Potential microRNA biomarkers for acute ischemic stroke

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Abstract. Acute ischemic stroke is a significant cause of high morbidity and mortality in the aging population globally. However, current therapeutic strategies for acute ischemic stroke are limited. Atherosclerotic plaque is considered an independent risk factor for acute ischemic stroke. To identify biomarkers for carotid atheromatous plaque, bioinformatics analysis of the gene microarray data of plaque and intact tissue from individuals was performed. Differentially expressed genes (DEGs) were identified using the Multtest and Limma packages of R language, including 56 downregulated and 69 upregulated DEGs. Enriched microRNA (miRNA or miR) DEGs networks were generated using WebGestalt software and the STRING databases, and the miRNAs were validated using serum from acute ischemic stroke patients with reverse transcription quantitative PCR (RT-qPCR). Four confirmed differentially expressed miRNAs (miR-9, -22, -23 and -125) were associated with 28 upregulated DEGs, and 7 miRNAs (miR-9, -30, -33, -124, -181, -218 and -330) were associated with 25 downregulated DEGs. Gene ontology (GO) function suggested that the confirmed miRNA-targeted DEGs predominately associated with signal transduction, the circulatory system, biological adhesion, striated muscle contraction, wound healing and the immune system. The confirmed miRNA-targeted genes identified serve as potential therapeutic targets for acute ischemic stroke.

Introduction

Stroke is one of the most common causes of mortality and disability worldwide (1-3). Stroke is a clinical syndrome with pathological types including ischemic, intracerebral hemorrhage, and sub-arachnoid hemorrhage types (4,5). Stroke

risk increases with age (6). Ischemic stroke, which accounts for 87% of all stroke subtypes presented clinically (7), is prevalent in populations of European origin and Chinese populations (5,8,9). Acute ischemic stroke is the major cause of high morbidity and mortality in the aging population worldwide (10). The prognosis is worse for stroke, with up to 50%of the individuals worldwide confirmed to have suffered a stroke succumbing or being dependent on carers 6 months after the event (5). Current therapeutic strategies for acute ischemic stroke are limited (3). Intravenous thrombolysis with aspirin improves the outcome for only a small proportion of patients presenting early after symptom onset with acute ischemic stroke (11). Thus, seeking clinical diagnosis, prognostic biomarkers and therapeutic targets for ischemic stroke is indispensable. Approximately 50% of ischemic strokes are attributed to large-artery atherothrombotic disease, 25% to disease of the small intracranial arteries, 20% to cardiac emboli, and 5% to various rare causes (e.g., extracranial artery dissection) (4,5). The most common characteristic of acute ischemic stroke is an occlusion of the artery. Plaque composition is thought to be an independent risk factor for acute ischemic stroke (12).

The thickness of the fibrous cap, the size of the necrotic core and intraplaque hemorrhage, and the extent of inflammatory activity within the plaque are key factors in advanced plaque that are most likely to lead to complications (13). However, it was not possible to demonstrate how the complicated plaques associate with symptoms, since it appears that complicated plaques may occur at any time, irrespective of symptoms (14). Additionally, the exact mechanisms causing plaque rupture remain to be determined (15). Detection of vulnerable plaque is important for preventing future clinical events and remains to be resolved.

MicroRNAs (miRNAs or miRs) are non-coding RNAs that regulate gene expression by translation inhibition or messenger RNA degradation (16). miRNAs that are highly stable in serum (17) and urine (18) are potential biomarkers of pathology, such as stroke (19,20) and age-associated pathologies (21). The differential expression of miRNAs in peripheral blood of young ischemic stroke patients in Malaysia has been previously examined (22). However, the miRNAs involved in the formation and progression of atherosclerotic plaque have yet to be thoroughly investigated.

An intra-patient comparison on the transcript profile of atheromatous plaque and intact tissue samples was performed by Ayari and Bricca (23). However, those authors only analyzed

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Jp-/Downregulated Enriched miRNAs		Targets DEGs	
Downregulated	hsa_TGTTTAC, miR-30	LGI1 ACTC1 PRUNE2 NLGN1 GRIA2 FRK TTLL7 LPHN3 PCDH20	0.0006
	hsa_GTGGTGA, miR-197	NLGN1 GRIA2 TTLL7 LPHN3	0.0006
	hsa_AAGCCAT, miR-135	THRBILRRN1 FRK PRUNE2 PDE8B NEGR1	0.0029
	hsa_TGCCTTA, miR-124	RYR2ITHRBICNTN3IGRIA2ICNTN1INEGR1	0.0261
	hsa_TGAATGT, miR-181	THRBILRRN1 CNTN4 GRIA2 NEGR1	0.0563
	hsa_TGCTTTG, miR-330	PDZRN3IPCDH11YIFRKIPRUNE2	0.0609
	hsa_CAATGCA, miR-33	LGI1ICNTN4	0.0864
	hsa_TAGCTTT, miR-9	THRBICNTN4INEGR1	0.0864
	hsa_AAGCACA, miR-218	PLD5 GRIA2 FREM1 NPY1R	0.0864
	hsa_TGCAAAC, miR-452	PLCB4ITHRB	0.0915
Upregulated	hsa_AATGTGA, miR-23	PRDM1ICD163	0.1423
	hsa_ACCAAAG, miR-9	VAV3IPRDM1	0.1423
	hsa_GGCAGCT, miR-22	DPP4IAQP9	0.1423
	hsa_ACTGTGA, miR-27	VAV3IRGS1	0.1423
	hsa_CTTTGTA, miR-524	VAV3IPRDM1	0.1423
	hsa_CTCAGGG, miR-125	PRDM1IANPEP	0.1423

Table I. Top 10 enriched miRNA with target DEGs.

Top 10, 10 most significant categories; *Adj*.P, P-value from hypergeometric test adjusted by multiple test adjustment (Benjamini-Hochberg method); DEGs, differentially expressed genes; miRNA or miR, microRNA.

the expression of CD163 and heme oxygenase (decycling) 1 (HMOX1), which are involved in the homeostasis of iron and heme in atherosclerotic plaques (23).

In the present study, we aimed to identify which miRNAs were associated with the formation and progression of plaque, to investigate the potential mechanism of plaque vulnerability to rupture, and to examine potential biomarkers and therapeutic targets for acute ischemic stroke. The same microarray data as that used by Ayari and Bricca (23) were downloaded, and differentially expressed genes (DEGs) in paired atheromatous plaque and intact tissue samples were identified using the Multtest and Limma packages of R language. Enriched miRNAs that regulate the DEGs were then predicted using WebGestalt software. The expression of those predicted miRNAs in serum was confirmed using reverse transcription quantitative PCR (RT-qPCR). Protein-protein interactions (PPIs) of DEGs were constructed from STRING databases (http://string-db.org/), and confirmed miRNA-targeted DEGs interactions were visualized using Cytoscape software (http://www.cytoscape.org/). Gene ontology (GO) function and pathway analysis of confirmed miRNA-targeted DEGs were performed using the Bingo plug-in of Cytoscape software.

Materials and methods

Microarray data and data preprocessing. The gene microarray data GSE43292 (23) was downloaded from the Gene Expression Omnibus (GEO), which includes paired atheromatous plaque and intact tissue samples collected from 32 patients. Endarterectomy specimens were characterized

histologically according to the classification proposed by the Nomenclature Committee of the American Heart Association (24). The sections consisted of the intima and a majority of tunica media, although the tunica adventitia was excluded. Stage IV and later were considered as atheromatous plaques, and intact tissues were almost exclusively composed of stage I and II lesions (23). The platform of GSE43292 was GPL6244 (HuGene-1_0-st) Affymetrix Human Gene 1.0 ST Array [transcript (gene) version].

The mean expression of a total of 33,297 probes was derived from GEO, and accurate annotations were made. Probes matching >1 gene ID were discarded. All the probes were then mapped to Entrez gene IDs.

Screening of the DEGs. The log2 transformation of the expression value was performed for each probe (25). Up- and downregulated probes were determined according to their fold differences (26). DEGs were identified using the Multtest and Limma packages of R language (27). The false discovery rate (FDR) correction was calculated using the Benjamini- Hochberg (BH) method (FDR < 0.05) (28). A two-fold change method was used to identify DEGs (llog FC (Fold change)|> 1).

Prediction of enriched miRNAs in carotid atheroma. The WebGestal, a web-based gene set analysis toolkit, was used to predict miRNA associated with DEGs (29,30). The hypergeometric test was widely used to investigate the significance of functional terms enrichment within a list of genes. The 10 most significant categories (Top 10) and a minimum of two genes for a category were selected as the threshold.

Table II. DEGs in the confirmed miRNA-target DEGs interaction networks.

Gene symbol	Entrez ID	Adj.P	logFC	Gene name
CNTN1	1272	2.00E-05	-1.91103	Contactin 1
CNTN4	152330	1.70E-05	-1.79233	Contactin 4
CASQ2	845	1.91E-05	-1.66766	Calsequestrin 2 (cardiac muscle)
PCDH11Y	83259	2.55E-05	-1.38541	Protocadherin 11 Y-linked
CNTN3	5067	3.89E-05	-1.36174	Contactin 3 (plasmacytoma associated)
NPY1R	4886	8.70E-05	-1.27438	Neuropeptide Y receptor Y1
LRRN1	57633	2.85E-05	-1.27233	Leucine rich repeat neuronal 1
RYR2	6262	5.41E-05	-1.24144	Ryanodine receptor 2 (cardiac)
LGI1	9211	6.74E-05	-1.15644	Leucine-rich, glioma inactivated 1
PRUNE2	158471	3.53E-05	-1.11997	Prune homolog 2 (Drosophila)
PCDH20	64881	2.81E-05	-1.11425	Protocadherin 20
PLD5	200150	3.62E-05	-1.11138	Phospholipase D family, member 5
NEGR1	257194	1.97E-05	-1.10062	Neuronal growth regulator 1
GRIA2	2891	1.42E-03	-1.08966	Glutamate receptor, ionotropic, AMPA 2
NPR1	4881	8.88E-05	-1.07677	Natriuretic peptide receptor A/guanylate cyclase A
ACTC1	70	2.93E-04	-1.07237	Actin, α , cardiac muscle 1
PDZRN3	23024	2.55E-05	-1.07182	PDZ domain-containing ring finger 3
LPHN3	23284	3.67E-04	-1.06682	Latrophilin 3
PLN	5350	9.84E-05	-1.06111	Phospholamban
NLGN1	22871	6.43E-05	-1.03802	Neuroligin 1
THRB	7068	1.16E-04	-1.0368	Thyroid hormone receptor, β
TTLL7	79739	7.12E-05	-1.02607	Tubulin tyrosine ligase-like family, member 7
GRIA1	2890	3.08E-04	-1.02562	Glutamate receptor, ionotropic, AMPA 1
FRK	2444	4.00E-04	-1.01427	Fyn-related kinase
FREM1	158326	1.57E-04	-1.00531	FRAS1 related extracellular matrix 1
ACP5	54	4.63E-04	1.00142	Acid phosphatase 5, tartrate resistant
PRDM1	639	3.75E-05	1.005106	PR domain containing 1, with ZNF domain
CR1	1378	7.47E-04	1.011272	Complement component (3b/4b) receptor 1 (Knops blood group)
VAV3	10451	3.72E-05	1.026726	Vav 3 guanine nucleotide exchange factor
CXCL10	3627	5.23E-04	1.051734	Chemokine (C-X-C motif) ligand 10
NPL	80896	5.61E-05	1.066364	N-acetylneuraminate-pyruvate lyase
ITGAX	3687	1.91E-04	1.070598	Integrin, α X (complement component 3 receptor 4 subunit)
RGS1	5996	5.42E-03	1.094815	Regulator of G-protein signaling 1
CYTIP	9595	1.22E-04	1.099515	Cytohesin 1 interacting protein
PLEK	5341	4.59E-05	1.104737	Pleckstrin
CD163	9332	3.84E-05	1.130551	CD163 molecule
ADAMDEC1	27299	2.95E-03	1.138718	ADAM-like, decysin 1
SELE	6401	2.36E-04	1.157189	Selectin E
ANPEP	290	8.68E-05	1.173833	Alanyl (membrane) aminopeptidase
CCR1	1230	3.94E-05	1.179454	Chemokine (C-C motif) receptor 1
PLIN2	123	1.37E-04	1.18298	Perilipin 2
AQP9	366	1.82E-03	1.252998	Aquaporin 9
CD52	1043	1.35E-04	1.263187	CD52 molecule
CHI3L1	1116	4.86E-03	1.289639	Chitinase 3-like 1 (cartilage glycoprotein-39)
IL1RN	3557	5.61E-05	1.394489	Interleukin 1 receptor antagonist
HMOX1	3162	4.75E-05	1.419502	Heme oxygenase (decycling) 1
MME	4311	8.26E-05	1.485	Membrane metallo-endopeptidase
MMP12	4321	3.59E-03	1.574106	Matrix metallopeptidase 12
DPP4	1803	3.69E-05	1.610873	Dipeptidyl-peptidase 4
CD36	948	1.99E-04	1.802205	CD36 molecule (thrombospondin receptor)
MMP9	4318	1.86E-04	1.817804	Matrix metallopeptidase 9
MMP7	4316	5.72E-04	1.840231	Matrix metallopeptidase 7
FABP4	2167	2.39E-05	2.454461	Fatty acid binding protein 4, adipocyte

Adj.P, P-value from hypergeometric test adjusted by multiple test adjustment (Benjamini-Hochberg method); DEGs, differentially expressed genes; miRNA or miR, microRNA.

Patient samples and RNA isolation. Serum specimens were collected from 10 patients with acute ischemia stroke (average age of 69 ± 10) and 10 healthy individuals (no disease) who received a health check-up. There were no significant differences in age and gender distributions between the patients with acute ischemic stroke and healthy controls (P>0.05).

The acute ischemic stroke patients were associated with significant infract lesions. Serum samples prior to miRNA analysis were stored at -80°C within 4 h following collection. The present study adhered to the tenets of the Declaration of Helsinki and was approved by the Medical Ethics Committee of Sichuan University (Sichuan, China). Informed consent was obtained from participants or their close relatives for the use of their blood in this study.

RT-qPCR. Total RNA was extracted from serum samples according to a method described previously (31). Synthetic *C. elegans* miRNA (cel-miR-39; Qiagen, Hilden, Germany) was spiked in each extracted RNA at a final concentration of 4 fmol as an internal control. The concentration and quality of RNA were measured by UV absorbance at 260 and 280 nm (A260/280 ratio).

Ten nanograms of total RNA was reverse transcribed and qPCR was performed using an All-in-OneTM miRNA qRT-PCR Detection kit (GeneCopoeia, Rockville, MD, USA) according to the manufacturer's instructions. The validated All-in-One miRNA forward primers were purchased from GeneCopoeia. Forward primers specific for cel-miR-39 were designed based on a sequence of mature miRNA obtained from the miRbase (32). The levels of miRNAs were normalized with cel-miR-39. The 2^{- $\Delta\Delta$ CT} method, described by Livak *et al* (33), was used to analyze data. miR-9 in each serum sample and no-template controls were run in triplicate.

Statistical analysis. Statistical analysis was performed using the two-tailed paired Student's t-test using IBM SPSS 21.0 (SPSS, Inc., Chicago, IL, USA). Differences were considered statistically significant at P<0.05.

Confirmed miRNA-targeted gene interaction network. PPIs of DEGs were constructed from the STRING databases (34), and interactions associated with confirmed miRNAs were retained. miRNA-targeted DEGs interaction networks were visualized using the Cytoscape software version 3.1.1 (35).

Hierarchical clustering of expressional values of DEGs. Hierarchical clustering of expression data of the signature DEGs and the confirmed miRNA-targeted DEGs that were altered in atheromatous plaque was performed in a dChip Analyzer (36). The clustering algorithms with Euclidean distance (37) metric and centroid linkage rule were used (36).

GO function and pathway analysis of confirmed miRNAtargeted DEGs interaction. The Cytoscape software with plug-in Bingo was run to determine which biological processes are statistically overrepresented in the set of genes corresponding to the predicted miRNA-targeted DEGs interaction networks (38). The hypergeometric test was employed with a BH-FDR-based multiple testing correction (corr P<0.01).



Figure 1. The expression of identified serum microRNAs (miRNAs or miRs) in acute ischemic stroke. Compared to the healthy controls, the expression of serum miRNAs in patients with acute ischemic stroke was tested using reverse transcription quantitative PCR (RT-qPCR). *P<0.05.

Results

Screening of the DEGs. The expression value was derived for each probe, and an accurate annotation was made. DEGs were identified using the Multtest and Limma packages of R language. In the present study, 56 and 69 genes that were significantly differentially down- and upregulated, respectively, were identified (data not shown).

Prediction of enriched miRNAs. The top 10 miRNAs significantly associated with the screened up- and downregulated DEGs were predicted using the WebGestalt toolkit (Table I). Six miRNAs were connected with the upregulated DEGs including miR-9, -22, -23, -27, -125 and -524, and 10 miRNAs were connected with the downregulated DEGs including miR-9, -30, -33, -124, -135, -181, -197, -218, -330 and -452. A list of target DEGs associated with these miRNAs is provided in Table I. In the DEGs provided in Table II, the expression of vav 3 guanine nucleotide exchange factor (VAV3) and PR domain containing 1 (PRDM1) was 2.0-fold higher than that in the intact tissue, and the expression of β -thyroid hormone receptor (THRB) and contactin 4 (CNTN4) in atheromatous plaque was a notable 2.1- and 3.5-fold lower than that in the intact tissue, respectively. Notably, miR-9 was associated with both down- and upregulated DEGs.

Validation of predicted serum miRNA in acute ischemic stroke. The expression of predicted serum miRNAs in acute ischemic stroke was validated by RT-qPCR (Fig. 1). It was confirmed that the expression of serum miR-9, -30, -33, -124, -181, -218, -330, -22, -23 and -125 was significantly altered in ischemic stroke, indicating these miRNAs are important in acute ischemic stroke.

Confirmed miRNA-targeted DEGs interaction networks and important DEG nodes. PPIs of DGEs were constructed from the STRING databases. Interactions associated with confirmed miRNAs were retained, and confirmed miRNA-targeted DEGs interaction networks were subsequently visualized



Figure 2. Confirmed microRNA (miRNA or miR)-targeted differentially expressed gene (DEG) interaction networks. Protein-protein interactions (PPIs) of (A) downregulated and (B) upregulated DEGs regulated by miRNAs. The nodes of miRNAs were emphasized in different shapes. Arrows show the associated DEGs.

using Cytoscape software (Fig. 2). Four miRNAs were linked to 25 downregulated DEGs (Fig. 2A) and 7 miRNAs were linked to 28 upregulated DEGs (Fig. 2B) forming miRNAs-DEGs interaction networks. Table II shows the DEGs in the interaction networks. The most important nodes in the miRNA interaction networks were downregulated DEGs including THRB, CNTN4, NEGR1 and glutamate receptor 2 (GRIA2) (Fig. 1A), and upregulated DEGs including VAV3, PRDM1, CD163, matrix metallopeptidase 9 (MMP9), pleckstrin (PLEK), CD36, chemokine (C-X-C motif) ligand 10 (CXCL10), chemokine (C-C motif) receptor 1 (CCR1), regulator of G-protein signaling 1 (RGS1), HMOX1, tartrate resistant acid phosphatase 5 (ACP5), dipeptidyl peptidase 4 (DPP4) and selectin E (SELE) (Fig. 2B).

Hierarchical clustering of expressional values of DEGs. Using the algorithm based on Euclidean distance metric and centroid linkage rule, the hierarchical clustering of expressional values of DEGs and the confirmed miRNA-targeted DEGs were divided into the atheromatous plaque and intact tissue groups (Fig. 3). Fig. 3 shows atheromatous plaque and intact tissue samples separated with the exception of 13 paired samples that were not well classified. There were 10 intact tissues clustered in the atheromatous plaque group, and three atheromatous plaques in the intact tissue group, indicating that the differences in gene expression between intact tissues and atheromatous plaques from those patients are extremely close and difficult to distinguish, and that there was a high risk of atheromatous in the 10 patients. Nevertheless, two clustering maps showed identical resampling, suggesting that the confirmed miRNA-targeted DEGs are important representative genes in the interaction networks, and that screening is extremely significant.

GO function and pathway analysis of confirmed miRNAtargeted DEG interaction. GO function suggested that the



confirmed miRNAs targeting downregulated DEGs were predominately associated with signal transduction, the circulatory system, biological adhesion and striated muscle contraction, and that the targeted upregulated DEGs were most significantly associated with signal transduction, wound healing and the immune system (Table III).



-3.0 -2.7 -2.4 -2.1 -1.9 -1.6 -1.3 -1.0 -0.7 -0.4 -0.1 0.1 0.4 0.7 1.0 1.3 1.6 1.9 2.1 2.4 2.7 3.0

Figure 3. Hierarchical clustering of gene expression profiles. Groups of atheromatous plaque (P1-P32) and intact tissues (C1-C32) are represented by (A) the vertical rows and signatures of differentially expressed genes (DEGs), and (B) the microRNA (miRNA)-target DEGs are represented horizontally. On the bottom of the clustering image is the color scale, showing the standardized expression values, since the expression levels for each gene are standardized to have a mean 0 and standard deviation 1. Red, the expression level above the mean expression lower than the mean. The top hierarchical break distinguished a group of atheromatous plaque from the group of intact tissues.

Discussion

The predominant etiology of stroke is atherosclerosis (atheroma) (39). The present study provides a list of potential clinical diagnoses and prognostic biomarkers and therapeutic targets for acute ischemic stroke via the identification of a list of DEGs associated with miRNA targets, the validation of expression of predicted miRNAs in acute ischemic stroke, and the GO function analysis of the confirmed miRNAs targeted DEGs in the advanced plaque using the microarray data from Ayari and Bricca (23). Furthermore, a new molecular mechanism causing atherosclerotic plaque rupture and intraplaque hemorrhage, and subsequently ischemic stroke remains to be determined.

Whole-transcript expression profile of human atherosclerotic arteries (endarterectomy specimens) was performed by Ayari and Bricca (23). As it is impossible to obtain normal artery tissue, the intra-patient comparison on the expression profile of atheromatous plaque and intact tissues from the individual was carried out to examine the atherogenic process per se (23). Ayari and Bricca analyzed the expression of two genes (CD163 and HMOX1) involved in the homeostasis of

Table III. GO enrichment analysis of confirmed miRNAs and targeted DEGs interaction networks.

Downregulated DEGs

GO-ID	Description	Genes in test set
44057	Regulation of system process	PLNINLGN1IRYR2INPR1ICNTN4INPY1RILGI1
8015	Blood circulation	ACTC1IPLNIRYR2INPR1INPY1R
3013	Circulatory system process	ACTC1IPLNIRYR2INPR1INPY1R
7155	Cell adhesion	PCDH11YIFREM1PCDH20INLGN1ICNTN1ICNTN4ICNTN3INEGR1
22610	Biological adhesion	PCDH11YIFREM1IPCDH20INLGN1ICNTN1ICNTN4ICNTN3INEGR1
6941	Striated muscle contraction	ACTC1IRYR2ICASQ2

Upregulated DEGs

GO-ID	Description	Genes in test set
9611	Response to wounding	CR1 CD36 PLEK HMOX1 CCR1 IL1RN MME SELE MMP12 CD163 CXCL10
2376	Immune system process	CR1 RGS1 PLEK AQP9 CCR1 MMP9 IL1RN ACP5 SELE DPP4 CXCL10
6950	Response to stress	CR1 CD36 PLEK AQP9 HMOX1 CCR1 IL1RN MME ACP5 SELE MMP12 DPP4 CD163 CXCL10
6954	Inflammatory response	CR1 HMOX1 CCR1 IL1RN SELE CD163 CXCL10
7229	Integrin-mediated signaling pathway	VAV3 PLEK ITGAX ADAMDEC1
48583	Regulation of response to stimulus	CR1 PLEK HMOX1 FABP4 ACP5 SELE DPP4 CXCL10
42221	Response to chemical stimulus	PLIN2 AQP9 HMOX1 CCR1 IL1RN FABP4 MME ACP5
	-	SELE MMP12 DPP4 CXCL10
30155	Regulation of cell adhesion	CD36 VAV3 CYTIP ADAMDEC1 DPP4
6952	Defense response	CR1 HMOX1 CCR1 IL1RN ACP5 SELE CD163 CXCL10
32101	Regulation of response to external stimulus	PLEK FABP4 ACP5 SELE CXCL10
42060	Wound healing	CD36 PLEK HMOX1 MME MMP12
60054	Positive regulation of epithelial cell proliferation involved in wound healing	MME MMP12
2685	Regulation of leukocyte migration	HMOX1 SELE CXCL10
34383	Low-density lipoprotein particle clearance	CD36 HMOX1
50896	Response to stimulus	CR1 AQP9 PLEK CCR1 IL1RN ACP5 MME MMP12 CD163 CXCL10 CD36 RGS1 PLIN2 HMOX1 FABP4 SELE DPP4
15718	Monocarboxylic acid transport	CD36 PLIN2 AQP9
48518	Positive regulation of biological process	CR1 CD36 VAV3 PLEK HMOX1 MMP9 FABP4 MME PRDM1 SELE MMP12 DPP4 CXCL10
42493	Response to drug	PLIN2 AQP9 FABP4 MME MMP12
6955	Immune response	CR1 RGS1 AQP9 CCR1 IL1RN ACP5 CXCL10
10033	Response to organic substance	PLIN2 AQP9 HMOX1 IL1RN FABP4 ACP5 SELE CXCL10
8284	Positive regulation of cell proliferation	HMOX1 FABP4 MME MMP12 DPP4 CXCL10

GO, gene ontology; DEG, differentially expressed genes; miRNA, microRNA. corr P<0.01.

iron and heme, and their role in atheromatous plaques (23). In the present study, we identified 56 downregulated DEGs and 69 upregulated DEGs (including CD163 and HMOX1). A 2-fold change with FDR<0.05 was set as the threshold criterion. Using these DEGs, we screened the associated Top 10 miRNA. Six miRNAs were linked to the upregulated DEGs including miR-9, -22, -23, -27, -125 and -524, and 10 miRNAs were linked to the downregulated DEGs including miR-9, -30, -33,

-124, -135, -181, -197, -218, -330 and -452 (Table I). Of these miRNAs, miR-33 and miR-125 were found to associate with hyperlipidemia (19). Notably, miR-9 is an important miRNA associated with down- and upregulated DEGs. The significant change in expression of predicted serum miRNAs (miR-9, -30, -33, -124, -181, -218, -330, -22, -23 and -125) in acute ischemic stroke was validated by RT-qPCR. The results confirmed that miRNAs may be important in acute ischemic stroke.

PPIs of DGEs were constructed from the STRING databases, and any interactions that were not associated with any miRNA targets were discarded, such as the interaction between calponin 1 (CNN1) and myocardin (MYOCD), which played crucial roles in cardiogenesis and differentiation of the smooth muscle cell lineage (40), as well as the interaction between tryptophan 2,3-dioxygenase (TDO2) and kynureninase (KYNU), which were involved in tryptophan metabolism (41). Interactions associated with confirmed miRNAs were retained, and the confirmed miRNA-targeted DEGs interaction networks were constructed. In total, 25 downregulated DEGs and 28 upregulated DEGs were involved in the miRNAs-DEGs interaction networks.

In the present study, the intact tissues were type I and II lesions, and the atheromatous plaque was type IV lesion and later lesion according to the definition and classification suggested by the Nomenclature Committee of the American Heart Association (24). Of note, type I and II lesions may be combined under the term early lesions, and type IV-VI lesions combined under the term advanced lesions. The early lesions generally are those that occur in infants and children, although they also occur in adults (24). Following the early lesions onset, advanced lesions may subsequently evolve following thickening of the arterial wall and therefore narrowing of the lumen or obstruction or modification of the blood flow. Thus, common gene expression in early and advanced lesions from some individuals may be identical. This hypothesis was supported by the hierarchical clustering maps which did not separate all the endarterectomy specimens into intact tissue and atheromatous plaque groups. Nevertheless, two clustering maps showed identical resampling, indicating that the miRNA-target DEGs were important representative genes in the interaction networks, and that screening is essential.

In the confirmed miRNAs-targeted DEGs interaction networks, the most important nodes identified were THRB, CNTN4, NEGR1 and GRIA2 in the downregulated network, and VAV3, PRDM1, CD163, MMP9, PLEK, CD36, CXCL10, CCR1, RGS1, HMOX1, ACP5, DPP4 and SELE in the upregulated network. Of note, the miR-9 target DEGs including THRB, CNTN4, NEGR1, VAV3 and PRDM1 were important nodes in the miRNAs-target DEGs interaction networks, suggesting that miR-9 plays a central role in the atheroma. The important nodes were enriched in the GO term.

Ischemic stroke occurs when an artery is obstructed (19), which is most often caused by atheroma. Atheroma (atherosclerosis), which is also known as the type VI lesion, is characterized by larger, confluent and more disruptive core of extracellular lipid (24). Almost 70% of plaque specimens (carotid endarterectomy specimens) demonstrated fissures, hematoma or hemorrhage, and/or thrombus, 64% demonstrated neovascularization (14,24). Arteries with thinned or ruptured fibrous caps, intraplaque hemorrhage, larger lipid-rich necrotic cores and larger wall thickness were associated with the occurrence of subsequent clinical events, especially the stroke (19,42). In addition, recurrent stroke is associated with a higher incidence of large-artery atherosclerosis than the first stroke (19). The exact mechanisms causing plaque are not yet completely known (15). The plaques that are rich in soft extracellular lipid (43), neoformed vessels and inflammatory infiltration (44) are vulnerable. The fibrous cap at the site of the rupture/erosion had an eroded surface characterized by loss of the endothelial lining (45). As demonstrated in GO annotations: CD36 was associated with lipid storage (GO: 19915); and HOMOX1, CCR1, SELE, CD163 and CXCL10 were associated with the inflammatory response (GO: 6950). miR-9 targeted downregulated DEGs including CNTN4, NEGR1 and THRB were predominately associated with biological adhesion and regulation of the neurological system, and targeted upregulated DEGs including VAV3 and PRDM1 were most significantly associated with signal transduction and vasculature development, showing a crucial role of miR-9 in the vulnerability of plaque. By contrast, the miRNA expression profile in young stroke patients (18-49 years) have not shown a difference in miR-9 (22). Our results supported that miR-9 is a potential biomarker for acute ischemic stroke. We also observed significant changes in miR-30, -33, -124, -181, -218, -330, -22, -23 and -125 expression. However, further investigation focusing on their role in acute ischemic stroke should be conducted. Furthermore, investigation into the molecular mechanism underlying the formation and rupture of atherosclerotic plaque may provide a new therapeutic strategy for the prevention, diagnosis, treatment and prognosis of ischemic strokes.

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