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ORIGINAL ARTICLE

Using Extracellular Circulating microRNAs to Classify the Etiological Subtypes of Ischemic Stroke

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Abstract

There is no effective biological method to classify ischemic stroke subtypes. In this study, we first performed a systematical gene array study on serum microRNAs with different ischemic stroke subtypes including 13 normal control subjects (NCs) and 87 ischemic stroke (IS) patients including 23 cardioembolism (CARD), 26 large artery atherosclerosis (LAA), 27 lacunar infarct (LAC), and 11 stroke of undetermined etiology (SUE). Validation was performed by using an independent cohort of 20 NCs and 85 IS patients including 28 CARD, 23 LAA, 18 LAC, and 16 SUE. In the pilot discovery gene array study, we found specific serum microRNA signatures between different ischemic stroke subtypes (CARD, LAA, LAC, and SUE). We further validated 6 microRNAs [miR-125b, miR-125a, let-7b, let-7e, miR-7-2-3p, miR-1908] in a different group of ischemic stroke subtypes by using an independent cohort of 20 NCs, 28 CARD, 23 LAA, 18 LAC, and 16 SUE. Moreover, these circulating miRNAs were further detected to be differentially expressed between pre- vs. post-stroke in different ischemic stroke subtypes. The ROC analysis showed that miR-125b, miR-125a, let-7b, and let-7e could discriminate CARD patients from normal controls and other subtypes. Furthermore, ROC curves shown that miR-7-2-3p and miR-1908 showed significant area-under-the-curve values in both LAA and LAC patients. In conclusion, these results demonstrated that circulating miRNAs in sera could be potentially novel risk factors that involve in the pathogenesis of ischemic stroke subtypes.

Keywords Ischemic stroke · Subtype · MicroRNA

Introduction

Ischemic strokes occur when blood supply is cut off to part of the brain. Nowadays, ischemic stroke is becoming a leading cause of long-term disability and death around the world [16]. Over two million people in China have stroke, and one million die from stroke-related causes each year [22]. Ischemic stroke is recently known as the possible etiologies including intracranial small-vessel disease, cardioembolism, and prothrombotic disorders [9, 10]. The molecular characteristics of ischemic stroke subtypes remain elusive.

MicroRNAs (miRNAs) are a class of small noncoding RNAs (~ 22 nucleotides in length) that are potent regulators of message RNA (mRNA) [4]. MiRNAs are known to participate in a large number of physiological and pathological processes, including neuronal death, neurodegeneration, or ischemic stroke [11, 15, 25]. For example, miR-195 is reported to protect against dementia via its anti-amyloidogenic effect [2]. In addition, Jeyaseelan et al. reported that miR-30a-3p was downregulated in the early reperfused MCAo rat brains but was subsequently upregulated [14]. The polymorphisms of miR-146a were found to be associated with ischemic stroke pathogenesis [13]. Endothelium-specific miRNA, miR-126, was reduced in young stroke patients [30]. Moreover, let-7 is recently known as a regulator activating toll-like receptor 7 that contributes to the spread of CNS damage [17]. However, the expression of serum circulating miRNAs in ischemic stroke has as yet not been evaluated in different ischemic stroke subtypes. The aim of this study was to (1) identify differentially regulated circulating microRNAs in ischemic

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stroke subtypes such as cardioembolism (CARD), large artery atherosclerosis (LAA), lacunar infarct (LAC), and stroke of undetermined etiology (SUE), (2) validate several miRNAs that was subsequently confirmed in independent cohorts of ischemic stroke subtypes, and (3) compare the circulating miRNAs expression pre- vs. post-stroke.

Materials and Methods

Ethics Statement

Written informed consent for participation in the study was obtained from either directly or from his or her guardian in all subjects and the work received approval from the institution ethics committee of Zhejiang University School of Medicine and in accordance with the tenets of the Declaration of Helsinki.

Study Population

Patients with suspected ischemic stroke were recruited within 24 h of symptom onset through the emergency department of our hospital. The enrollment period was January 2014 to December 2017. All patients had a final diagnosis of ischemic stroke as defined by an acute focal neurological deficit in combination with a diffusion-weighted imaging-positive lesion on magnetic resonance imaging or a new lesion on a delayed computed tomography (CT) scan. For the discovery and validation sample, we excluded ischemic stroke patients and normal controls (NCs) with active malignant disease, inflammatory or infectious diseases, surgery within the last 3 months and prior medication with low-molecular or unfractionated heparin within the last month. For the validation sample, we excluded patients with prior medication with low-molecular or unfractionated heparin within the last month. No study subjects suffered from polycythemia vera or essential thrombocythemia. The discovery sample included 13 NCs and 87 IS patients including 23 CARD, 26 LAA, 27 LAC, and 11 SUE. Validation was done in 20 NCs and 85 IS patients including 28 CARD, 23 LAA, 18 LAC, and 16 SUE. Stroke etiology was classified according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) [1]. NCs from the discovery and validation sample were matched to patients with IS for age, sex, hypertension, smoking history, hypercholesterolemia, obesity, diabetes mellitus, family history, and use of anti-platelet therapy.

Patient Blood Sampling and Processing

Blood samples from IS patients were collected at hospital arrival in the emergency department. Whole blood was drawn into EDTA-containing tubes (BD Biosciences). Plasma was separated by differential centrifugation at 2000 g for 10 min and 2500 g for 15 min at 15 °C. Samples were aliquoted in screw cap vials and kept at − 80 °C.

RNA Isolation

Plasma RNA was isolated according to the manufacturer's protocol using the miRNeasy Mini kit (Qiagen). RNA quality was assessed by using UV 260/280 and 230/260 absorbance ratios obtained by using Nanodrop (Thermo Scientific), resulting in a mean 260/280 ratio of 1.95.

MicroRNA Expression Profiling

The TaqMan Low-Density Array Human miRNA Panel v1.0 (Applied Biosystems) was utilized for global miRNA profiling. The panel includes two 384-well microfluidic cards (human miRNA pool A and pool B) that contain primers and probes for 746 different human miRNAs in addition to six small nucleolar RNAs that function as endogenous controls for data normalization. Quantitative PCR was performed on an Applied BioSystems 7900HT thermocycler (Applied Biosystems) using the manufacturer's recommended cycling conditions.

MicroRNA Target Prediction and Pathway Analysis

DIANA-mirPath [27] was employed to perform the enrichment analysis of predicted target genes by one or more miRNAs in biological pathways. The statistical significance associated with the identified biological pathways was calculated by the mirPath (http://microrna.gr/mirpath).

TaqMan miRNA Assay for Individual miRNAs

Individual miRNA analysis was performed using TaqMan miRNA assays (Applied Biosystems). A synthetic RNA spike-in, *C. elegans* miR-39 miRNA mimic, was added prior to cDNA synthesis as a control for variation in reverse transcription efficiency. The PCR amplifications were carried out by incubation for 10 min at 95°C, followed by 40 amplification cycles at 95°C for 10 s and 60°C for 1 min. The spike-in amplification values were used for quality control of each sample. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Raw data from TaqMan Low-Density Array was analyzed by using the SDS software version 2.2.2 (Applied Biosystems). Assays that had Ct values > 35 were removed from the analysis. The group comparison was analyzed using Mann–Whitney *U* tests. The following values were considered

Table 1 Demographic data, baseline characteristics, and risk factors for stroke, by ischemic stroke subtype

Characteristics	NC ($n = 13$)	CARD ($n = 23$)	LAA ($n = 26$)	LAC ($n = 27$)	SUE ($n = 11$)
Age, years[a]	58 (11)	60 (10)	60 (12)	62 (13)	61 (13)
Male, n (%)	6 (46)	10 (43)	13 (50)	13 (48)	5 (45)
Risk factors, *n* (%)					
Atrial fibrillation	0 (0)	14 (61)	–	–	2 (18)
Hypertension	0 (0)	8 (35)	14 (54)	18 (67)	7 (64)
Diabetes	0 (0)	5 (22)	8 (31)	7 (26)	3 (27)
Smoking	3 (23)	3 (13)	11 (42)	11 (41)	4 (36)
Prior TIA or stroke	0 (0)	3 (13)	2 (8)	3 (11)	2 (18)
Hypercholesterolemia[b]	0 (0)	4 (17)	9 (35)	5 (18)	4 (36)
CNS score > 6.5[c]	0 (0)	10 (43)	15 (58)	19 (70)	5 (45)

CARD cardioembolism, *LAA* large artery atherosclerosis, *LAC* lacunar infarction, *SUE* stroke of undetermined etiology, *TIA* transient ischemic attack

[a] Mean (SD)

[b] Cholesterol > 220 mg/dl

[c] Canadian neurological scale score on admission

significant: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). For evaluation of sensitivity and specificity, a receiver operating characteristics (ROC) curve was used to determine cut off points and to calculate the area under the curve (AUC) by using GraphPad PRISM 6.0.

Results

Differential Expression Pattern of Serum microRNAs in Ischemic Stroke Subtypes

To determine the profile of circulating microRNAs associated with IS in the acute phase, we first performed TaqMan microRNA array in the discovery sample including 13 NCs, 23 CARD, 26 LAA, 27 LAC, and 11 SUE (Table 1). Patients and control subjects included in the discovery samples were matched with respect to all variables between IS subtypes. Initial experiments were designed to minimize the impact of covariates by matching the samples for key confounding factors. Therefore, we used a univariate test to screen for differentially expressed miRNAs, and validated the result with a separate set of samples. The results showed that there were 198 miRNAs (26.5%) that can be detected (assays giving Ct values < 35 in over 50% subjects defined as detectable). The relative abundance of detected serum miRNAs was compared by ischemic stroke patients to normal control subjects. Results from these comparison revealed that there were 69 miRNAs differentially expressed in serum of CARD patients compared to NCs. Among them, we found that 35 serum miRNAs were significantly upregulated and 34 miRNAs were downregulated significantly (fold change > 2, $p < 0.05$) in CARD serum (Table 2). In addition, we found 66 miRNAs differentially expressed in serum of LAA patients compared to NCs, including 35 upregulated miRNAs and 31 downregulated miRNAs (fold change > 2, $p < 0.05$) in LAA patients (Table 2). Furthermore, 64 circulating miRNAs were dysregulated in LAC patients compared to NC. There were 33 higher expressed miRNAs and 31 low expressed miRNAs in LAC patients (Table 2). Moreover, the plots for ischemic stroke subtypes (NC, CARD, LAA, LAC, and SUE) were performed as principal component analysis (PCA) among all samples based on miRNA profiles (Supplemental Fig. 1). NC and SUE were not correlated with the first and second principal components. CARD, LAA, and LAC were correlated with the first PC ($p < 0.01$), which suggests that the statistical results from differential miRNA expression profiling would be affected by principal components when testing differential serum miRNAs expression between CARD, LAA, LAC, and NC. However, LAC and LAA were not correlated with the first principal components, while CARD was correlated with the second principal components when compared to LAA and LAC. These data indicated that extracellular circulating miRNAs were differentially expressed in different ischemic stroke subtypes, which further provide important clues to pathogenesis of different ischemic stroke progression.

Comparative Pathway Analyses

We further identified which biologic pathways were affected in ischemic stroke subtypes [CARD, LAA, LAC, and SUE], we applied DIANA-mirPath on the dysregulated serum miRNAs between CARD and NC, and 54 KEGG pathways were significantly enriched ($p < 0.05$) in CARD-associated miRNAs after false discovery rate was corrected. GABAergic synapse (4.516E-07), Hippo signaling pathway (4.516E-07), axon guidance (1.153E-06), glutamatergic synapse (4.193E-06), endocrine and other factor-regulated calcium reabsorption (0.002),

Table 2 Differential microRNAs expression between CARD vs. NC, LAA vs. NC, and LAC vs. NC

CARD vs. NC miR-Name	Fold change	Adjusted *p* value	LAA vs. NC miR-Name	Fold change	Adjusted *p* value	LAC vs. NC miR-Name	Fold change	Adjusted *p* value
hsa-miR-335	0.257	0.0239	hsa-miR-23a	0.608	0.0000	hsa-miR-23a	0.321	0.0000
hsa-miR-382	0.534	0.0208	hsa-miR-4797	1.44	0.0000	hsa-miR-4797	1.389	0.0001
hsa-miR-126	0.54	0.0313	hsa-miR-4706	1.123	0.0005	hsa-miR-4706	1.251	0.0000
hsa-miR-30a	0.598	0.0207	hsa-miR-6776	7.155	0.0000	hsa-miR-6776	7.772	0.0000
hsa-miR-6745	1.313	0.0231	hsa-miR-6732	1.928	0.0009	hsa-miR-1469	1.339	0.0006
hsa-miR-6875-3p	0.136	0.0331	hsa-miR-1469	1.471	0.0000	hsa-miR-6732	1.961	0.0016
hsa-let-7b	0.693	0.0237	hsa-miR-6511b	2.5	0.0028	hsa-miR-6511b	2.16	0.0000
hsa-let-7i	0.662	0.0287	hsa-miR-3148	5.538	0.0058	hsa-miR-1273 g-3p	2.65	0.0005
hsa-let-7e	1.361	0.0147	hsa-miR-7-2-3p	2.123	0.0410	hsa-miR-221	0.589	0.0017
hsa-miR-125a	1.364	0.0147	hsa-miR-2277	0.132	0.0738	hsa-miR-3148	6.386	0.0001
hsa-miR-125b	1.372	0.0189	hsa-miR-4539	3.063	0.0481	hsa-miR-2277	0.116	0.0011
hsa-miR-4529-3p	0.92	0.0297	hsa-miR-4763-3p	1.889	0.0087	hsa-miR-7-2-3p	2.05	0.0001
hsa-miR-223	1.555	0.0205	hsa-miR-1273 g-3p	2.893	0.0302	hsa-miR-223	4.059	0.0001
hsa-miR-143-3p	1.386	0.0323	hsa-miR-607	3.837	0.0004	hsa-miR-21	5.006	0.0018
hsa-miR-363-3p	0.595	0.0140	hsa-miR-1910	0.011	0.0007	hsa-miR-7108	0.536	0.0003
hsa-miR-4735-3p	0.994	0.0207	hsa-miR-6803	0.963	0.0042	hsa-miR-124	3.937	0.0011
hsa-miR-6750	0.494	0.0249	hsa-miR-4787	0.843	0.0067	hsa-miR-4763-3p	2.291	0.0133
hsa-miR-4793-3p	2.106	0.0082	hsa-miR-6729	3.923	0.0115	hsa-miR-6085	1.501	0.0009
hsa-miR-2277	0.102	0.0485	hsa-miR-6085	1.462	0.0173	hsa-miR-4507	0.975	0.0018
hsa-miR-2861	2.556	0.0091	hsa-miR-6791	0.492	0.0175	hsa-miR-6791	0.396	0.0020
hsa-miR-4707	0.51	0.0197	hsa-miR-877	0.688	0.0205	hsa-miR-210	3.055	0.0027
hsa-miR-551b	0.546	0.0307	hsa-miR-1281	0.385	0.0275	hsa-miR-6803	0.84	0.0004
hsa-miR-210	1.19	0.0145	hsa-miR-2861	4.122	0.0408	hsa-miR-4787	0.805	0.0111
hsa-miR-378 h	2.891	0.0435	hsa-miR-21	4.124	0.0100	hsa-miR-607	3.685	0.0122
hsa-miR-619	1.034	0.0197	hsa-miR-221	0.139	0.0130	hsa-miR-145	2.903	0.0003
hsa-miR-3910	1.705	0.0501	hsa-miR-548a-3p	0.824	0.0203	hsa-miR-6729	2.947	0.0004
hsa-miR-3148	5.866	0.0272	hsa-miR-4507	0.617	0.0352	hsa-miR-29b	2.647	0.0004
hsa-miR-4507	0.867	0.0126	hsa-miR-7108	0.424	0.0494	hsa-miR-4707	0.612	0.0007
hsa-miR-4508	0.534	0.0164	hsa-miR-124	3.973	0.0007	hsa-miR-4529-3p	0.943	0.0020
hsa-miR-3136	0.345	0.0355	hsa-miR-3656	0.349	0.0010	hsa-miR-4466	0.511	0.0022
hsa-miR-6727	1.226	0.0069	hsa-miR-4471	0.552	0.0012	hsa-miR-548a-3p	0.832	0.0030
hsa-miR-663a	0.785	0.0579	hsa-miR-4466	0.511	0.0015	hsa-miR-4487	0.681	0.0046
hsa-miR-23a	0.585	0.0124	hsa-miR-1908	0.386	0.0015	hsa-miR-1281	0.342	0.0046
hsa-miR-1228-3p	1.726	0.0205	hsa-miR-8071	1.237	0.0020	hsa-miR-1908	0.653	0.0059
hsa-miR-6088	0.287	0.0108	hsa-miR-6756	1.276	0.0022	hsa-miR-638	4.58	0.0061
hsa-miR-29b	1.275	0.0203	hsa-miR-4786-3p	2.075	0.0022	hsa-miR-6756	1.495	0.0069
hsa-miR-6511b	2.138	0.0150	hsa-miR-4529-3p	0.976	0.0025	hsa-miR-4801	0.091	0.0072
hsa-miR-638	3.727	0.0095	hsa-miR-6089	2.789	0.0026	hsa-miR-8075	1.681	0.0074
hsa-miR-145	1.234	0.0134	hsa-miR-1228-3p	1.457	0.0027	hsa-miR-6794	0.267	0.0076
hsa-miR-6089	2.952	0.0379	hsa-miR-8075	1.815	0.0028	hsa-miR-5787	0.218	0.0078
hsa-miR-1281	0.459	0.0180	hsa-miR-5787	0.307	0.0028	hsa-miR-143-3p	1.503	0.0083
hsa-miR-6729	3.871	0.0136	hsa-miR-663a	0.902	0.0030	hsa-miR-363-3p	0.616	0.0085
hsa-miR-6777	0.464	0.0153	hsa-miR-6724	0.96	0.0031	hsa-miR-4785	0.271	0.0086
hsa-miR-6787	0.255	0.0259	hsa-miR-4632	0.732	0.0041	hsa-miR-6819-3p	4.136	0.0088
hsa-miR-3613-3p	1.1	0.0060	hsa-miR-6819-3p	7.291	0.0055	hsa-miR-548c	1.106	0.0095
hsa-miR-1910	0.011	0.0151	hsa-miR-5093	1.095	0.0066	hsa-miR-5093	0.914	0.0122
hsa-miR-6803	0.993	0.0554	hsa-miR-143-3p	1.449	0.0069	hsa-miR-1910	0.009	0.0124

Table 2 (continued)

CARD vs. NC miR-Name	Fold change	Adjusted *p* value	LAA vs. NC miR-Name	Fold change	Adjusted *p* value	LAC vs. NC miR-Name	Fold change	Adjusted *p* value
hsa-miR-548c	1.387	0.0072	hsa-miR-363-3p	0.601	0.0072	hsa-miR-2861	2.655	0.0188
hsa-miR-21	2.938	0.0147	hsa-miR-4801	0.11	0.0074	hsa-miR-8072	3.3	0.0229
hsa-miR-4668	0.139	0.0084	hsa-miR-4707	0.6	0.0078	hsa-miR-3201	0.481	0.0245
hsa-miR-4706	1.345	0.0197	hsa-miR-4487	0.699	0.0080	hsa-miR-668	0.636	0.0266
hsa-miR-124	2.303	0.0223	hsa-miR-4785	0.358	0.0086	hsa-miR-877	0.482	0.0312
hsa-miR-297	0.802	0.0183	hsa-miR-3613	2.105	0.0089	hsa-miR-3656	0.413	0.0315
hsa-miR-3613	1.845	0.0564	hsa-miR-6794	0.267	0.0089	hsa-miR-4632	0.882	0.0352
hsa-miR-4787	0.901	0.0074	hsa-miR-6858	1.386	0.0101	hsa-miR-4539	2.1	0.0361
hsa-miR-4471	0.634	0.0249	hsa-miR-3921	1.679	0.0107	hsa-miR-6850	1.057	0.0397
hsa-miR-6732	2.108	0.0221	hsa-miR-455-3p	1.564	0.0111	hsa-miR-3921	0.947	0.0433
hsa-miR-221	0.502	0.0089	hsa-miR-223	1.711	0.0120	hsa-miR-6724	1.443	0.0436
hsa-miR-548a-3p	0.969	0.0301	hsa-miR-6785	1.932	0.0120	hsa-miR-4786-3p	1.995	0.0438
hsa-miR-6776	8.228	0.0174	hsa-miR-1184	0.46	0.0133	hsa-miR-1184	0.629	0.0444
hsa-miR-5093	1.038	0.0101	hsa-miR-548b-3p	2.879	0.0149	hsa-miR-6785	1.932	0.0444
hsa-miR-7108	0.514	0.0256	hsa-miR-6850	1	0.0200	hsa-miR-4750-3p	0.544	0.0455
hsa-miR-4722-3p	1.564	0.0153	hsa-miR-3613-3p	1.039	0.0270	hsa-miR-6858	0.836	0.0468
hsa-miR-4466	0.584	0.0397	hsa-miR-548c	0.807	0.0285	hsa-miR-548b-3p	1.971	0.0493
hsa-miR-607	3.935	0.0220	hsa-miR-3201	0.455	0.0338			
hsa-miR-6779	0.569	0.0216	hsa-miR-6877-3p	0.71	0.0465			
hsa-miR-1469	1.389	0.0190						
hsa-miR-6085	1.36	0.0231						
hsa-miR-7704	2.196	0.0088						

adrenergic signaling in cardiomyocytes (0.002), dorso-ventral axis formation (0.009), and circadian entrainment (0.009) were top ranked as the most prominent pathways enriched in quantiles with the serum miRNA signature of CARD patients (supplemental Table 1). Hippo signaling pathway (1.338E-10), FoxO signaling pathway (9.290E-08), endocrine and other factor-regulated calcium reabsorption (4.625E-05), AMPK signaling pathway (6.536E-05), glycosaminoglycan biosynthesis (8.618E-05), Rap1 signaling pathway (8.618E-05), mucin type O-glycan biosynthesis (9.547E-05), Ras signaling pathway

Table 3 Demographic data, baseline characteristics, and risk factors for stroke for independent cohort of ischemic stroke patients

Characteristics	NC (*n* = 20)	CARD (*n* = 28)	LAA (*n* = 23)	LAC (*n* = 18)	SUE (*n* = 16)
Age, years[a]	62 (13)	57 (14)	63 (16)	65 (11)	60 (8)
Male, *n* (%)	10 (50)	14 (50)	12 (52)	9 (50)	8 (50)
Risk factors, *n* (%)					
Atrial fibrillation	0 (0)	16 (57)	–	–	1 (6)
Hypertension	0 (0)	10 (35)	10 (43)	12 (67)	10 (62)
Diabetes	0 (0)	8 (28)	5 (22)	6 (33)	5 (31)
Smoking	5 (25)	6 (21)	11 (48)	10 (55)	6 (37)
Prior TIA or stroke	0 (0)	2 (7)	1 (4)	2 (11)	3 (19)
Hypercholesterolemia[b]	0 (0)	3 (11)	8 (34)	3 (17)	7 (43)
CNS score > 6.5[c]	0 (0)	15 (53)	16 (69)	14 (78)	8 (50)

CARD cardioembolism, *LAA* large artery atherosclerosis, *LAC* lacunar infarction, *SUE* stroke of undetermined etiology, *TIA* transient ischemic attack

[a] Mean (SD)

[b] Cholesterol > 220 mg/dl

[c] Canadian neurological scale score on admission

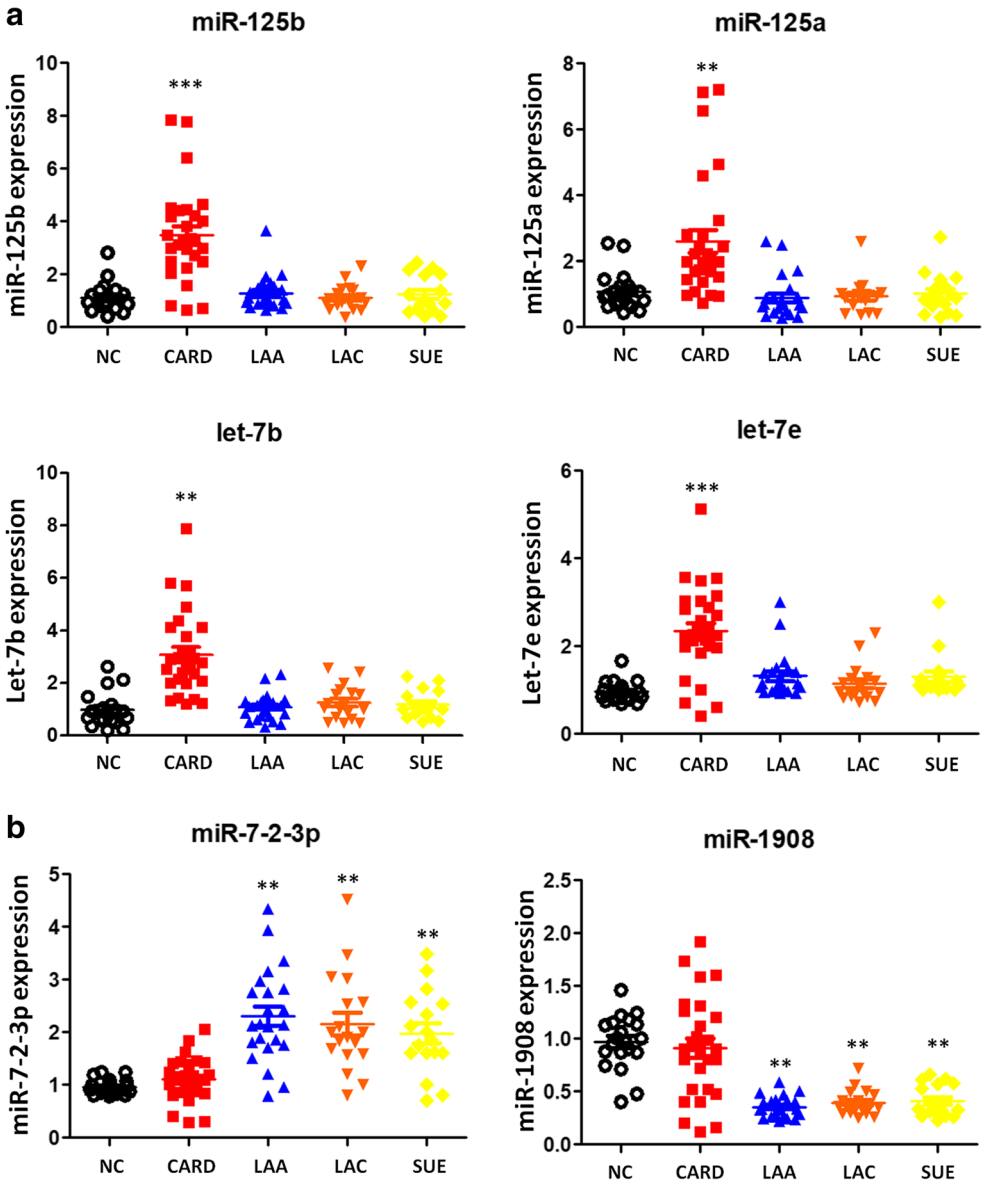

Fig. 1 Validation of miRNA expression using independent samples. TaqMan real-time RT-PCR to validate the expression levels of miR-125b, miR-125a, let-7b, let-7e, miR-7-2-3p, and miR-1908 using an independent cohort of 20 normal control subjects (NCs) and 85 IS patients including 28 CARD, 23 LAA, 18 LAC, and 16 SUE. Data shown are as mean ± SEM

(9.802E-05), and endocytosis (2.463E-04) were enriched in LAA-associated miRNAs (supplemental Table 2). Further, FoxO signaling pathway (4.038E-08), Ras signaling pathway (2.856E-05), ErbB signaling pathway (3.261E-05), Hippo signaling pathway (3.688E-05), AMPK signaling pathway (4.389E-05), TGF-beta signaling pathway (1.438E-04), mucin type O-glycan biosynthesis (1.441E-04), Rap1 signaling pathway (2.840E-04), fatty acid biosynthesis (3.913E-04), endocytosis (6.675E-04), Wnt signaling pathway (1.767E-03), and glycosaminoglycan biosynthesis (0.002) were found to be dysregulated by LAC-associated miRNAs (supplemental Table 3).

Validation of Differentially Expressed microRNAs

Next, we examined these 6 microRNAs [miR-125b, −125a, let-7b, let-7e, miR-7-2-3p, miR-1908] in an independent validation sample using quantitative real-time polymerase chain reactions. These miRNAs were selected for further validation according to their significance of the difference (fold change, *p* value), previous observations and biological plausibility. A further validation assay was performed using an independent cohort of 20 NCs and 85 IS patients including 28 CARD, 23 LAA, 18 LAC, and 16 SUE (Table 3). The validation samples were matched for demographic and vascular risk factors as well as previous use of anti-platelet medication. We found that miR-125b, miR-125a, let-7b, and let-7e were significantly increased in CARD patients, while there was no significance of LAA, LAC, and SUE when compared to NC (Fig. 1). However, miR-7-2-3p showed higher levels, miR-1908 with lower levels, in LAA, LAC, and SUE patients but not in CARD patients (Fig. 1). These findings confirmed that certain dysregulated

Table 4 Demographic data, baseline characteristics, and risk factors for stroke for16 CARD, 16 LAA, and 16 LAC patients

Characteristics	CARD ($n = 16$)	LAA ($n = 16$)	LAC ($n = 16$)
Age, years[a]	52 (5)	65 (10)	63 (10)
Male, *n* (%)	12 (75)	8 (50)	10 (63)
Risk factors, *n* (%)			
Atrial fibrillation	10 (63)	–	–
Hypertension	6 (37)	7 (45)	12 (75)
Diabetes	4 (25)	4 (25)	5 (31)
Smoking	3 (18)	5 (31)	9 (56)
Hypercholesterolemia[b]	2 (13)	8 (50)	3 (18)
CNS score > 6.5[c]	8 (50)	13 (81)	13 (81)

CARD cardioembolism, *LAA* large artery atherosclerosis, *LAC* lacunar infarction

[a] Mean (SD)

[b] Cholesterol > 220 mg/dl

[c] Canadian neurological scale score on admission

serum miRNAs represented as potential noninvasive biomarkers for ischemic stroke subtypes.

Serum microRNAs Were Differentially Expressed in Sequential Progression of Ischemic Stroke Subtypes

We further aimed to identify and assess the serum miRNAs that could detect the presence of clinical and pre-clinical ischemic stroke in at-risk patients. In the experiment, we recruited 16 CARD, 16 LAA, and 16 LAC patients and thus we performed paired comparison between pre-IS and post-IS. Demographic data, baseline characteristics, and risk factors for stroke are included in Table 4. We identified four miRNAs [miR-125b, miR-125a, let-7b, let-7e] to detect CARD by comparing these miRNA levels between pre-stroke and post-stroke of 16 CARD patients (all p values < 0.01). Further miR-7-2-3p was over expressed in 16 LAA and 16 LAC patients after stroke compared to those before stroke, and miR-1908 were under expressed in 16 LAA and 16 LAC patients compared to that before stroke onset (Fig. 2). These data demonstrated that serum miRNA classifier is a potential biomarker for ischemic stroke subtypes in patients.

ROC Analysis of Circulating miRNAs Involved in IS Subtypes

We performed an ROC analysis to calculate the relationship between sensitivity and specificity for combined cohorts (discovery and validation cohorts), 51 CARD patients, 49 LAA patients, 45 LAC patients versus 33 normal controls. The ROC analysis showed that miR-125b, miR-125a, let-7b, and let-7e could discriminate CARD patients from normal controls [miR-125b: AUC = 0.906, CI95% = (0.888–0.956), sensitivity = 86%, specificity = 87%; miR-125a: AUC = 0.866, CI95% = (0.795–0.963), sensitivity = 87%, specificity = 82%; let-7b: AUC = 0.833, CI95% = (0.763–0.925), sensitivity = 83%, specificity = 85%; let-7e: AUC = 0.923, CI95% = (0.859–0.998), sensitivity = 89%, specificity = 90%, $p < 0.001$ for all comparisons] (Supplemental Fig. 2). Similarly, ROC curves were performed by comparing with LAA and LAC patients vs. normal controls to evaluate the relationship between sensitivity and specificity for miR-7-2-3p and miR-1908. miR-7-2-3p and miR-1908 showed significant area-under-the-curve values in both LAA and LAC patients (LAA: miR-7-2-3p: AUC = 0.874, CI95% = (0.795–0.945), sensitivity = 86%, specificity = 87%; LAC: miR-7-2-3p: AUC = 0.849, CI95% = (0.777–0.917), sensitivity = 84%, specificity = 83%. LAA: miR-1908: AUC = 0.811, CI95% = (0.734–0.879), sensitivity = 79%, specificity = 83%; LAC: miR-1908: AUC = 0.789, CI95% = (0.715–0.867), sensitivity = 77%, specificity = 81% (Supplemental Fig. 3)). Therefore, these results strongly suggest circulating miRNAs will be of potential value as a diagnostic marker for different ischemic stroke subtypes.

Discussion

Clarifying the ischemic stroke subtypes is of importance in choosing treatment alternatives and predicting the outcome. There are two classification systems used in clinical trials: OCPS (Oxfordshire community stroke project) [3] and TOAST (Trial of Org 10172 in Acute Stroke Treatment) [1]. In this study, we used TOAST for the high interobserver advantage for etiological diagnoses. The institute neurologist (Yaxing Gui) reviewed the clinical history, neurological examination, diagnostic studies, and brain imaging studies of all patients and assigned infarct subtype classifications according to TOAST classification system: 1, cardioembolism (CARD); 2, large artery atherosclerosis (LAA); 3, lacunar infarct (LAC); and 4, stroke of undetermined etiology (SUE). In the current study, we performed a systematical gene array study with different ischemic stroke subtypes, and found that extracellular circulating miRNAs were differentially expressed in ischemic stroke subtypes, biologic pathways were affected in ischemic stroke subtypes [CARD, LAA, LAC, and SUE], we further validated several serum miRNAs in a different group of ischemic stroke subtypes, and these circulating miRNAs were further detected differentially expressed between pre- vs. post-stroke. Furthermore, we performed an ROC analysis to calculate the relationship between sensitivity and specificity by using 51 CARD patients, 49 LAA patients, 45 LAC patients versus 33 normal controls. The ROC analysis showed that miR-125b, miR-125a, let-7b, and let-7e could discriminate CARD patients from normal controls (Supplemental Fig. 2). Similarly, ROC

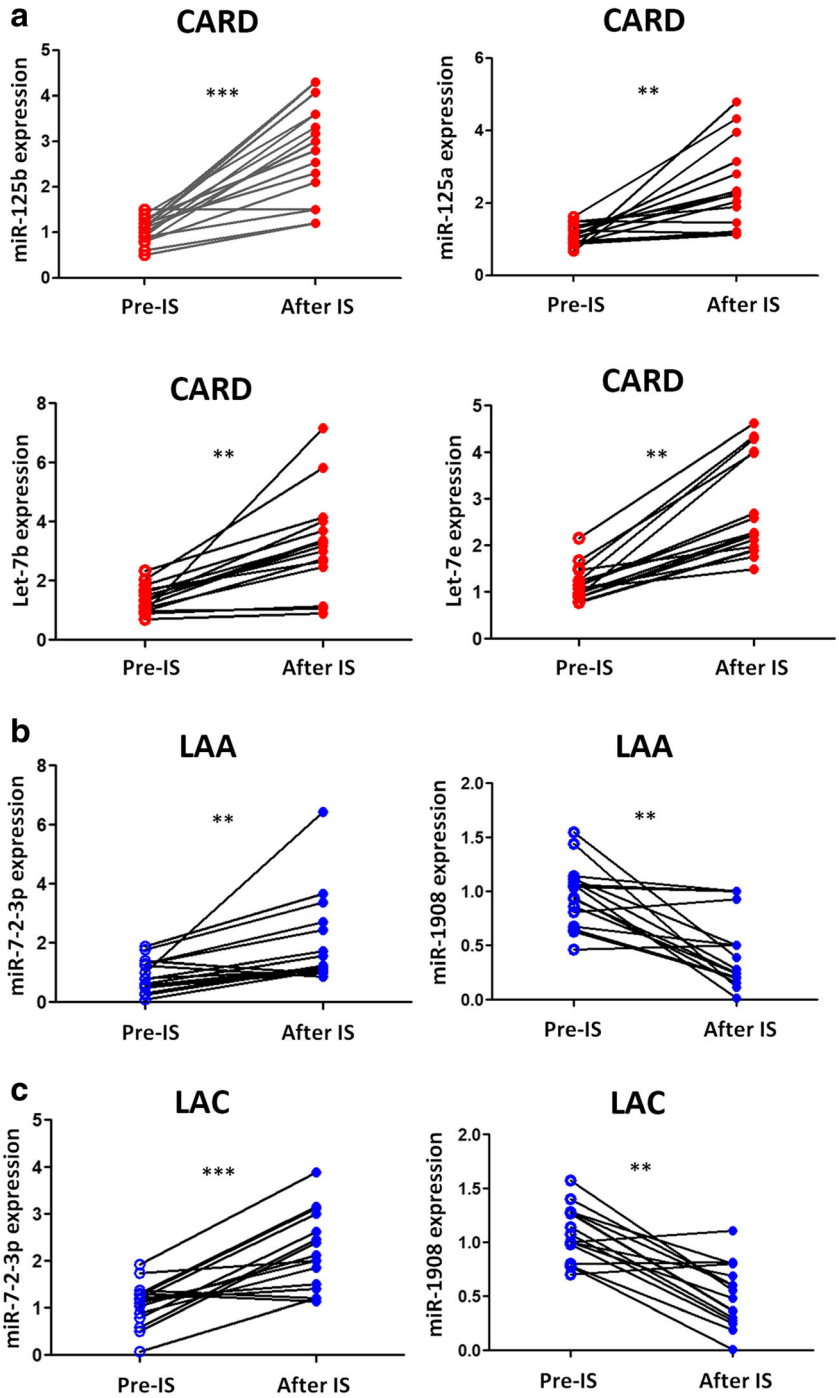

Fig. 2 Differential expressed serum microRNAs pre- vs. post-stroke. **a** miR-125b, miR-125a, let-7b, and let-7e were compared by real-time PCR in 16 CARD patients between pre-stroke and post-stroke. **b** miR-7-2-3p and miR-1908 expression levels in 16 LAA patients after stroke compared to that before stroke. **c** miR-7-2-3p and miR-1908 expression levels in 16 LAC patients after stroke compared to that before stroke

curves were performed by comparing with LAA and LAC patients vs. normal controls. Results showed that miR-7-2-3p and miR-1908 showed significant area-under-the-curve values in both LAA and LAC patients (Supplemental Fig. 3). Taken all together, these results strongly suggest circulating miRNAs could not only be of potential value as a diagnostic marker for different ischemic stroke subtypes, they further demonstrated specific dysregulated miRNAs contribute to different mechanism or etiologies for different ischemic stroke subtypes.

MicroRNAs specific signatures vary according to stroke type reflect potential biochemical status of the ischemic lesion and thereby function as a prognostic predictor of ischemic strokes. Ischemic stroke might impact on expression levels of circulating microRNAs through various simultaneously occurring pathophysiological processes, including platelet aggregation, endothelial dysfunction, neuronal and glial injury, as well as immunologic reactions [5, 18, 19, 30, 31]. For example, let-7c and miR-221-3p were found upregulated in

stroke patients and miR-523-3p were expressed more frequently among stroke patients [29]. miR-140-5p was also found to be upregulated in the blood of stroke patients [29]. In addition, miRNAs expression changes in response to middle cerebral artery occlusion and varies according to the duration of ischemia in rat brain tissues [7, 14, 20, 21, 23, 33]. Tan et al. [30] also found the differential miRNA expression profiles in the blood of young patients with ischemic stroke. Furthermore, miR-125a-5p and miR-125b-5p was shown to support endothelial barrier properties [28] and to the differentiation of inflammatory cells [26], miR-125b-5p further known as a regulator for synaptic morphology and function [8]. Interestingly, we found the expression levels of miR-125a and miR-125b specifically higher as expressed in patient with CARD IS, not LAA and LAC. Therefore, potential biological effects of miR-125a and miR-125b might have more sustained relevance after acute stroke of cardioembolism.

The expression levels of the target genes CASP3 and NLK, which were reduced in ischemic stroke patients, were further negatively correlated with let-7e-5p expression in stroke patients [12]. The let-7b expression level in the sera of patients with ischemic stroke was upregulated after the onset of IS symptoms [24]. Results from our current study further demonstrated that these two microRNAs, let-7b and let-7e, were highly expressed in patient with CARD IS, not LAA and LAC. The biogenesis of brain-enriched miR-7 is regulated post-transcriptionally, and inhibited by cell type-specific factors [6]. MiR-1908 was previously known to function as a glioblastoma oncogene by suppressing PTEN tumor suppressor pathway [32]. In our study, we found that miR-7-2-3p was highly expressed in LAA, LAC, and SUE, while not in CARD ischemic strokes, demonstrating that miR-7-2-3p was a potential growth and regulated risk factor in LAA, and LAC subtypes but not in CARD. MiR-1908 was found to be downregulated in LAA, LAC, and SUE, while not in CARD.

Our study also has limitations. First, the small sample size, and it is necessary to investigate the question in a larger group of patients whether miRNA profiling aids categorization of the different ischemic stroke subtypes with respect to etiology. Second, we did not perform any functional investigation of the identified miRNAs. Future experimental validation on miRNA-targets will be performed in both in vitro and in vivo animal model of ischemic strokes.

In conclusion, our findings strongly support the role of serum miRNAs in ischemic stroke subtypes. The results of this investigation may open avenues for more in-depth understanding of the pathogenesis of different ischemic stroke subtypes.

Author Contribution YXG, ZPX, TJ, LSZ, LLC, and BH performed the experiments and data analysis. FX, WL, and XYH collected the subject's samples and clinical data. All the authors contributed to the manuscript writing and revision.

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Compliance with Ethical Standards

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

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Written informed consent for participation in the study was obtained from either directly or from his or her guardian in all subjects and the work received approval from the institution ethics committee of Zhejiang University School of Medicine and in accordance with the tenets of the Declaration of Helsinki.

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