyses, ARG1 protein expression was significantly reduced in Raw 264.7 cells treated with a *miR-340-5p* mimic as compared to cells treated with either mock (vehicle) and negative controls (Fig. 4a). We next investigated the effect of *miR-340-5p* on NO metabolism in RAW 264.7 cells after LPS stimulation. Treatment with LPS robustly increased NO release from macrophages 48 h after treatment. Transfection with *miR-340-5p* prior to LPS treatment resulted in higher NO level at 48 h after LPS treatment as compared to controls (Fig. 4b). These results suggest that *miR-340-5p* influences NO metabolism by regulating *ARG1* expression in mouse macrophage.

Discussion

In the present study, we found that acute ischemic stroke dynamically altered the expression of miRNAs in peripheral blood. In a miRNA microarray study, a total of 30 miRNAs were differentially regulated in peripheral blood during the acute phase of ischemic stroke. Among 30 miRNAs analyzed in the present microarray study, eight miRNAs (*let-7e*, *miR-186-5p*, *miR-142-3p*, *miR-19a-3p*, *miR-301a-3p*, *miR-140-5p*, *miR-18b-5p*, and *miR-877-5p*) were previously reported to be differentially expressed in the blood of stroke patients [17, 18]. The expression pattern of *let-7e*, *miR-186-5p*, *miR-142-3p*, *miR-18b-5p*, *miR-19a-3p*, and *miR-877-5p* we observed was similar to that reported in a previous study [17, 18, 30],

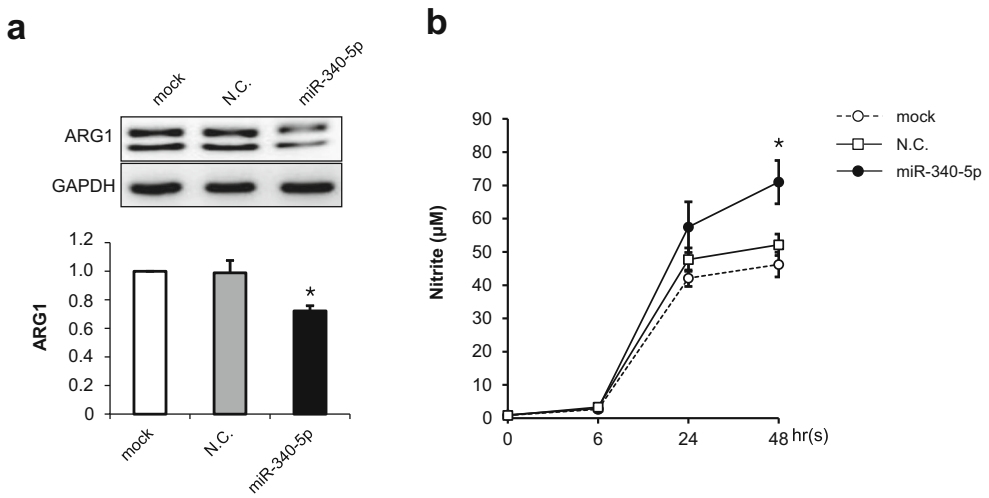

Fig. 4 Effect of *miR-340-5p* overexpression on protein expression of ARG1 and NO production in a mouse macrophage cell line. **a** Western blot for ARG1 protein after transfection with mock, negative control (N.C.), and a *miR-340-5p* mimic in Raw 264.7 cells. Expression levels were normalized to GAPDH. NIH Image J was used for quantification of the western blot. $*p < 0.05$ by Kruskal-Wallis test with a post hoc Conover's test. **b** Nitric oxide assay after transfection with mock, negative control (N.C.), and *miR-340-5p* mimic in Raw 264.7 cells. $*p < 0.05$ by Mann-Whitney *U* test

suggesting that a distinct alteration in expression of miRNAs in peripheral blood occurs in response to acute ischemic stroke. However, the expression pattern of *miR-140-5p* and *miR-301a-3p* differed between the previous study and our own [17, 30]. This discrepancy may be due to differences in patients' characteristics, the time interval between stroke and blood sampling, and the methodology used for statistical analysis. Another possible explanation for the different results is the dynamic alteration of miRNA expression depending on the time course of cerebral ischemia [14]. Despite these differences, there were several miRNAs in peripheral blood cells that altered in response to brain ischemia between the previous studies and ours. Therefore, these miRNAs could serve as a molecular fingerprint for acute ischemic stroke.

Among the altered miRNAs, we found that *miR-340-5p* was downregulated in the peripheral blood of patients with acute ischemic stroke; furthermore, *miR-340-5p* regulates *ARG1* expression. ARG1 is a cytosolic enzyme that is predominantly expressed in the liver as a component of the urea cycle. Recent studies have shown that *ARG1* is also expressed in immune cells in the peripheral blood and is involved in the immune response to injury [21, 28, 31]. ARG1 exerts its anti-inflammatory action via ARG1-mediated depletion of L-arginine, which suppresses Th1 cell proliferation and augments Th2 cell proliferation [21, 22, 32]. ARG1 expression in murine macrophages inhibited the production of NO by competing with iNOS for L-arginine [33, 34], which can suppress the inflammatory response. The anti-inflammatory effect of ARG1 is further supported by the finding that alternative M2-like macrophages enhanced the expression of *ARG1* [35]. In addition to intracellular ARG1, circulating ARG1 plays a role in the regulation of the peripheral immune response. Under inflammatory conditions, a large amount of ARG1 is released from macrophages [36], polymorphonuclear neutrophils [37], and myeloid-derived suppressor cells into the extracellular space where circulating ARG1 protein suppresses T-cell proliferation [37]. Although the clinical implications of ARG1 production during stroke are still unknown, recent microarray studies demonstrated that *ARG1* mRNA is consistently upregulated in white blood cells (especially polymorphonuclear cells) in patients with acute ischemic stroke [7, 23]. These data suggest an important role for ARG1 on the pathophysiology of stroke in human. Our study showed that *miR-340-5p* is downregulated whereas *ARG1* was upregulated in peripheral blood after acute ischemic stroke. Overexpression of *miR-340-5p* downregulates ARG1 protein expression in human neutrophils and mouse macrophages. These findings suggest that *miR-340-5p* participates in the peripheral immune response to acute ischemic stroke by fine-tuning *ARG1* expression.

Based on these findings, a plausible molecular mechanism by which *miR-340-5p* and *ARG1* interact during acute ischemic stroke is suggested. In acute ischemic stroke, downstream signals from the injured brain upregulate *ARG1* mRNA and downregulate the expression of *miR-340-5p* in circulating immune cells. The downregulation of *miR-340-5p* further enhances ARG1 expression via relief from translational repression. The net effect is a robust increase in ARG1 protein in the peripheral blood during the acute phase of ischemic stroke. A recent study has reported another role for *miR-340-5p* in the immune system. Guerau-Arellano et al. [38] demonstrated that *miR-340-5p* was upregulated in T-cells of patients with multiple sclerosis; upregulated *miR-340-5p* caused $CD4^+$ T cells to polarize toward proinflammatory Th1 cells by

targeting interleukin-4 (*IL4*), one of the major anti-inflammatory cytokines released from T-cells. Furthermore, increased IL-4 protein binds to IL-4 receptor on macrophages, which induces *ARG1* mRNA transcription via Jak/STAT6 signaling [39]. Therefore, it is conceivable that *miR-340-5p* plays a major role in the pathogenesis of stroke and other inflammatory CNS diseases, acting as an endogenous regulator of the peripheral immune response by targeting *ARG1* and/or *IL4*. This post-stroke molecular change may be associated with a compensatory response to local inflammation in the CNS, acting as defense machinery against activation of pro-inflammatory signals after stroke. Another possible clinical implication of the increase in *ARG1* is stroke-induced immunodepression (SID), in which the patient is more vulnerable to infection and poor outcome [40, 41]. ARG1 protein released from neutrophils suppresses T lymphocyte proliferation; in addition, circulating ARG1 protein released from neutrophils induces lymphopenia in a murine model of stroke [37]. A recent study showed that blood *ARG1* mRNA expression is correlated with stroke severity and poor outcome along with a high neutrophil-to-lymphocyte ratio [42]. As ARG1 is a target of *miR-340-5p*, it is possible that altered expression of *miR-340-5p* may contribute to and serve as a biomarker for SID.

The limitations of the present study should be addressed. Our results must be interpreted cautiously because our sample size was small. The clinical characteristics of our stroke and control subjects were not representative of the general stroke population. Furthermore, our study did not include patients with lacunar stroke, one of the common types of ischemic stroke. Therefore, further studies are needed to clarify the biological and clinical significances of altered *miR-340-5p* levels in the general stroke population. Another consideration is the possible existence of other miRNAs and targets, in addition to *miR-340-5p*/ARG1, that are specifically expressed in stroke patients. In the present study, we specifically focused on the miRNAs that binds *ARG1* or *IL18R1*, because these genes are reported to be exhibit the greatest amount of differential expression in the peripheral blood of acute stroke patients as compared to controls [7, 19]. It is generally known that a single miRNA can have multiple target genes; thus, there would be many candidate genes that can bind to differentially expressed miRNAs from our microarray data. It is extremely difficult for us to find all of the target genes of the differentially expressed miRNAs we identified. We suggest that other differentially expressed miRNAs in our study, such as *let-7e*, *miR-19a-3p*, *miR-186-5p*, and *miR-32-5p*, should be investigated in the future as well. Recent emerging evidence provides valuable clues to the role of these miRNAs in the immune system. *Let-7e* was found to regulate the immune response by targeting Toll-like receptor 4 (*TLR4*) [43] and interleukin-10 (*IL10*) [44]. *miR-186-5p* was predicted to target more than 50% of candidate genes for autoimmune disease in a bioinformatic model [45]. *miR-32-5p* strongly regulates the immune response by targeting tumor necrosis factor-receptor associated factor 3 (*TRAF3*) [46]. Hence, these miRNAs may also participate in the pathogenesis of stroke.

Conclusions

The present study demonstrated that alteration of expression of specific miRNAs in peripheral blood after acute ischemic stroke. *miR-340-5p* regulates the immune response by targeting the ARG1 protein. Although further studies are needed, we conclude that altered expression of *miR-340-5p* and its target gene, *ARG1*, may provide an important molecular signature of acute ischemic stroke.

Authors' Contribution Yoo H, Kim J, Kim JK, and Oh SH designed the study concept, design, and wrote the draft. Yoo H, Kim J, and Oh SH performed the acquisition of data and analysis. Lee JM, Lee AR, Kim OJ, and Kim JK conducted the interpretation. Yoo H, Kim J, Oh SH, and Kim JK made substantial revision of the report. All authors have given final approval of the manuscript for publication.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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