

Circulating mRNA and microRNA profiling analysis in patients with ischemic stroke

SUJUAN SUN, LITAO LI, LIPENG DONG, JINMING CHENG, CONGYING ZHAO, CHU BAO and HEBU WANG

Department of Neurology, Hebei General Hospital, Shijiazhuang, Hebei 050050, P.R. China

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Abstract. To provide insight into molecular diagnosis and individualized treatment of ischemic stroke (IS), several available datasets in IS were analyzed to identify the differentially expressed genes and microRNAs (miRNAs). Series matrix files from GSE22255 and GSE16561 (mRNA profiles), as well as GSE110993 (miRNA profile) were downloaded from the Gene Expression Omnibus database. System-level clustering was performed with GeneCluster 3.0 software, and gene annotation and pathway enrichment were performed with gene ontology analysis and Database for Annotation, Visualization and Integrated Discovery software. For a protein-protein interaction (PPI) network, Biological General Repository for Interaction Datasets and IntAct interaction information were integrated to determine the interaction of differentially expressed genes. The selected miRNA candidates were imported into the TargetScan, miRDB and miRecords databases for the prediction of target genes. The present study identified 128 upregulated and 231 downregulated genes in female stroke patients, and 604 upregulated and 337 downregulated genes in male stroke patients compared with sex- and age-matched controls. The construction of a PPI network demonstrated that male stroke patients exhibited YWHAE, CUL3 and JUN as network center nodes, and in female patients CYLD, FOS and PIK3R1 interactions were the strongest. Notably, these interactions are mainly involved in immune inflammatory response, apoptosis and other biological pathways, such as blood coagulation. Female and male upregulated genes were cross-validated with another set of Illumina HumanRef-8 v3.0 expression beadchip (GSE16561). Functional item association networks, gene function networks and transcriptional regulatory networks were successfully constructed, and the relationships between miRNAs and target genes were successfully predicted. The present study identified a number of transcription factors, including DEFA1, PDK4,

SDPR, TCN1 and MMP9, and miRNAs, including miRNA (miR)-21, miR-143/145, miR-125-5p and miR-122, which may serve important roles in the development of cerebral stroke and may be important molecular indicators for the treatment of IS.

Introduction

Ischemic stroke (IS), which is a major cause of disability and mortality worldwide, is a cerebrovascular disease caused by cerebral blood supply disorder or hemorrhage and results in the formation of an infarction zone with tissue necrosis and softening (1,2). It can lead to the severe impairment of sensory and motor dysfunction, neurodegenerative diseases and sudden mortality (3). Cerebral infarction not only seriously affects the life quality of patients, but also imposes a huge burden and pressure on public health care and the social economy (4). A previous study reported that the impact caused by IS will become more apparent in the next two decades as the world's population ages and lifestyles change, especially in developing countries (5). An epidemiological study has showed that >70% of patients with IS currently have mental and physical functional disorders (6). Tissue plasminogen activator is the only thrombolytic drug approved by the US Food and Drug Administration for the treatment of acute cerebral infarction, but its clinical application is limited by the timing of stenosis (onset) and side effects such as bleeding (7,8). A number of other previous studies have reported that transient cerebral ischemia/hypoxia can lead to neuronal death, but the mechanism is not fully understood (9,10). Therefore, further understanding of the pathogenesis of cerebral infarction, finding biomarkers that can be used to diagnose and predict prognosis, and eliciting new therapeutic targets have become a significant and urgent task.

MicroRNAs (miRNAs) are highly conserved, endogenous non-coding single-stranded small RNAs that are ~22-24 nucleotides long and can bind to target mRNAs with a complementary nucleic acid sequence. In general, miRNAs act post-transcriptionally to inhibit the translation of the target mRNAs or degrade the target mRNA (11). Each miRNA can regulate the expression of multiple target genes, and the same gene can also be regulated by several different miRNAs (12). Currently, >2,000 human miRNAs have been identified, and it is hypothesized that these small molecules regulate about one-third of human genes (13). Therefore, miRNA changes can lead to various diseases. A number of studies have demonstrated that miRNAs serve important biological roles in cell

Correspondence to: Professor Hebo Wang, Department of Neurology, Hebei General Hospital, 348 Hepingxi Road, Shijiazhuang, Hebei 050050, P.R. China
E-mail: wanghbhope@hebmh.edu.cn

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growth, proliferation, differentiation and apoptosis, as well as reconstruction of hematopoietic and damaged tissues (14,15). Therefore, the role of miRNAs in neurological diseases has attracted increasing attention. In the nervous system, miRNAs are closely related to the growth and differentiation of neurons, memory and learning functions, as well as neurological degenerative diseases and mental disorders (16,17). In addition, abnormal miRNA expression is detected in brain tumors, Parkinson's disease, Alzheimer's disease and IS (18-20).

Studies on the identification of target genes for miRNAs associated with cerebral infarction suggest that miRNAs play a key role in the development and progression of cerebral infarction (21,22). Among them, apoptosis is the central link of this process, and related studies have indicated that apoptosis is a highly regulated and controlled death process induced by miRNAs (23). In the present study, bioinformatics analysis was performed to investigate the putative genes and miRNAs involved in the pathogenesis and disease process of IS, and the mechanism was studied at the molecular level, which provided a reference for molecular diagnosis and individualized treatment of IS.

Materials and methods

Microarray data. Two mRNA microarray datasets, GSE22255 and GSE16561, and one miRNA profile, GSE110993, of IS were obtained from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo>). GSE22255 is based on the GPL570 (HG-U133_Plus_2) Affymetrix Human Genome U133 Plus 2.0 Array. Gene expression profiling was performed on peripheral blood mononuclear cells (PBMCs) of 20 patients with IS (10 male; 10 female) and 20 sex- and age-matched healthy control (CTL) patients using Affymetrix microarrays. GSE16561 contained a total of 63 samples based on the platform of GPL6883 Illumina HumanRef-8 v3.0 expression beadchip, in which peripheral blood samples were collected in Paxgene Blood RNA tubes from patients who were >18 years of age with MRI diagnosed IS (n=39; 22 females and 17 males) and CTL (n=24; 14 females and 10 males) who were non-stroke neurologically healthy. As for GSE110993, RNA sequencing results were analyzed to study expression changes of circulating miRNAs in a discovery sample of 20 patients (10 males and 10 females) with IS and 20 sex- and age-matched healthy control (CTLs) patients was further applied in independent samples for validation (40 patients with IS and 40 CTL patients) and replication (200 patients with IS, 100 CTLs).

Identification of differentially expressed genes (DEGs). Background correction and quartile data normalization of the downloaded data were performed using the robust multi-array average algorithm (24). Principal component analysis was subsequently conducted to reduce the dimension of multivariate data to two or three principal components, which can be visualized graphically with the least information loss. We used R software to calculate and visualize the principal component analysis (25). Probes without a corresponding gene symbol were then filtered and the average value of gene symbols with multiple probes was calculated using R software (26). DEGs between two groups were screened by Student's t-test and

fold-change (FC) filtering with R software limma package (27). With a threshold of $P < 0.05$ and $|FC| > 2$, volcano plots were constructed to identify the DEGs with statistical significance between two groups by R software ggplot2 package (data not shown) (28). In addition, hierarchical clustering and combined analyses were performed for the DEGs. The GSE16561 dataset was used to validate top 20 of DEGs in the GSE22255 dataset to identify overlapping DEGs in two microarrays.

Protein-Protein Interaction (PPI) network construction and analysis. The PPI network was created by integrating Biological General Repository for Interaction Datasets (BioGRID) and IntAct databases at EMBL-EBI. First, the PPI pairs were used to construct the PPI network. Second, the regulatory relationship between genes was visualized by using Cytoscape (version 3.4.0) (29), and analyzed through a topological property of computing network including the degree distribution of network by using the CentiScape app version 2.2 (30).

Functional enrichment analysis. The Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics resource (<http://david.abcc.ncifcrf.gov>) was used to conduct functional enrichment analysis to understand the underlying biological processes and pathways of aberrantly expressed intersecting genes.

Gene Ontology (GO) terms for the biological process (BP) were enriched. The most enriched term within a cluster was chosen as the one to represent the cluster. Only terms with $P < 0.05$ and the concentrated gene numbers ≥ 1.5 were considered meaningful (≥ 1 gene number exists).

Prediction of miRNA targets. The target genes for differentially expressed (DE) miRNAs in GSE110993 were predicted using three established programs: TargetScan version 7.2.0 (31), miRTarBase version 7.0 (32) and miRDB version 5.03 (33), which are the most popular databases of experimentally validated miRNA interactions. The genes that were predicted by all three programs were chosen as the targets of DE miRNAs and the miRNA-gene network was constructed using miRWalk 2.0 (34). Cytoscape (<https://cytoscape.org>; version 3.4.0) was used to visualize the miRNA regulatory network.

Results

Identification of differentially expressed genes in female and male IS patients. A total of 10 female and 10 male patients with IS, and 20 sex- and age-matched respective CTLs from the data in the GEO database (GSE22255) were clustered and divided into four groups (Fig. 1A): i) PBMCs_IS_M group, ii) PBMCs_IS_F group, iii) PBMCs_CTL_M group and iv) PBMCs_CTL_F group, as depicted in Fig. 1A. Principle component analysis of genes in patients with IS and CTL is presented in Fig. 1B. Following data preprocessing, the unique genes in the four groups of samples were analyzed and it was determined that a total of 1,300 genes were differentially expressed in the IS samples compared with the CTL samples. Among these DEGs, 128 genes were upregulated in females, 603 genes were upregulated in males, 232 were genes downregulated in females and 337 genes were downregulated in

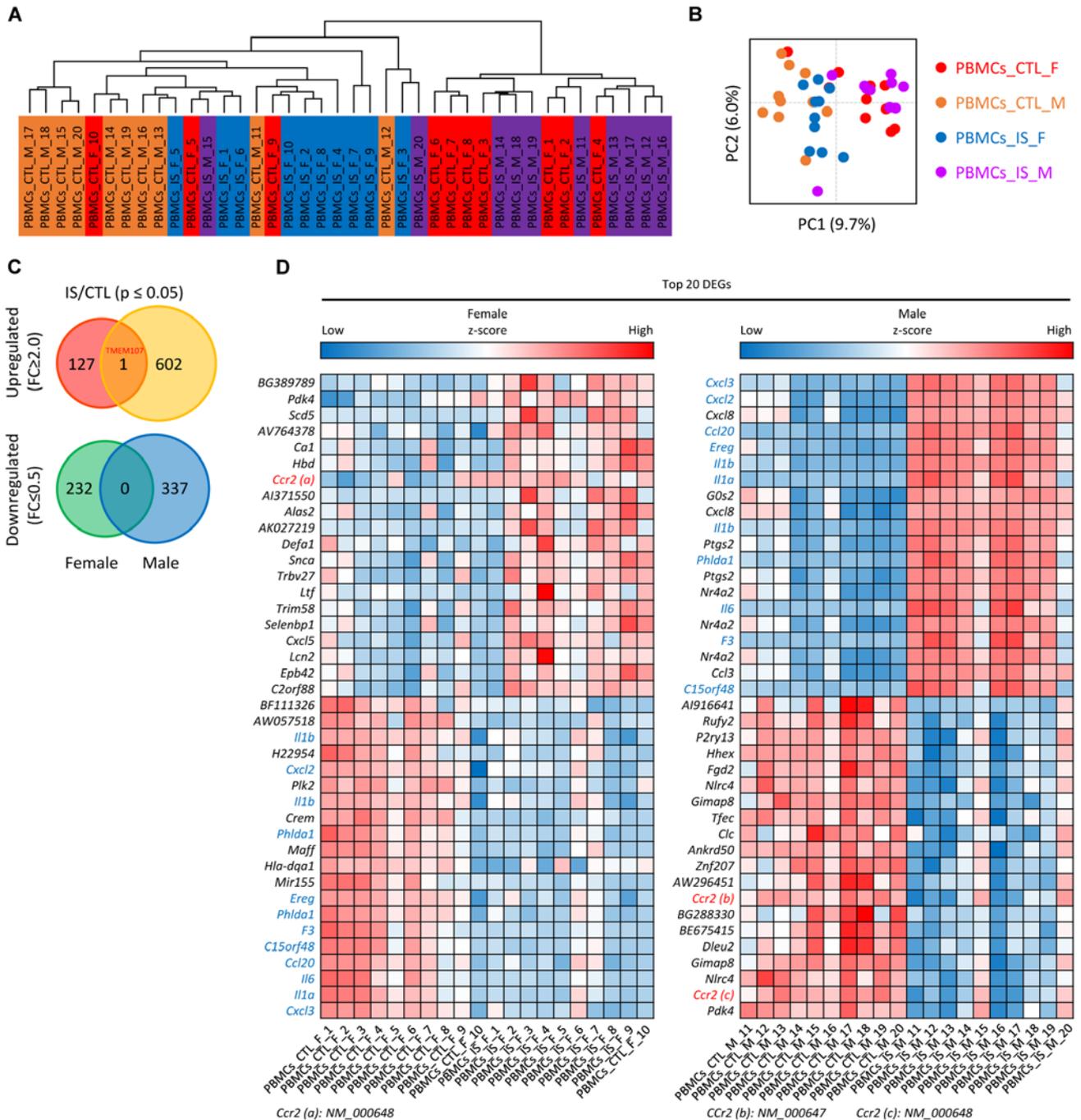


Figure 1. Identification of DEGs in female and male patients with IS. (A) Bidirectional hierarchical clustering and (B) principal component analysis of samples based on differentially expressed mRNAs. (C) Venn diagram of differentially expressed mRNAs from IS/CTL group. (D) Heatmap showing the expression levels of the top 20 mRNAs in the female (left) and male (right) dataset. The expression levels are from blue (low expression) to red (high expression). CTL, control; DEG, differentially expressed genes; F, female; FC, fold-change; IS, ischemic stroke; M, male; PBMC, peripheral blood mononuclear cells.

males. For instance, it was found that the chemokines C-X-C motif chemokine (CXCL)3, CXCL2 and CXCL8 were significantly upregulated in male IS patients. However, TMEM107 was the only overlapped upregulated DEG between the female and male group, which is displayed in a Venn diagram (Fig. 1C). A heat map of the top 20 up- and downregulated DEGs in male and female groups is shown in Fig. 1D.

Functional and pathway enrichment analyses. DAVID was used to perform GO term enrichment analyses to explore the function and pathways of 1,300 identified DEGs (Fig. 2).

GO-BP term enrichment analysis demonstrated that the upregulated genes were significantly enriched in ‘blood coagulation’ and ‘inflammatory response’ in the female IS compared with the CTL group. In addition, ‘positive regulation of transcription from RNA polymerase II promoter’ and ‘neutrophil chemotaxis’ were enriched in the male IS compared with the CTL group (Fig. 2A). Downregulated genes were mainly involved in ‘positive regulation of transcription’ and ‘immune response’ in the female IS group (Fig. 2B). It was also revealed that most of the downregulated genes were involved in ‘inflammatory response’ and ‘protein ubiquitination’ in the male IS group,

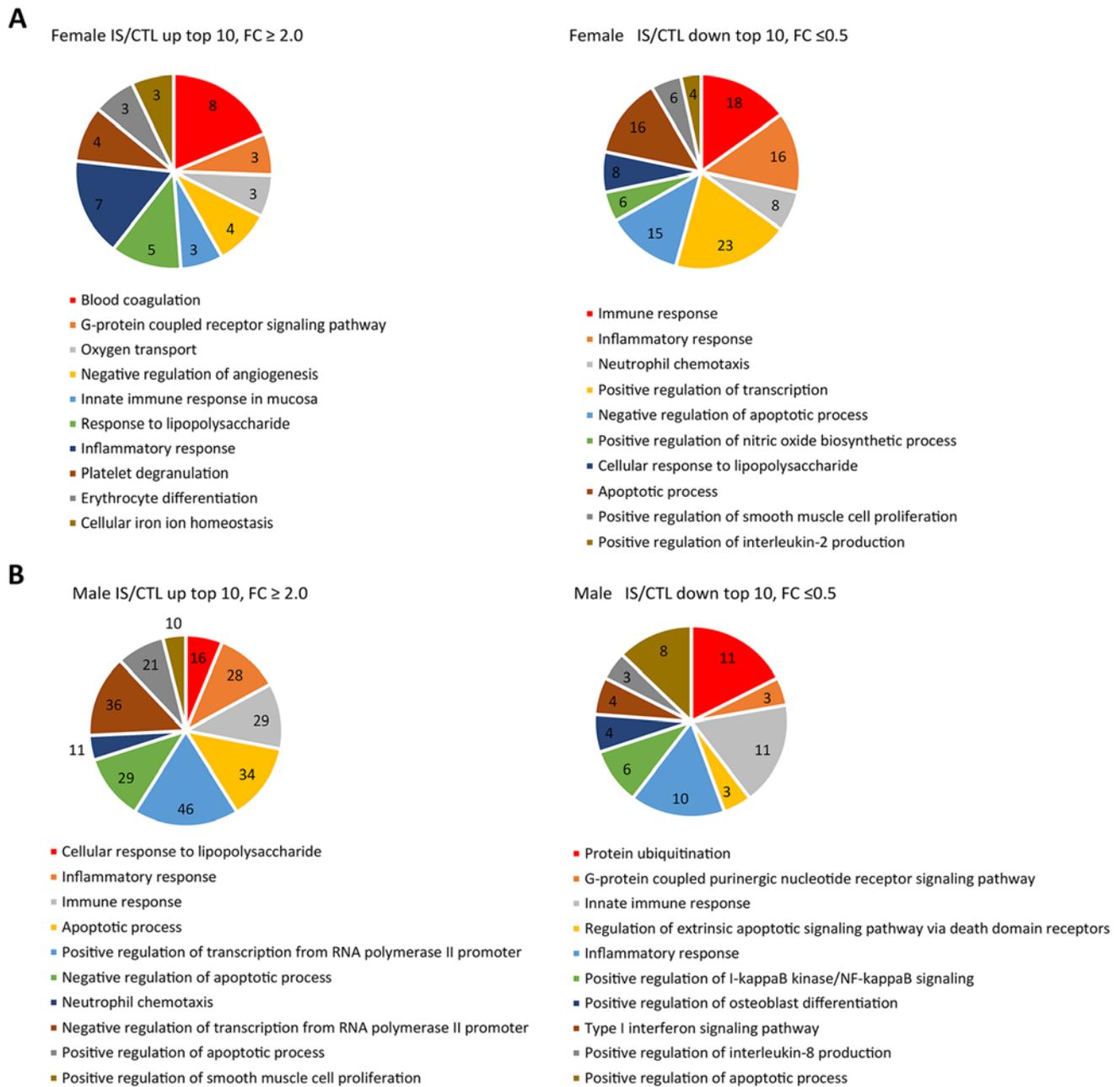


Figure 2. Gene Ontology biological function term enrichment analyses from IS compared with the CTL group in the (A) female and (B) male patients. The numbers in the pie slices represent the number of differentially expressed genes involved in the function. CTL, control; IS, ischemic stroke.

respectively. Of note, in both the male and female groups, the pathways of differential expression were mainly involved in ‘immune response’, ‘inflammatory response’, ‘apoptotic process’ and ‘neutrophil chemotaxis’.

Construction of PPI network and module analysis. To investigate the associations between the DEGs and BPs, a comprehensive analysis of protein interactions of the top up- and downregulated DEGs was conducted using BioGRID, IntAct database and Cytoscape software (Fig. 3). Direct or indirect interactions were identified, and the highest-scoring nodes were screened as central genes in the female IS/CTL group, including FOS, CLU, LTF, PF4, CXCL5 and CYLD (Fig. 3). In the male IS/CTL group, the top 3 genes with the most significant expression were CUL3, YWHAE and

JUN (Fig. 4). The associations between BP and gene expression were categorized into 4 processes, namely inflammatory response, immune response, apoptotic process and neutrophil chemotaxis.

miRNA target regulatory network analysis. DE miRNAs obtained from GSE110993 database were analyzed. For the female IS/CTL group, six DE miRNAs were identified, including let-7f-5p, let-7i-5p, miR-17-5p, miR-20a-5p, miR-19b-3p and miR-205-5p (Fig. 5). In the male IS/CTL group, there were 11 DE miRNA identified, such as miR-205-5p, miR-17-5p, miR-20a-5p, miR-125a-5p, miR-92a-3p, miR-18a-5p and miR-143-3p (Fig. 6). Their target genes were predicted using TargetScan, miRTarBase and miRDB. The upregulated targets were STRADB, PDK4, SCD5, SNCA, RARA and SHBGRL2

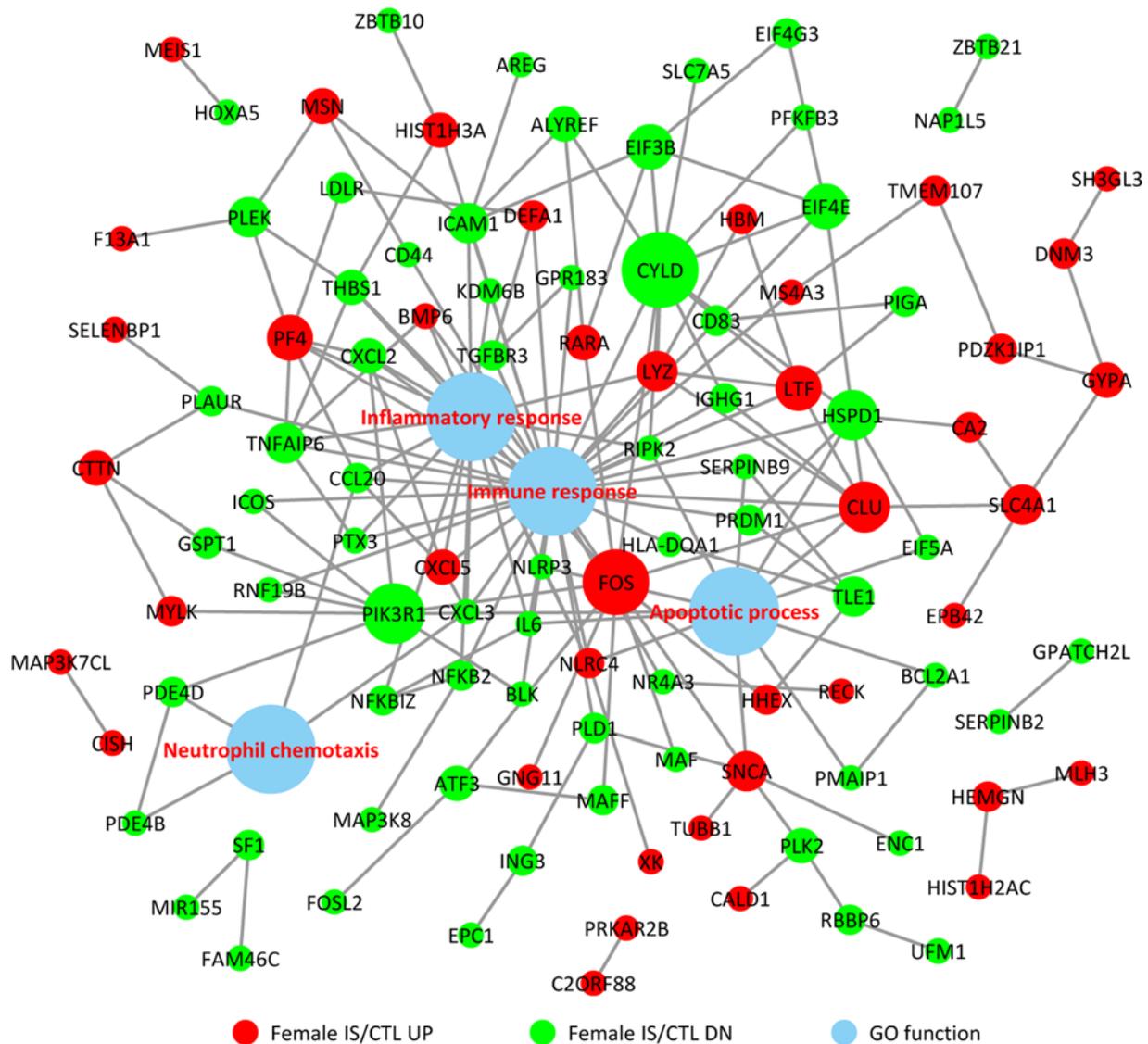


Figure 3. Protein-protein interaction of the upregulated and downregulated genes in female patients with IS and their association with immune response, inflammatory response, apoptotic process and neutrophil chemotaxis. CTL, control; DN, downregulated; GO, Gene Ontology; IS, ischemic stroke; UP, upregulated.

in the female IS group (solid red circles in Fig. 5); the down-regulated target genes are also indicated, including TGFBR3, ETNK1, LDLR, RORA and RBBP6 (solid green circles in Fig. 5). The targets of miRNAs for the male IS/CTL group are shown in Fig. 6 (solid green circles represent downregulated target genes, such as RAB11FIP1, C5ORF51, GAB1 and SMAD5, and red circles represented upregulated target genes such as PPP1R15B, ELK4, MXD1 and LDLR).

Validation of DEGs in IS. Crosscheck validation analysis of female and male IS patient DEGs from GSE22255 and another set of Illumina HumanRef-8 v3.0 expression beadchip (GSE16561) are shown in Fig. 7. There were 4 genes that are upregulated in both datasets in the female group including defensin α 1 (DEFA1), pyruvate dehydrogenase kinase 4 (PDK4), caveolae associated protein 2 (SDPR) and transcobalamin 1 (TCN1) and only one shared upregulated gene in the male group, matrix metalloproteinase 9 (MMP9). These findings suggested that the differential expression targets

selected had certain repeatability. Although numerous studies have been published regarding DEGs as candidate biomarkers for IS (35-37), the reliability of these findings remains uncertain, as they were generated from the studies without sufficient evidence of reproducibility and independent clinical validation.

Discussion

As a high-throughput genomics research technology, gene chips serve an important role in datamining of potential biological information and exploring the structure and function of the genome (38). Gene expression profiling experiments are mainly used to determine DEGs under different physiological or pathological conditions (39). Such analysis will facilitate the exploration of the relationship between health and disease at the molecular level and the potential mechanism through network regulation. However, the etiology and physiological pathology of IS are still not fully understood. Therefore, further clarifying the pathogenesis of IS, determining its risk

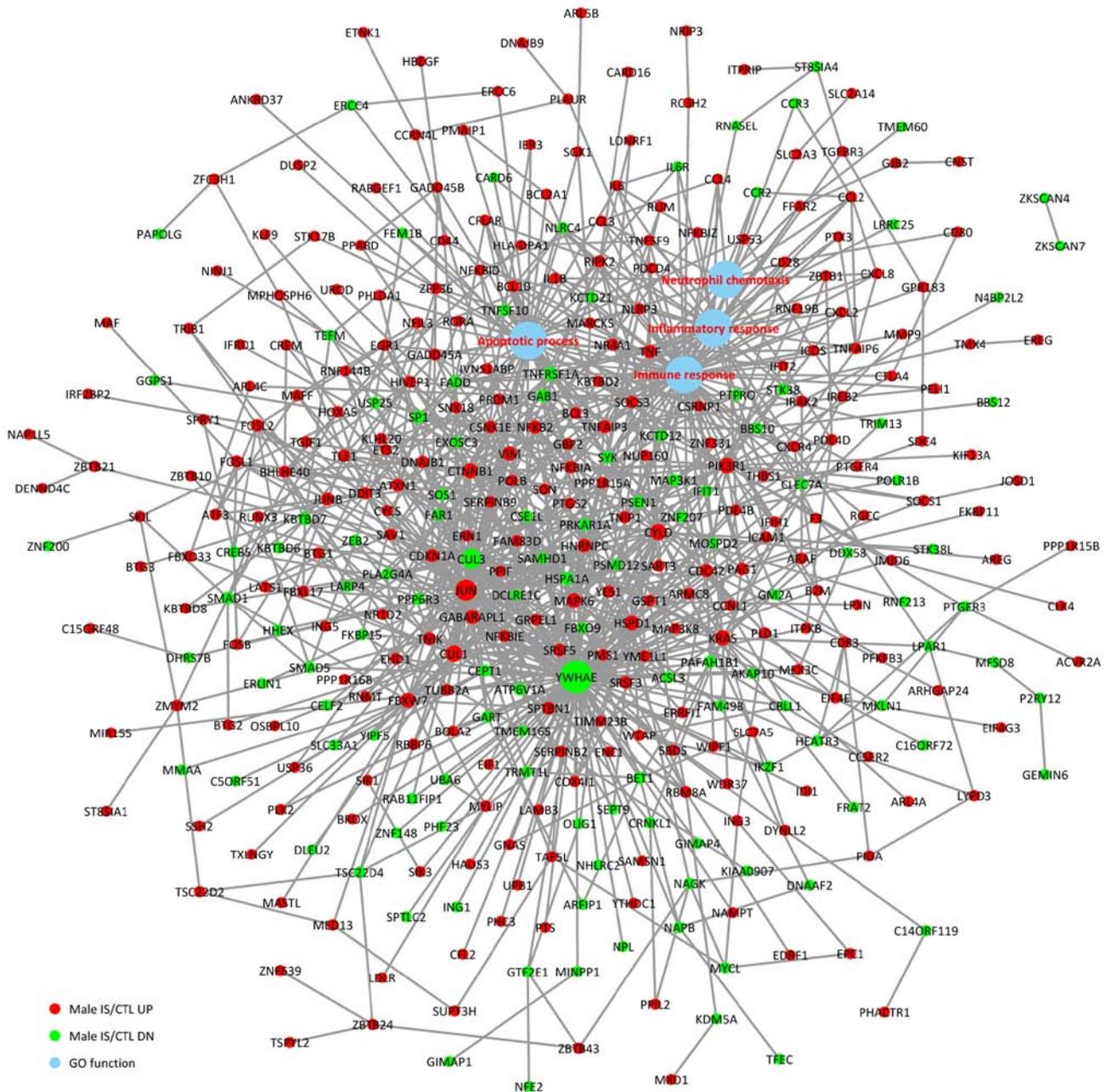


Figure 4. Protein-protein interaction of the upregulated and downregulated genes in male patients with IS and their association with immune response, inflammatory response, apoptotic process and neutrophil chemotaxis. CTL, control; DN, downregulated; GO, Gene Ontology; IS, ischemic stroke; UP, upregulated.

factors and developing effective therapeutic strategies will significantly improve the clinical outcome of stroke treatment.

The present study used bioinformatics tools and methods to screen the key DEGs and their functions in stroke. A total of 128 upregulated genes and 231 downregulated genes were found in female patients with IS, and 604 upregulated genes and 337 downregulated genes in male patients with IS. These DEGs were mainly associated with inflammatory response and apoptosis, and other biological pathways. Combining PPI and GO term enrichment results, it was also found that the genes involved in the GO analysis pathway were almost identical to the PPI network node-related genes, especially the CXCL family; tumor necrosis factor and PDK were most closely related to these pathways, which was consistent with the reported results.

Following a stroke, the damaged brain tissue releases proinflammatory chemokines, which trigger the inflammatory pathway in immune cells (40). Depending on the sequence of N-terminal residues, chemokines can be divided into several different types, including CXC, CC, XC and CX3C, where C is an N-terminal cysteine residue and X represents any amino acid residue (41). The chemokine ligands and receptors are emerging as potential therapeutic targets for cardiovascular diseases and have become increasingly studied in recent years. Brait *et al* (42) found that the expression of CXCR2 and its ligands CXCL1 and CXCL2 is significantly upregulated and peaks 1-3 days after injury in a middle cerebral artery occlusion (MCAO) rat model. Villa *et al* (43) inhibited CXCR1 with interleukin (IL)-8/CXCL8 receptor inhibitors in MCAO model rats and demonstrated that CXCR2, myeloperoxidase and



Figure 5. miRNA target network of the upregulated and downregulated genes of female patients with IS with differentially expressed miRNAs. CTL, control; diffexp, differentially expressed; DN, downregulated; IS, ischemic stroke; miRNA, microRNA; UP, upregulated.

IL-1A expression levels decreased, which reduced the symptoms of neurological deficits. In the present study, it was found that the chemokine receptors CXCL3, CXCL2 and CXCL8 were significantly upregulated in male patients with IS, and this was consistent with the previous reports.

PDK4 is a key enzyme that regulates pyruvate dehydrogenase complex (PDC) activity and is a key regulator of pyruvate oxidation and glucose maintenance *in vivo* (44). In calcified vascular smooth muscle cells and calcified vessels of patients with atherosclerosis, PDK4 expression is found to be upregulated alongside increased PDC phosphorylation, suggesting that PDK4 serves an important role in vascular calcification (45). In phosphate-treated vascular smooth muscle cells, PDK4 expression is upregulated, inducing mitochondrial dysfunction and, subsequently, apoptosis. Atherosclerosis is the result of cholesterol and lipid deposition on the arterial wall, often related to calcification, which is formed by the

deposition of inorganic calcium on the arterial wall. Thus, the upregulation of PDK4 increased osteogenic markers and enhanced vascular calcification without significant effects on bone formation (46). This indicated that PDK4 may be a new target for the treatment of vascular calcification (47,48).

In the pathogenesis of various injuries, the immune inflammatory signaling pathway caused by ischemia serves a major role, whereas ischemia itself serves a secondary role (49). In IS the key role of the immune inflammatory response has been widely recognized, but whether it plays a role in brain damage or brain protection may depend on the degree and stage of ischemia. Secondary injuries are caused by the inflammatory cascade following ischemia. Various inflammatory mediators, including cytokines, chemokines and cell adhesion molecules, aggravate ischemic brain damage (27,28). In the early stage of ischemia, the inflammatory response exacerbates brain damage, and in the late

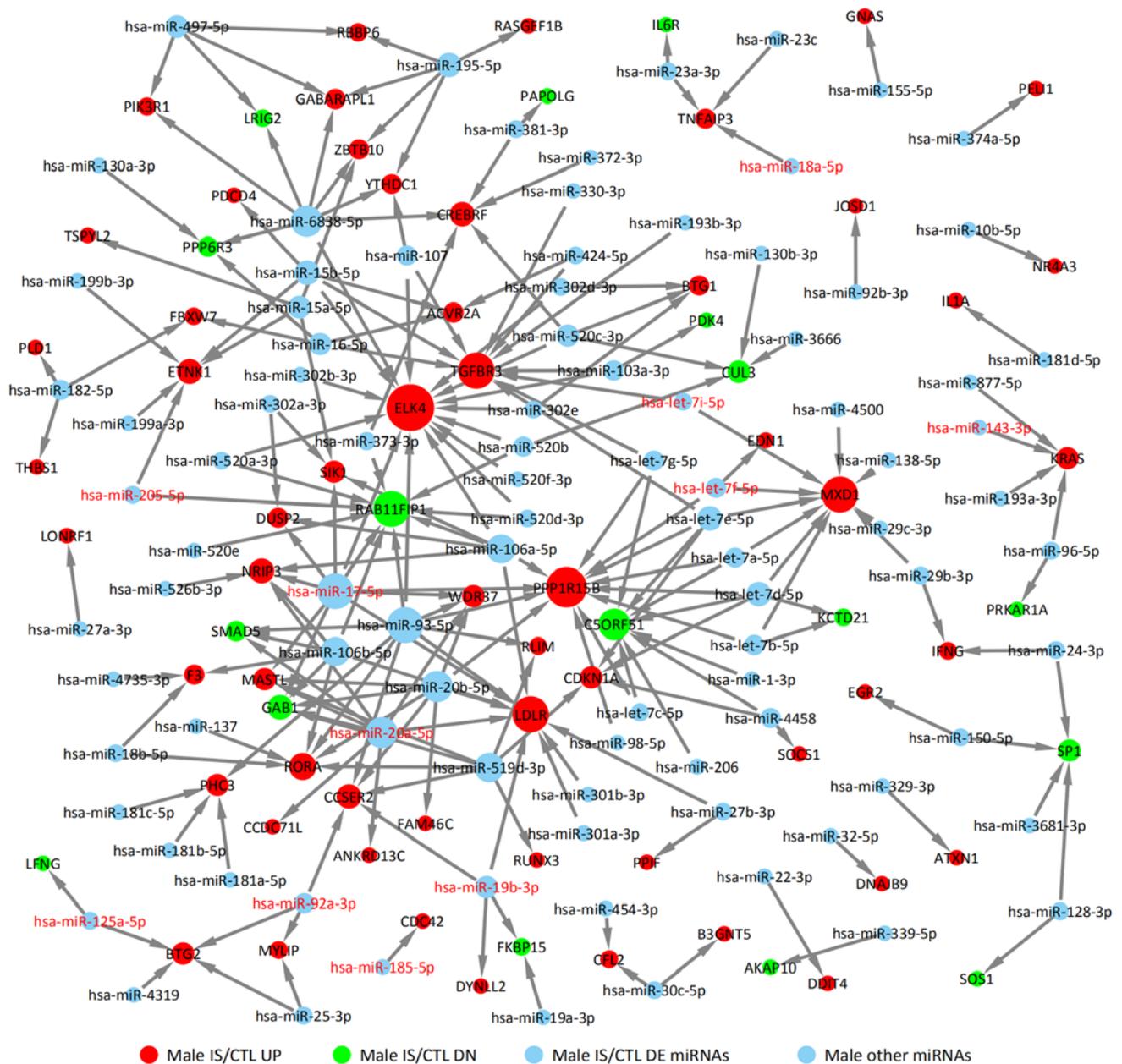


Figure 6. miRNA target network of the upregulated and downregulated genes of male patients with IS with differentially expressed miRNAs. CTL, control; diffexp, differentially expressed; DN, downregulated; IS, ischemic stroke; miRNA, microRNA; UP, upregulated.

stage of ischemia, it serves an important role in the recovery of nerve function and tissue repair. In addition, patients with IS are prone to a hypercoagulable state and advanced fibrinolysis. Most patients have coagulation changes in the clinic, which may lead to adverse events such as thrombosis and infarction, which affects the prognosis (50).

miRNAs are important post-transcriptional regulators that coordinate the regulation of target genes with multi-target mRNA. Risk factors involved in IS include hypertension, atherosclerosis, atrial fibrillation, diabetes and dyslipidemia. The role of miRNAs in the pathophysiology of stroke has become a focus of interest in recent research (51). A previous study reported that circulating miRNA expression in peripheral blood is relatively stable (52), suggesting that differential expression of specific miRNAs following stroke is expected to be a potential biomarker for diagnosis and prognosis, providing

an effective theoretical basis for elucidating the pathogenesis of IS (53).

In the present study, the GSE110993 dataset was analyzed and 17 differentially expressed miRNAs (6 miRNAs in females and 11 miRNAs in males) were screened. According to previous studies, there are differences in the expression patterns of miRNAs in IS and in normal healthy people (54). For example, the abnormal expression of miR-221 is related to metabolic syndrome, whereas the expression of miR-221 in healthy people was significantly higher compared with expression levels in patients with IS (55). Studies have demonstrated that the abnormal expression level of miR-221 is significant for distinguishing healthy individual and those with cardiovascular and cerebrovascular diseases, tumor populations and endocrine populations (56,57). Other previous studies have shown that miR-143/145 (57) and miR-125-5p (58) can also be used as

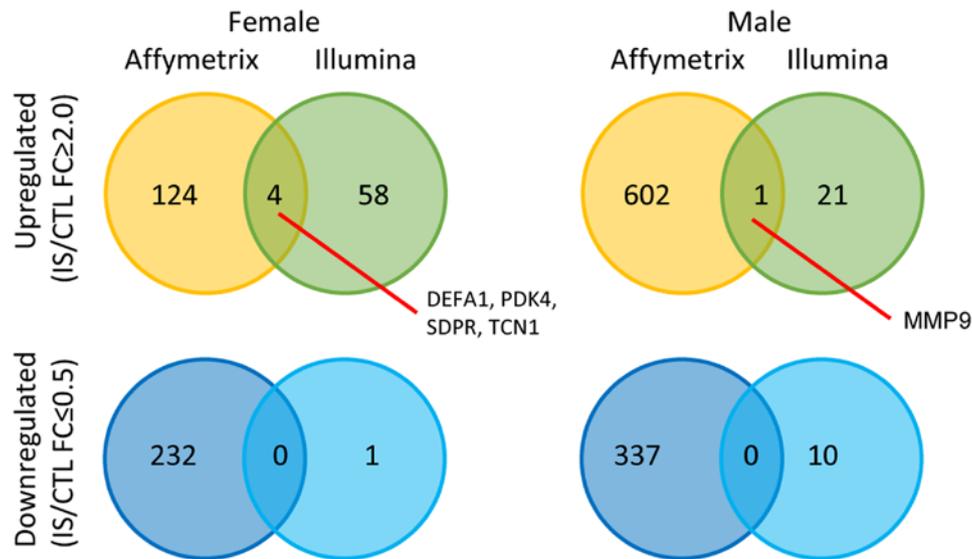


Figure 7. Overlapping upregulated and downregulated genes from two different beadchips (Affymetrix Human Genome U133 Plus 2.0 array and Illumina HumanRef-8 v3.0 expression beadchip) in female and male patients. CTL, control; FC, fold-change; IS, ischemic stroke; DEFA1, defensin alpha 1; PDK4, pyruvate dehydrogenase kinase 4; SDPR, caveolae associated protein 2; TCN1, transcobalamin 1; MMP9, matrix metalloproteinase 9.

markers for the risk of IS, which is consistent with the findings of the present study. miR-143/145 is most abundant in normal vascular tissues compared with other miRNAs and is selective in vascular smooth muscle cells expression. miR-125-5p has been found to be significantly upregulated in atherosclerosis (58). Inhibition of miR-125-5p expression significantly increases the uptake of lipids and enhances the clearance of human expression and secretion of inflammatory cell molecules (59). Some of these inflammatory cell molecules (60) may be involved in the inflammatory response of ox-LDL-stimulated monocytes, whereas ox-LDL may be taken up by macrophages, converted into foam cells that secrete pro-inflammatory factors and leads to cell necrosis and plaque formation. This series of chronic inflammatory reactions is atherosclerosis.

There are several limitations to the present study. First, it is notable that only one upregulated gene overlapped and none of downregulated genes overlapped between male and female groups. This may be due to differences in the effects of different types of sex hormones on platelet function. Androgens increase platelet activity, whereas estrogen inhibits its function (61). This may have an effect on the relative expression levels of mRNA and miRNA in the blood, suggesting that doctors might consider different medications for stroke patients of different sexes (62). Secondly, the data in Fig. 7 do not give confidence in the high-throughput genomics analyses because there are only 4 genes that are upregulated in both dataset in the female group and only one shared gene upregulated in the male group. This phenomenon may be because GSE22255 is based on GPL570 Affymetrix Human Genome U133 Plus 2.0 Array, whereas GSE16561 contained a total of 63 samples based on the platform of GPL6883 Illumina HumanRef-8 v3.0 expression beadchip. On the one hand, these two different gene platforms contain different numbers and different types of genes; on the other hand, there may be detection errors in chips from Illumina and Affymetrix.

In summary, all of the miRNAs described above have a certain correlation with the pathogenesis and development of

IS, which is consistent with the findings of stroke researchers worldwide in recent years. The correlation between some miRNAs selected in this study have not been reported previously in the literature to the best of our knowledge. The possibility that these miRNAs are novel biomarkers for IS and its biological significance still needs further study. Further studies on the role of miRNAs will provide a theoretical basis for understanding the molecular mechanism of the pathogenesis and development of IS, as well as new research programs for prevention and treatment.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LL designed the experiments; HW and SS performed the experiments; LD and JC conducted the statistical analysis of the data and drafted the manuscript; CZ and CB conceived the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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