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Original Article

Unique MicroRNAs Signature of Lymphocyte of Yang and Yin Syndromes in Acute Ischemic Stroke Patients*

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ABSTRACT **Objective**: To identify the differentially expressed microRNAs (miRNAs) profiles of yang and yin syndromes in patients with acute ischemic stroke, and to provide the molecular basis of the classification of these two syndrome types in acute ischemic stroke patients. **Methods**: A microarray assay was performed to assess the expression pattern of miRNAs in the lymphocyte of acute ischemic stroke patients. Target genes for the deregulated miRNAs were predicated using the online bioinformatic algorithms and functional annotation via Kyoto encyclopedia of genes and genomes pathway analysis for miRNAs predicted targets was carried out. Based on the predicted target genes of differentially expressed miRNAs, the miRNA-gene-network and miRNA-pathway-network were constructed. **Results**: Yang score based on tongue texture, urine, dejecta, and appearance, etc. showed that clinical symptoms were distinct between yang and yin syndromes. There were significantly higher total leukocyte number and lower total protein level in patients with yang syndrome compared with those in patients with yin syndrome ($P<0.05$). Comprehensive miRNA analysis identified 36 unique down-regulated miRNAs in yang syndrome group, and 20 unique down-regulated and 2 unique up-regulated miRNAs in yin syndrome group. The key regulatory miRNAs, gene, and pathways in the yang syndrome were hsa-miR-93-5p and -320b, enabled homolog, the metabolic pathways and mitogen-activated protein kinase signaling pathways, respectively, while those in the yin syndrome were hsa-miR-424-5p and -106b-5p, CNOT4, hepatitis B and pathways in cancer, respectively. **Conclusion**: These results offered insight into the molecular basis underlying the different pathogenesis of yang or yin syndrome, providing clues for the individualized therapeutic strategies of acute ischemic stroke.

KEYWORDS acute ischemic stroke, yang syndrome, yin syndrome, microRNAs profile, diagnosis, Chinese medicine

Acute ischemic stroke is a major public health problem throughout the world.(1) Thrombolysis or thrombectomy aimed at reopening the occluded blood vessel are currently used in clinic for treatment of the disease. However, the limited time window and reperfusion injury after the treatments remain challenges for clinician. The development of other evidence-based treatment for acute ischemic stroke is obstructed by our less understanding of the ischemic stroke etiology and pathology. A variety of risk factors for stroke are recognized, including atherosclerosis, hypertension, diabetes mellitus, hyperlipidemia, and age, among others.(2) These risk factors, together with post-stroke pathological changes, such as oxidative stress, inflammation, endoplasmic reticulum stress, apoptosis and autophagy,(3) make the mechanisms too complicated to find suitable target for the treatment.

The yin-yang theory of Chinese medicine (CM) is a way to describe the etiology and pathology of diseases, as well as the property of CM,(4) which links the diagnosis and treatment.(5) The main discrimination between the yin and yang syndromes has been characterized in acute ischemic stroke in a multicenter trial.(6) Moreover, it was found that this type of classification associated with the clinical characteristics especially the post-stroke inflammation of patients, showing that yang syndrome in

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acute ischemic stroke is correlated with serum cytokines levels interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), pituitariuno-adrenal axis hormones and cholecystokinin-8.(7-9) Although yang and yin syndromes have been used in China to evaluate prognosis and guide the therapy of acute ischemic stroke patients,(10-12) the scientific basis for this application remains to be elucidated.

Protein biomarkers from blood (serum/plasma) have been widely used for diagnosis and prognosis in clinic.(13) Recently, nucleic acids in peripheral blood samples have proven to be an alternative useful biomarker in the assessment of stroke pathogenesis, and microRNA (miRNA)-induced gene expression contributes extensively to the disease phenotype.(14,15) In the present study, the difference of miRNAs expression patterns in yin and yang syndromes of acute ischemic stroke patients was assessed, and the biological significance of this difference was further investigated.

METHODS

Inclusion and Exclusion Criteria

Male patients aged 50–60 years old with first-ever ischemic stroke and admission within 6 h after symptom onset were included, who met "China guideline for the diagnosis and treatment of acute cerebral ischemic stroke in 2010".(16) The patients excluded from the study were those who were with transient ischemic attack, cerebral hemorrhage, or subarachnoid hemorrhage; tumor or hematologic disease confirmed by auxiliary examination; severe diseases of heart, kidney, blood, endocrinium or osteoarthrosis; suffering infections; having psychosis or severe dementia that influenced nervous system functional assessment.

Participants

Twenty male acute ischemic stroke patients with first-ever ischemic stroke as diagnosed by computed tomography or magnetic resonance imaging, as well as clinical findings, were recruited between March of 2010 and June of 2010 from the Department of Neurology, Xuanwu Hospital of Capital Medical University. Two healthy men were recruited from Medical Examination Center of Xuanwu Hospital as normal control who matched with research group by age with no atherosclerosis, normal hepatic and renal function, blood glucose and fat level. This study was approved by the Ethical Committee of Xuanwu Hospital. Written informed consent forms were obtained from all participants.

Diagnostic Criteria of CM

It was conducted referring to standard for diagnosis and therapeutic effect evaluation of stroke and diagnosis standard for yang syndrome of stroke.(17,18) According to the yang and yin score (Table 1),(18) patients whose score ⩾7 were assigned to the yang group (10 cases) and patients whose score<7 were assigned to the yin group (10 cases).

Table 1. Symptoms of Yang and Yin (Score)

Observation item	Performance and score
Tongue texture	Red (5), purple-red (6)
Fur	Thin and yellow (2), thick and yellow (3), dry (4), grey-black and dry (5)
Feelings in mouth	Bitter taste of mouth and dry pharynx (1), thirsty and cool drink (2)
Concentrated and yellow urine	No (0), yes (1)
Dejecta	Dry stool and dyschezia (2), dry stool and no dejecta for 3 days (3), dry stool and no dejecta for 5 days (4)
Appearance	Upset and tantrum (2), irritable and inquietude (3), coma and delirium (4)
Face and breath	Hypso-voice, pykno-breath or dry-red lips (2), flushing, conjunctival congestion or tachypnea, halitosis (3)
Fever	Yes (3), no (0)
Pulse tracings	Rapid, great and powerful pulse or wiry and rapid pulse or slippery and rapid pulse (2)

Clinical Data and Blood Sample Collection

The National Institute of Health Stroke Scale (NIHSS), Modified Rankin Scale (mRS), Barthel Index (BI)(19) and yang scores were evaluated. Blood was collected from each patient for assessment of routine blood, fasting blood-glucose, glycosylated hemoglobin, homocysteine, blood clotting tetrachoric, platelet aggregation rate, C-reactive protein, and erythrocyte sedimentation rate.

miRNAs Microarray Assay

Lymphocytes were isolated from peripheral blood of each subject, from which RNAs were separated using Trizol reagent (Invitrogen, USA), and processed for miRNA microarray analysis using Agilent Human miRNA Microarray Rel 12.0 (Agilent Technologies, USA). A total of 851 homo sapiens-miRNAs were screened.

Differentially Expressed Genes and Prediction of Targets

The random variance model corrected *t*-test

(RVM-T test) was applied to filter the differentially expressed genes for the healthy control and 2 groups of patients. After the significance analysis and false discovery rate (FDR) analysis, the differentially expressed genes were selected according to the *P* value threshold. Hierarchical clustering was carried out using correlation distance as the distance metric and average linkage between clusters. Principal component analysis was performed using covariance for the dispersion matrix and normalized scaling. Targetscan (http://www.targetscan.org), miRanda (http://www.microRNA.org) and miRBase target prediction database (http://www.ebi.ac.uk/enrightsrv/microcosm) were used to determine the predicted target genes, and the intersection of 3 database prediction was collected. The intersection genes between the target genes and the different expressing genes analyzed by multiClassDif analysis, and corresponding miRNAs were analyzed in the next step.

Pathway Analysis, miRNAs-Gene-Network and miRNAs-Pathway-Network

The overlapping targets predicted by the 3 databases were subjected to Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis. Based on KEGG, pathway analysis was performed by the Fisher's exact test and chi-square test, and the threshold of significance was defined by *P* value and FDR. The selection criterion for significant KEGG pathway terms was *P* value <0.01.

On the basis of the attributes of the intersecting target genes and miRNAs, the miRNA-Gene-Network representing the critical miRNAs and their targets were established according to the miRNA degree (i.e., the contribution of one miRNA to the genes around or the contribution of one gene to the miRNAs around it). The key miRNA and gene in the network always have the largest degrees. In the miRNA-Pathway-Network, the circle represented gene and the square represented miRNA, and their relationship was represented by one edge. The center of the network was represented by degree. The key miRNAs in the network had the highest degrees.

Statistical Analysis

Data were presented as mean ± standard deviation ($\bar{x}\pm s$). SPSS 16.0 software (SPSS Inc., USA) was used for the statistical analyses. Statistical significance between groups was determined by unpaired *t*-test. All tests of statistical significance were 2-sided and $P<0.05$ was considered significant difference.

RESULTS

Comparison of Clinical Data between Yang and Yin Syndromes

To exclude the influence of severity of infarction and cerebrovascular disease risk factors on differentially expressed miRNAs profiles between yang and yin syndrome groups of acute ischemic stroke, only those patients were enrolled who showed no significant difference in NIHSS score, age, blood pressure, blood glucose and blood lipid between the two groups (Table 2). The yang and yin scores

Table 2. Comparison of Clinical Data between Yang and Yin Syndrome Groups ($\bar{x}\pm s$)

Index	Yang group (10 cases)	Yin group (10 cases)	*P* value
NIHSS (Score)	8.0±0.8	6.0±1.4	0.34
Age (Year)	56±4	53±2	0.62
Blood pressure (mm Hg)	143±11	140±13	0.51
Leucocyte (10^9/L)	9.28±0.95	6.68±1.14	0.04
Neutrophil (%)	72.00±11.26	67.83±2.90	0.57
Monocyte (%)	5.60±1.31	4.80±1.81	0.57
Lymphocyte (%)	21.97±9.84	24.33±2.32	0.71
Homocysteic acid (μ mol/L)	35.93±21.44	22.20±8.24	0.36
C reactive protein (mg/dL)	1.29±1.24	0.43±0.51	0.33
Total protein (g/L)	62.23±3.11	71.03±2.09	0.01
Globulin (g/L)	22.38±5.28	28.01±2.58	0.17
Albumin (g/L)	39.52±2.88	43.02±1.97	0.16
Prealbumin (mg/L)	2.56±26.10	2.48±47.35	0.82
Blood sedimentation (mm/h)	13.67±9.30	7.33±6.81	0.40
Hemoglobin (g/L)	146.00±13.53	159.67±11.68	0.26
Glycosylated hemoglobin (%)	5.50±0.40	6.13±0.25	0.08
Blood glucose (mmol/L)	5.73±1.32	6.54±2.70	0.67
Prothrombin time (s)	13.90±1.49	13.53±0.60	0.71
Activated partial thromboplastin time (s)	33.00±0.36	37.70±6.45	0.28
International normalized ratio	1.05±0.06	1.01±0.15	0.66
Platelet aggregation (%)	42.27±34.68	47.13±18.01	0.84
Fibrinogen (g/L)	2.97±0.85	3.18±1.36	0.83
Triglyceride (mmol/L)	1.98±0.90	2.40±1.66	0.72
Total cholesterol (mmol/L)	4.80±0.56	5.09±1.06	0.70
High-density lipoprotein (mmol/L)	1.24±0.25	1.11±0.13	0.47
Low-density lipoprotein (mmol/L)	2.51±0.42	2.72±0.82	0.71
ApoA (g/L)	0.90±0.12	0.88±0.16	0.91
ApoB (g/L)	0.86±0.21	1.04±0.49	0.59

were significantly higher in the yang syndrome group than that in the yin syndrome group (7.86±0.69 *vs.* 3.25±1.06, *P*<0.01). The result of blood biochemical examination is listed in Table 2. Compared with the yin syndrome group, the total leukocyte number in the yang syndrome group was significantly higher, while the level of total protein was significantly lower (*P*<0.05).

Differentially Expressed miRNAs in Lymphocyte of Yang and Yin Syndromes

Among the 851 homo sapiens-miRNAs, 105 were found detectable. Principal component analysis using 3 principal components, which describe 91.64% of the variance, showed miRNAs to be significantly altered across the 3 groups (Figure 1A). Moreover, hierarchical clustering of the miRNAs revealed 8 distinct clusters of the 3 groups. The dendrogram generated by the cluster analysis showed a clear separation among yang syndrome, yin syndrome and normal control groups (Figure 1B). Further comparative analysis of differentially expressed miRNAs found that 54 miRNAs significantly changed in yang syndrome group as compared with normal control group, of which 18 changed as well as in yin syndrome group, and 36 unique down-regulated in yang syndrome group (Figure 1C and Appendix 1). Similarly, 44 miRNAs markedly changed in yin syndrome group as compared with normal control group, of which 22 changed as well in yang syndrome group, while 20 unique down-regulated and 2 unique up-regulated in yin syndrome group (Figure 1D and Appendix 2).

Different KEGG Pathway in Yang and Yin Syndromes

Based on the predicted target genes of miRNAs that participate in signaling pathways, 11 and 25 significant pathways were specifically targeted by down-regulated miRNA in yang and yin syndrome patients, respectively (Figure 2A), and the 83 common

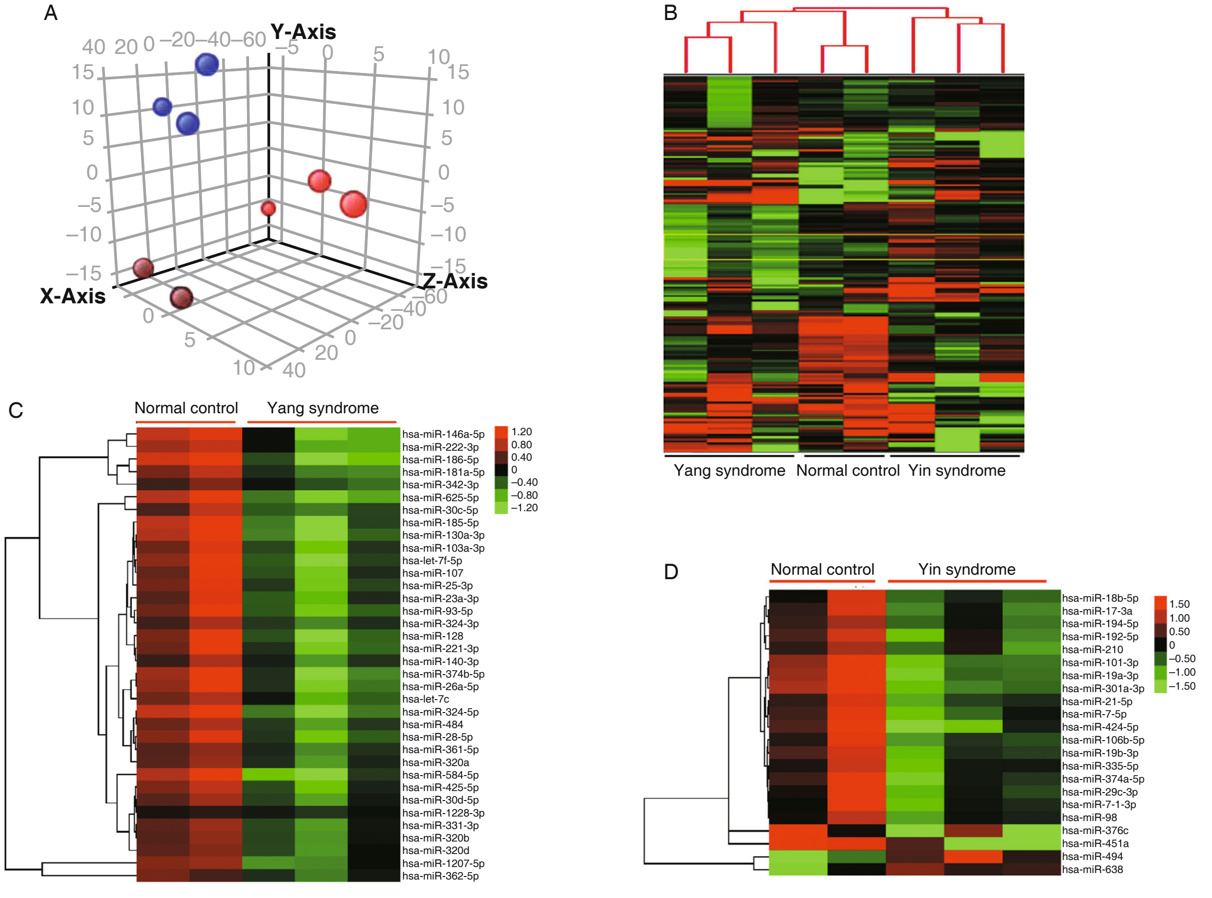

Figure 1. Cluster Analysis and Principal Component Analysis of Yang and Yin Syndrome Groups and Normal Control Group

Notes: (A) Principal component analysis. Brown, normal controls; red, yang syndrome group; blue, yin syndrome group. (B) Hierarchical clustering of total miRNAs. (C) Clustering analysis of 36 differentially expressed unique miRNAs in yang group compared with normal control group. (D) Clustering analysis of 22 differentially expressed unique miRNAs in yin group compared with normal control group. Each row represents a miRNA and each column represents a sample. Green: downregulation; red: upregulation.

significant pathways were divided into 4 groups based on their function (Figures 2B–D). Eleven specific pathways targeted by down-regulated miRNAs of yang syndrome group were involved in viral carcinogenesis, metabolic pathways, mRNA surveillance pathway, cell adhesion molecules (CAMs), etc. Twenty-five specific pathways targeted by down-regulated miRNAs of yin syndrome group were respectively related to biosynthesis and decomposition such as protein digestion and absorption, adipocytokine signaling pathway, long-term potentiation, and pathways involved in inflammation such as extracellular matrix (ECM)-receptor interaction, B cell receptor signaling pathway, as well as ion balance-regulated pathways such as aldosterone-regulated sodium reabsorption, endocrine and other factor-regulated calcium reabsorption, mineral absorption, and proximal tubule bicarbonate reclamation (Figure 2A).

Except for the unique pathways, it is worth noting that the neural function-related pathways implicated in axon guidance, cholinergic synapse, GABAergic synapse, and morphine addiction were influenced to different degree in the yang and yin syndromes (Figure 2B). In addition, the cell function-related pathways including focal adhesion, adherens junction, vasopressin-regulated water reabsorption and ubiquitin mediated proteolysis were also regulated to different degree in the yang and yin syndromes (Figure 2C). The modified cellular signaling pathways that targeted by down-regulated miRNAs in the yang and yin syndrome groups also included ErbB, phosphatidylinositol 3-kinase (PI3K)-protein kinase B (Akt), retrograde endocannabinoid, Wnt, and hypoxia-inducible factor 1 (HIF-1) signaling pathway (Figure 2D).

miRNA-Gene-Network in Yang and Yin Syndromes

The high degree of connectivity between the miRNA-mRNA pairs suggests that these miRNAs play critical roles during stroke progression. The key miRNAs in the network of yang syndrome group were identified as hsa-miR-93-5p, miR-181a-5p, miR-320b, miR-320a, miR-107, miR-103a-3p, miR-130a-3p, and miR-23a-3p (Appendixes 3A and 4) with the top four key mRNAs being enabled homolog

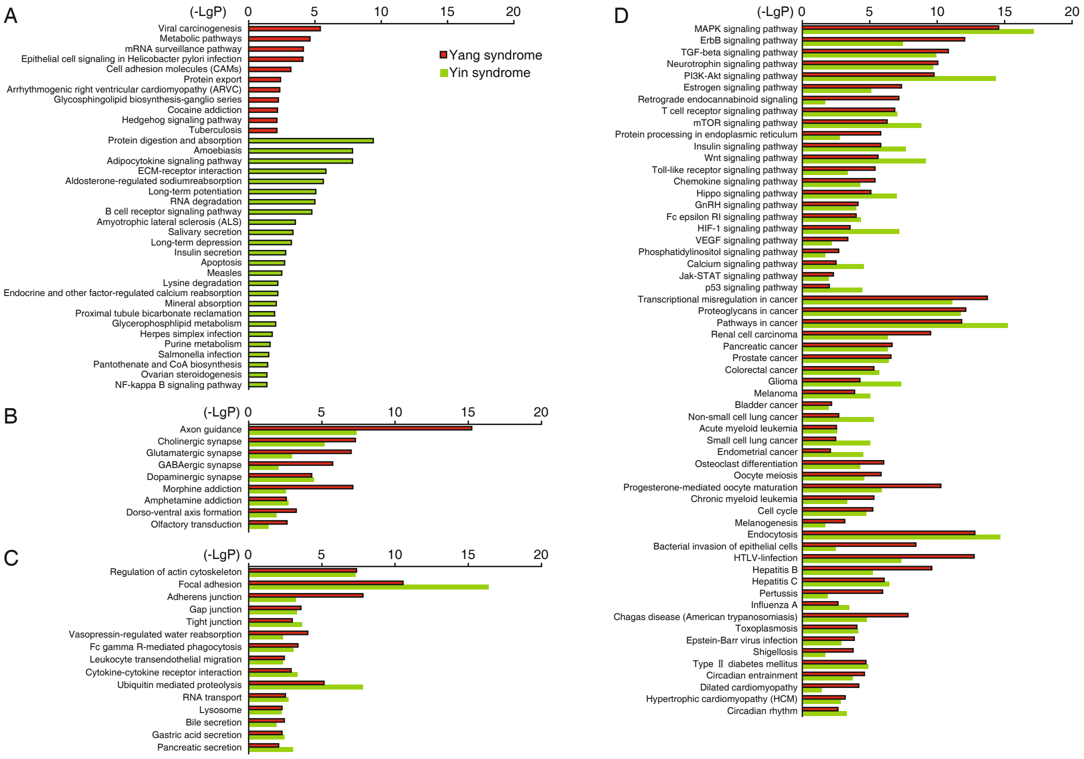

Figure 2. Pathway Analysis Based on miRNA-Targeted Genes of Yang and Yin Syndromes of Acute Ischemic Stroke Patients

Notes: The vertical axis is the pathway category and the horizontal axis is the -LgP of pathway; LgP is the logarithm of *P*-value, and *P*<0.01 is considered significant. (A) 11/25 significant pathways targeted by downregulated miRNA in yang/yin syndrome group. (B–D) 83 common significant pathways are divided into 4 groups according to their function including neural function-related pathways (B), cell function-related pathway (C), cellular signaling pathways and diseases-related pathways (D). The vertical axis is the pathway category, and the horizontal axis is the significance of pathways.

[ENAH (Drosophila)], SEMA3A [sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A], ACVR2B (activin A receptor, type ⅡB), and MYBL1 [v-myb myeloblastosis viral oncogene homolog (avian)-like 1] (Appendix 4). The key miRNAs in the network of yin syndrome group were identified as hsa-miR-424-5p, miR-106b-5p, miR-301a-3p, and miR-29c-3p, etc. (Appendixes 3B and 5) with the key pathways listed in the lower part of Appendix 5.

miRNAs-Pathway-Network in Yang and Yin Syndromes

The five key miRNAs in the network of yang syndrome group were identified to be hsa-miR-93-5p, miR-320b, miR-320a, miR-128, and miR-181a-5p (Appendixes 6A and 7), most of them overlapped the key miRNAs in miRNA-Gene-Network. The key pathways included metabolic pathways, mitogen-activated protein kinase (MAPK) signaling pathway, pathways in cancer, human T-lymphotropic virus type-Ⅰ (HTLV-Ⅰ) infection, PI3K-Akt signaling pathway, regulation of actin cytoskeleton, endocytosis, glutamatergic synapse, and transforming growth factor-beta (TGF-β) signaling pathway (Appendix 7). Five key miRNAs in the network of yin syndrome group were identified to be hsa-miR-424-5p, miR-106b-5p, miR-7-5p, miR-19a-3p, and miR-301a-3p (Appendixes 6B and 8), with all overlapping the key miRNAs in miRNA-Gene-Network. The identified key pathways included hepatitis B, pathways in cancer, endocytosis, focal adhesion, insulin signaling pathway, MAPK signaling pathway, PI3K-Akt signaling pathway, TGF-β signaling pathway, and transcriptional misregulation in cancer (Appendix 8).

DISCUSSION

Classification of ischemic stroke into yang and yin syndromes has been used as a guideline in CM for treatment of the disease in clinic. The present study, based on examination of tongue texture, urine, dejecta, and appearance, etc., revealed that the acute ischemic stroke patients with yang and yin syndromes have different blood biochemical parameters with significant higher total leukocyte number and lower total protein in patients with yang syndrome than in patients with yin syndrome, implying a relatively stranger inflammation reaction occurring in the former case. Interestingly, the yang and yin syndromes exhibited distinctly expressed profile of miRNAs, genes and signaling pathways, providing molecular basis for the classification of the two types of acute stroke patients.

miRNAs play important regulatory roles in cell proliferation, apoptosis, development and differentiation, and different sets of expressed miRNAs are found in different cell types and tissues. Recent studies have investigated the profile of miRNAs in cerebrospinal fluid and blood plasma of ischemic stroke patients(20) and in brain microvascular endothelium of middle cerebral artery occlusion animal model.(21) The present study revealed a differentially expressed miRNAs profile in lymphocytes of acute ischemic stroke patients with both yang and yin syndromes, as compared with control participants. The identified differentially expressed miRNAs in the yang syndrome have been reported to play roles in some conditions. For example, miR-93 promotes the malignant phenotypes of human glioma cells and induces their chemoresistance to temozolomide.(22) miR-181a promotes proliferation and inhibits apoptosis by suppressing CFIm25 in osteosarcoma.(23) Hsa-miR-320b was found to be a possible regulator of human-specific neural development,(24) and a potential biomarker for individual progressive multifocal leukoencephalopathy risk assessment.(25) miR-107 was implicated in protection of excitatory neurotoxicity(26) and glioma angiogenesis.(27) Given the function of miR-93 and miR-181a in promoting cell proliferation, their downregualtion in lymphocyte in yang syndrome patients might contribute to the reduced percentage of lymphocyte compared with yin syndrome patients, as demonstrated in our result. Likewise, the top five differentially expressed miRNAs in the yin syndrome have been found to be involved in some disorders. To this end, hsa-miR-424-5p was identified to alleviate oxidative injury in neurons or suppress microglia-related inflammation following stroke.(28,29) Has-miR-106-5p may serve as potential diagnostic biomarkers for ischemic stroke,(30) promote neuronal and neuroendocrine differentiation of neural crest cells,(31) implicate in berberine-treated multiple myeloma cells.(32) Since miR-424-5p and miR-106-5p could inhibit cell proliferation,(28,33) their downregulation in lymphocyte could upregulate the number of lymphocyte in yin syndrome patients compared with yang syndrome patients, which is consistent with our result. Taken together, the differential miRNA expression might contribute to the distinct number of total leukocyte in stroke patients with yang and yin syndrome. Although some of the known functions are suggestive, the significance of the differentially expressed mRNAs in yang and yin syndromes remains elusive.

Further clue regarding the significance of the present finding comes from the analysis of pathways of all the miRNA-targeted genes. The eleven specific pathways targeted by down-regulated miRNAs of yang syndrome include metabolic pathways, mRNA surveillance pathway, CAMs, etc. The down-regulated miRNAs-targeted 25 specific pathways of yin syndrome are related to metabolism including protein digestion and absorption, adipocytokine signaling, as well as related to pathways involved in inflammation including ECM-receptor interaction and B cell receptor signaling pathway. It was demonstrated that changes in the immune system happened in both syndromes, however, different immune-related pathways were stimulated in two syndromes, the CAMs pathway B cell receptor signaling pathway were activated respectively in yang and yin syndromes. In addition, the differential pathways related to ion balance such as aldosterone-regulated sodium reabsorption, endocrine and other factor-regulated calcium reabsorption, and proximal tubule bicarbonate reclamation suggested that the renal function of the two syndromes is different, which need further identification. Moreover, pathways related to protein biosynthesis and decomposition including protein digestion and absorption, protein export, lysine degradation is also implicated, which might contribute to the significantly different of plasma total protein levels of yang and yin syndromes. These results highly suggest that it is the distinct metabolic pathways and immune related pathways that lead to the differences in yang and yin score and clinical biochemical index. The links between these pathways and clinical manifestation need further investigation.

In summary, the classification of acute ischemic stroke as yang and yin syndromes in CM is based on the experience and lacks sufficient scientific evidence and thus has not been fully accepted by modern Western medicine. The present study showed a deferentially expressed miRNAs profiles and pathway in yang and yin syndromes, providing scientific evidence for the theory of yin-yang in acute ischemic stroke. In addition, the methodology used in the present study may potentially be developed as strategies for diagnosis and treatment of stroke that are specifically tailored for yang and yin syndromes patients.

Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contributions

Zhao HP participated in the design and coordination of the study, created the figures and drafted the manuscript; Liu P, Xu CM and Li GW helped collect clinical samples, created the figures and drafted the manuscript; Gao L and Luo YM participated in the design and coordination of the study. All authors read and approved the final manuscript.

Electronic Supplementary Material: Supplementary materials (Appendixes 1–8) are available in the online version of this article at http://dx.doi.org/10.1007/s11655-018-2843-3

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