Bioinformatics analysis of microarray data to reveal the pathogenesis of brain ischemia

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Abstract. Brain ischemia leads to energy depletion, mitochondrial dysfunction and neuronal cell death. The present study was designed to identify key genes and pathways associated with brain ischemia. The gene expression profile GSE52001, including 3 normal brain samples and 3 cerebral ischemia samples, was downloaded from the Gene Expression Omnibus database. Differentially expressed genes (DEGs) were identified using the limma package. Then functional and pathway enrichment analyses were performed by the MATHT tool. Protein-protein interaction (PPI) network, module selection and microRNA (miRNA)-target gene network were constructed utilizing Cytoscape software. A total of 488 DEGs were identified (including 281 upregulated and 207 downregulated genes). In the PPI network, Rac family small GTPase 2 (*RAC2*) had higher degrees. *RAC2* was significantly enriched in the FcγR-mediated phagocytosis pathway. *miR-29A/B/C* had a higher degree in the miRNA-target gene network. Insulin like growth factor 1 (*Igf1*) was identified as the target gene for *miR-29A/B/C*. *RAC2* may function in brain ischemia through mediating the FcγR-mediated phagocytosis pathway. Meanwhile, *miR-29A/B/C* and their targets gene *Igf1* may serve important roles in the development and progression of brain ischemia.

Introduction

Ischemic stroke, the third leading cause of death, leads to neuronal cell death by necrosis or apoptosis, mitochondrial dysfunction, energy depletion, and its complications such as coma, and hemiplegia (1-3). The study shows that the incidence of ischemic stroke decrease over time among men, but it is stable among women (4). It is report that the incidence of ischemic stroke is 170/100 thousand in adult women (5,6), and it is 212/100 thousand in men. Notably, the incidence of ischemic stroke is 91.3/100 thousand-263.1/100 thousand in China (7). Usually, the middle cerebral artery is related to ischemic stroke (8). Despite some clot lysing drugs have applied to ameliorate cerebral ischemic according to clinical experience, the treatment efficacy is limited by the narrow therapeutic safety and time window (1,9). In addition, reperfusion also aggravates brain injury, such as neuronal apoptosis, reactive oxygen species overproduction, and neuro-inflammation (10,11). Thus, it is critical to explore the novel therapeutic agents and targets of ischemic stroke.

Currently, numerous studies involved the pathophysiological of ischemic stroke are performed. For example, the Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) signaling pathway is suggested to play a vital role in central nervous system (12). In this pathway, hypoxia preconditioning (13), rhEPO (14), IL-6 (15) can activate JAK2-STAT3 pathway and promote neurological recovery (13,16). In addition, the suppressor of cytokine signaling (SOCS) family of proteins, including SOCS1 and SOCS3, can suppress cytokine activity by interacting with JAK (17), indicating JAK2/STAT3 pathway is associated with cerebral ischemia reperfusion injury. Moreover, studies show that Rac family small GTPase 2 (Rac2), a well-studied small GTPase, has the effect on hematopoietic and endothelial cell integrin and immunoreceptor signaling (18,19). Joshi *et al* demonstrate that Rac2 is related to macrophage autonomous process, which can control tumor growth (20). However, the relationship between Rac2 and cerebral ischemia need to be further investigated.

MicroRNA (miRNA), a small non-coding RNA molecule, has important functions in the RNA silencing and post-transcriptional regulation of gene expression (21). The study shows that miRNAs are involved in neuro protection, ischemia, and injury (21). Previous study indicated that miR-29a had the protective effect on reperfusion injury by targeting a pro-apoptotic family member (22). However, potential gene markers related to brain ischemia based on gene or miRNA expression remains unclear.

The GSE52001 is obtained on Agilent Array platform and firstly analyzed by Lai *et al* (23). However, based on the huge information of gene expression profile, the data about the role of potential gene markers in cerebral ischemia are limited. In the present study, a bioinformatics study was performed based on the microarray data deposited by Lai *et al* (23). On

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the basis of differentially expressed gene (DEGs) between sham brain samples (sham group) and cerebral ischemia brain samples (ischemia brain group), the function and pathways analyses were investigated. Protein-protein interaction (PPI) network analysis was also conducted. Then the analysis for potential miRNA-target regulation in the process of glioma was performed. We expected to explore a detailed mechanism of transcriptional regulation in the cerebral ischemia, and provide a novel strategy for cerebral ischemia therapy.

Materials and methods

Microarray data. The gene expression profiling GSE52001 was downloaded from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) database (24), which was based on the platform of GPL14746 Agilent-028282 Whole Rat Genome Microarray 4x44 K V3.0 expression beadchip. The organism of this dataset was *Rattus norvegicus*, including 3 normal brain samples (Sham brain, SB, SB1, SB2, SB3) and 3 brain samples of cerebral ischemia (ischemia brain, IB, IB1, IB2, IB3) (23). The IB samples were collected as follows: Male Sprague Dawley rats (220±20 g; 7-8 weeks old) were anesthetized with 10% chloral hydrate (3 ml/kg). Then a silicone-coated nylon monofilament was inserted from the left common carotid artery to the origin of the middle cerebral artery. After 2 h of occlusion, reperfusion was induced by withdrawing the filament. Sham animals were operated on in the same manner except that the middle cerebral artery was not occluded. The animal experiments were conducted in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study of Lai *et al* was approved by the Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine (23).

Pretreatment and differential analysis. The robust multi-array average (RMA) method in limma package (http://www.bioconductor.org/packages/2.9/bioc/html/limma.html) (25) was applied to preprocess the raw CEL data by performing background correction, data normalization, conversion of original data, and quartile data normalization. Then the DEGs were identified by the non-paired t-test in limma package (25). Here, the adjusted P-value <0.05 and |log fold change (FC) |≥1 were set as the threshold value. Finally, the heat map for DEGs was generated via the Pheatmap package (https://CRAN.R-project.org/package=pheatmap) (26) in R (version 3.3.2).

Functional and pathway enrichment analyses. Gene Ontology (GO) (http://www.geneontology.org) analysis (27) is used for analyzing the functions of a large number of genes. The Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.ad.jp/kegg/) (28,29) pathway is the major recognized pathway-related database which contains varieties of biochemical pathways (29). Multifaceted Analysis Tool for Human Transcriptome (MATHT) (www.biocloudservice.com) was used to perform GO term and KEGG pathway enrichment analyses for the DEGs. The setting of cut-off value was P-value <0.05.

PPI network and module analyses. The Search Tool for the Retrieval of Interacting Genes (STRING) (version 10.0) (30) (http://www.string-db.org/) is an online database providing experimental and predicted PPI information. Here, the STRING database (30) was applied to analyze the PPIs among the proteins encoded by the DEGs. The parameter was set as medium confidence score >0.4. Then the PPI networks for the upregulated genes and the downregulated genes were separately visualized by Cytoscape software (version 3.2.0) (http://www.cytoscape.org/) (31), and node degrees were determined. In addition, the significant modules were obtained using the MCODE plug-in (http://apps.cytoscape.org/apps/mcode) (32) in Cytoscape software. Additionally, the KEGG pathway enrichment analysis for nodes in the significant modules was performed using MATHT tool.

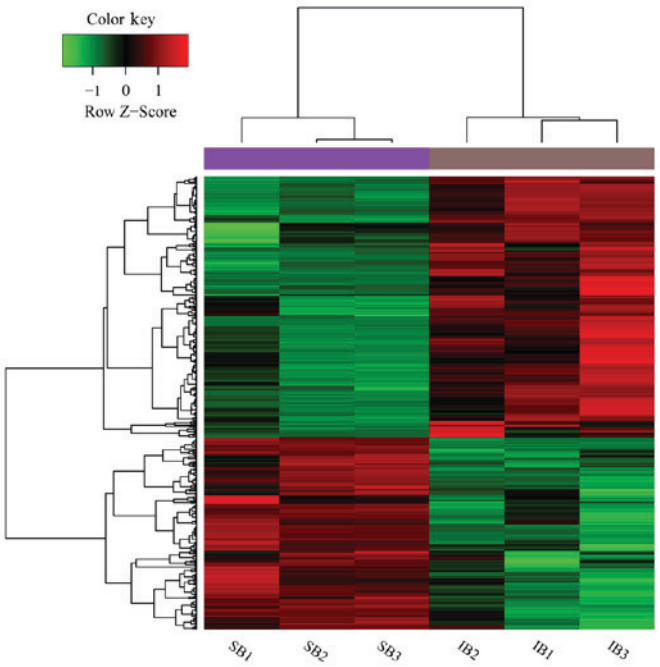

Figure 1. Cluster analysis of DEGs. DEGs, differentially expressed genes; IB, ischemia brain samples; SB, sham brain samples. Cluster analysis was performed at both gene level (vertical) and sample level (horizontal).

miRNA-target gene regulatory network analyses. With the discovery of RNA interference (RNAi), the function of noncoding RNAs in gene expression and regulation was widely focused (33). MiRNAs regulate the expression of genes by interacting with their target genes at the post transcription stage (33). In the present study, the miRNAs associated with DEGs were searched utilizing WebGestal (http://www.webgestalt.org/option.php) (34,35) online tool, and miRNA-DEG regulatory network was visualized by Cytoscape software (31).

Results

DEGs and clusters. In total, 488 DEGs were identified, including 281 upregulated and 207 downregulated DEGs. Thereafter, the 488 DEGs and 6 samples were clustered, and DEGs could well differentiate the IB samples from the SB controls (Fig. 1).

Functional and pathway enrichment analyses. The enriched GO terms and KEGG pathways for DEGs were identified.

Table I. KEGG pathways significantly enriched by DEGs.

A, Upregulated genes

Pathway ID	Pathway name	Count	P-value	Genes
rno05150	Staphylococcus aureus infection	12	8.15×10^{-10}	*C1QA, C1QB, C5AR1, FCGR2B, C3, LOC498276, C1R, ITGB2, C2, C1S, FCGR3A, C1QC*
rno05133	Pertussis	11	2.64×10^{-7}	*C1QA, C1QB, C3, PYCARD, SERPING1, C1R, ITGB2, C2, C1S, C1QC, CD14*
rno04610	Complement and coagulation cascades	10	2.36×10^{-6}	*C1QA, C1QB, A2M, C5AR1, C3, SERPING1, C1R, C2, C1S, C1QC*
rno04650	Natural killer cell mediated cytotoxicity	10	3.05×10^{-5}	*CD48, PTPN6, RAC2, FCER1G, ITGB2, VAV2, FCGR3A, IFNGR1, HCST, TYROBP*
rno05140	Leishmaniasis	7	1.13×10^{-3}	*PTPN6, CYBA, C3, LOC498276, ITGB2, FCGR3A, IFNGR1*
rno04145	Phagosome	11	1.41×10^{-3}	*RT1-A2, CYBA, RT1-A1, FCGR2B, C3, LOC498276, C1R, ITGB2, CTSS, FCGR3A, CD14*
rno05322	Systemic lupus erythematosus	9	1.44×10^{-3}	*C1QA, C1QB, C3, LOC498276, C1R, C2, C1S, FCGR3A, C1QC*
rno05152	Tuberculosis	10	3.01×10^{-3}	*LSP1, FCGR2B, C3, LOC498276, FCER1G, ITGB2, CTSS, FCGR3A, IFNGR1, CD14*
rno04666	FcγR-mediated phagocytosis	6	1.28×10^{-2}	*PTPRC, RAC2, FCGR2B, HCK, LOC498276, VAV2*
rno04142	Lysosome	7	1.81×10^{-2}	*CTSZ, GUSB, LGMN, CTSE, CTSC, CTSS, CD63*
rno04380	Osteoclast differentiation	7	1.94×10^{-2}	*CYBA, FCGR2B, LOC498276, TREM2, FCGR3A, IFNGR1, TYROBP*
rno04611	Platelet activation	7	2.37×10^{-2}	*P2RY12, ORAI1, TBXAS1, FERMT3, LOC498276, COL3A1, FCER1G*
rno00860	Porphyrin and chlorophyll metabolism	4	2.94×10^{-2}	*GUSB, HMOX1, HEPH, CP*
rno05146	Amoebiasis	6	3.70×10^{-2}	*ARG1, COL3A1, SERPINB1A, ITGB2, SERPINB1B, CD14*
rno04670	Leukocyte transendothelial migration	6	4.75×10^{-2}	*CYBA, RAC2, CLDN1, ITGB2, VAV2, MMP2*

B, Downregulated genes

PathwayID	Pathway name	Count	P-value	Genes
rno05143	African trypanosomiasis	4	4.70×10^{-3}	*LOC689064, HBB-B1, PLCB1, HBB*
rno04925	Aldosterone synthesis and secretion	4	3.96×10^{-2}	*CYP11B1, NR4A1, PLCB1, CACNA1S*

KEGG, Kyoto Encyclopedia of Genes and Genomes; DEGs, differentially expressed genes.

According to the P-values (ascending sort), the top 5 enriched terms were exhibited in Fig. 2. The upregulated genes were significantly enriched in the functions of aging (BP, $P=1.69\times10^{-7}$), extracellular exosome (CC, $P=3.58\times10^{-11}$), extracellular space (CC, $P=5.70\times10^{-14}$), protein homodimerization activity (MF, $P=6.23\times10^{-4}$), and identical protein binding (MF, $P=3.80\times10^{-3}$) (Fig. 2A). While the downregulated genes were dramatically enriched in the functions of response to drug (BP, $P=3.20\times10^{-3}$), extracellular space (CC, $P=1.25\times10^{-2}$), transcriptional activator activity (MF, $P=1.03\times10^{-4}$), and RNA polymerase II core promoter proximal region sequence-specific binding (MF, $P=1.03\times10^{-4}$) (Fig. 2B). Besides, the significantly enriched KEGG pathways were presented in Table I. For the upregulated genes, the significantly enriched KEGG pathways mainly include staphylococcus aureus infection (pathway, $P=8.15\times10^{-10}$), pertussis (pathway, $P=2.64\times10^{-7}$), and complement and coagulation cascades (pathway, $P=2.36\times10^{-6}$). There were only 2 significantly enriched KEGG pathways for the downregulated genes, which include African trypanosomiasis and Aldosterone synthesis (pathway, $P=4.70\times10^{-3}$), and secretion (pathway, $P=3.96\times10^{-2}$) (Table I).

PPI network and module analyses. The PPI network with 295 nodes and 827 edges was constructed (Fig. 3). Upregulated gene with higher node degree were Rac2, angiotensinogen (Agt), integrin β2 (Itgb2), protein tyrosine phosphatase, receptor

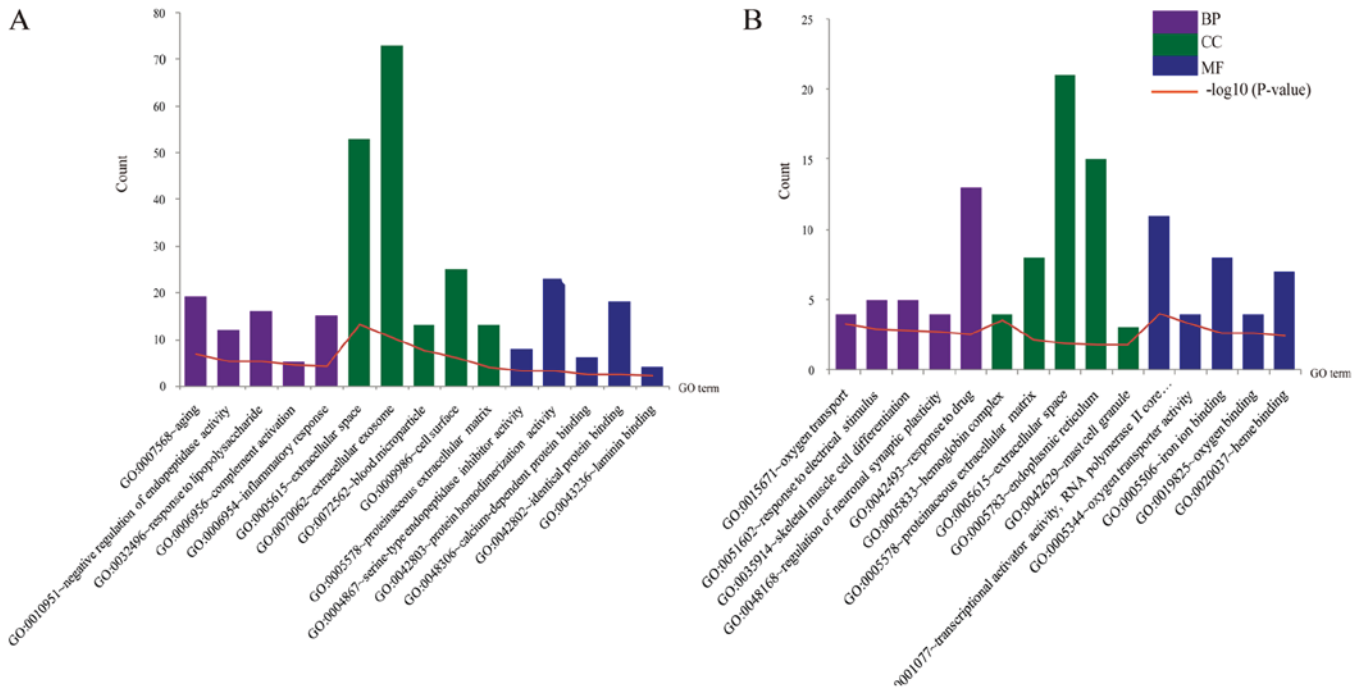

Figure 2. Top 5 GO terms enriched separately. GO terms separated according to (A) upregulated and (B) downregulated genes. MF, Molecular function; BP, Biological processes; CC, Cellular components; GO, gene ontology; The horizontal axis represents the enriched GO terms The vertical axis represents the count of enriched DEGs.

type, C (Ptprc), protein tyrosine phosphatase, non-receptor type 6 (Ptpn6), and hematopoietic cell kinase (Hck). Downregulated genes with higher degrees were Fos, Hras, and Junb. The degree of top 20 DEGs was listed in the Table II. In this study, 3 significant modules were obtained by MCODE plug-in, which included module A (10 nodes and 45 edges), module B (12 nodes and 33 edges), and module C (5 nodes and 10 edges) (Fig. 4).

In addition, 2 KEGG pathways were significantly enriched in module A, including neuroactive ligand-receptor interaction ($P=9.62x10^{-4}$), and inflammatory mediator regulation of TRP channels ($P=3.03x10^{-3}$). Meanwhile, 8 KEGG pathways were dramatically enriched in module B, such as FcγR-mediated phagocytosis ($P=1.60x10^{-6}$), B cell receptor signaling pathway ($P=5.40x10^{-5}$), and natural killer cell mediated cytotoxicity ($P=1.54x10^{-4}$) (Table III). However, no pathways were enriched for the nodes in module C.

miRNA-target regulatory network analysis. A total of 58 DEGs, 13 miRNAs, and 128 edges were contained in the miRNA-DEG regulatory network (Fig. 5). The nodes with top 10 degrees were listed in Table IV. Among them, the degrees of *miR-29A*, *miR-29B* and *miR-29C* were higher than other miRNAs in the miRNA-target regulatory network. Besides, target genes with higher degrees such as *Mycn*, *Plcb1*, *Igf1* were shown in Fig. 5.

Discussion

In the present study, a total of 488 DEGs were identified, including 281 upregulated and 207 downregulated DEGs. Rac2, with higher degree in the PPI network, was associated with FcγR-mediated phagocytosis pathway. In the miRNA-target gene network, the degrees of *miR-29A*, *miR-29B* and *miR-29C*

Table II. Degree of top 20 differentially-expressed genes in the protein-protein interaction network.

A, Upregulated

Gene	Degree
Rac2	35
Agt	34
Tyrobp	30
Itgb2	30
Ptprc	28
Timp1	27
Ptpn6	24
Lgals3	23
Hck	21
Igf1	20
Jak3	20
Anxa1	20
Cd53	20
Dcn	18
C1qb	16

B, Downregulated

Gene	Degree
Fos	34
Hras	32
Junb	20
Sst	19
Egr1	17

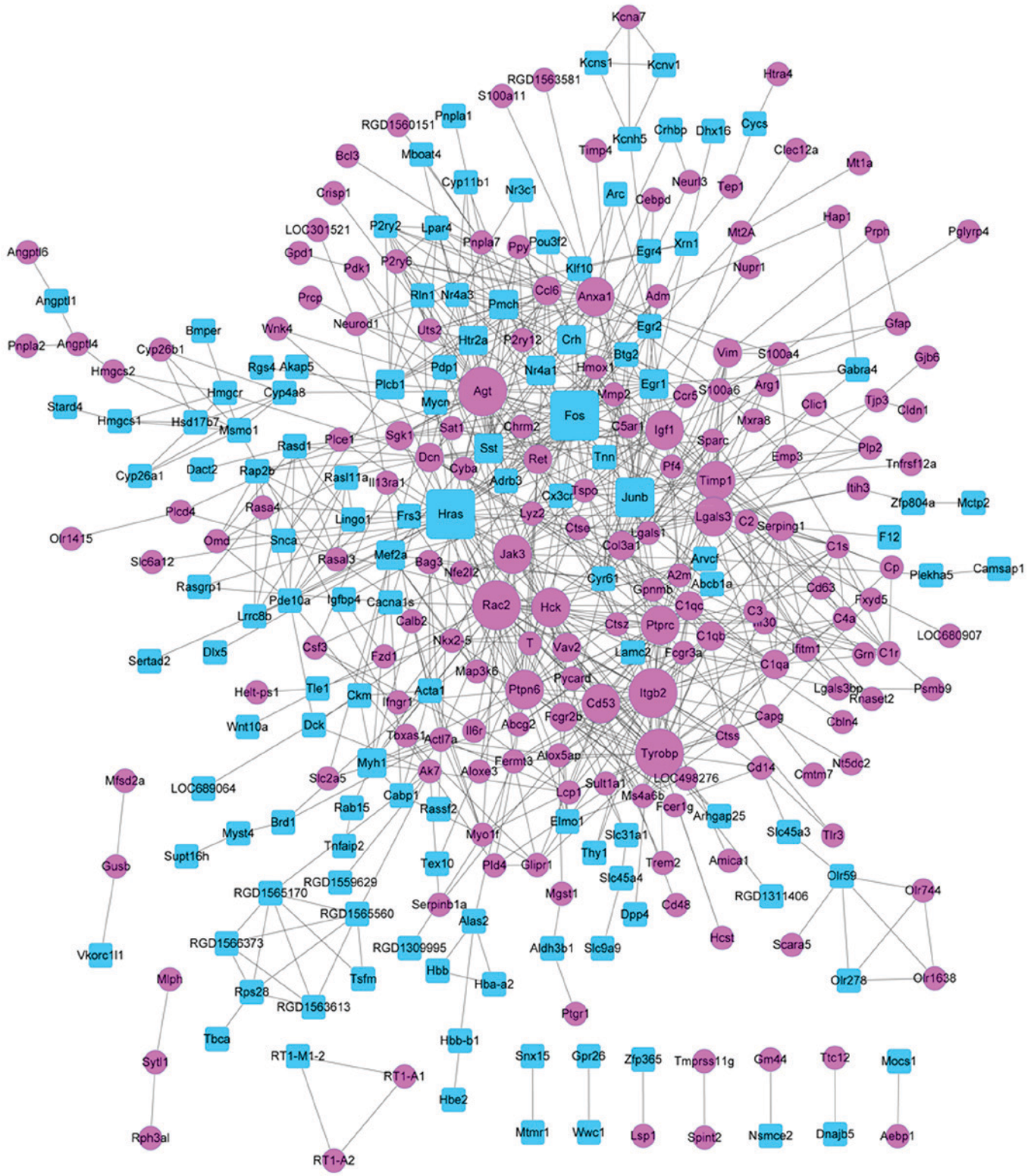

Figure 3. Protein-protein interaction network constructed for the DEGs. The pink circle and the blue square represent upregulated genes and downregulated genes, respectively. DEGs, differentially-expressed genes.

were higher than other miRNAs. Notably, the target gene *Igf1* was regulated by *miR-29A*, *miR-29B* and *miR-29C*.

Rac2, a member of Rac sub-class 3 proteins (including Rac1, Rac2 and Rac3), is a well-studied small GTPase (36,37). Among sub-class proteins, there are 92% sequence identity between Rac1 and Rac2, 83% identity between Rac2 and Rac3, and 77% identity between Rac1 and Rac3 (18). Rac2 is only expressed in hematopoietic and endothelial cells, while Rac1 and Rac3 are comprehensively expressed in mammalian systems (36-38). Rac2, the hematopoietic specific GTPase, plays an obligate role in endothelial integrin signaling and the postnatal neovascularization response *in vivo* (19). Additionally, some studies demonstrated that Rac2 regulates FcγR-mediated phagocytosis (39-41). Consistent with

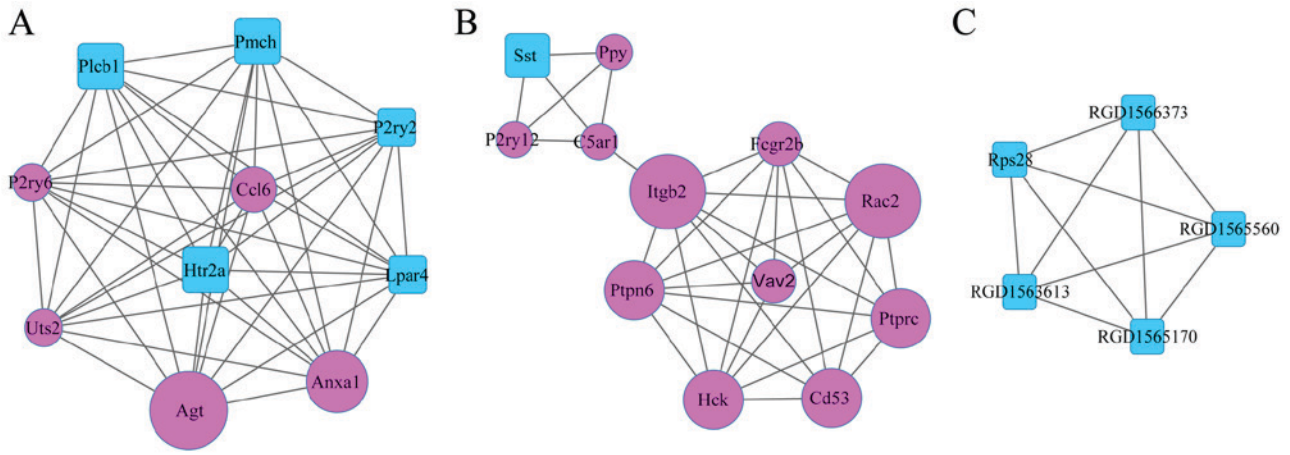

Figure 4. Module analyses of protein-protein interaction network. (A) The pink circles represent upregulated DEGs, and the blue squares represent downregulated DEGs. (B) The pink circles represent upregulated DEGs, and the blue square represents downregulated DEGs. (C) The blue squares represent downregulated DEGs. DEGs, differentially-expressed genes.

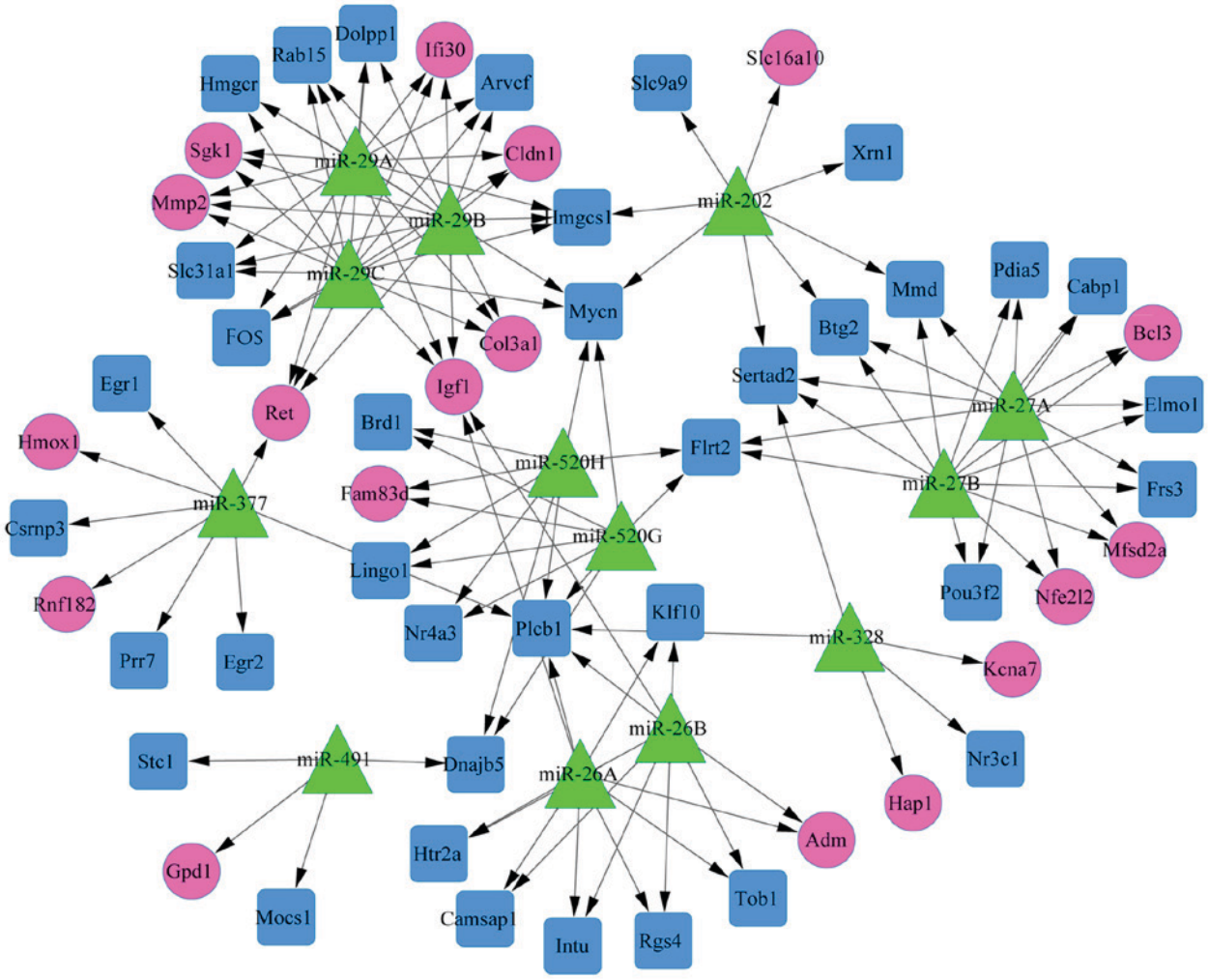

Figure 5. miRNA-target regulatory network. The pink circle and the blue square represent upregulated genes and downregulated genes, respectively. The green triangle represents miRNA. Arrow represents the miRNA-target relationship. DEGs, differentially-expressed genes; miRNAs, microRNAs.

Yang *et al* (42), this study also shows that RAC2 is related to FcγR-mediated phagocytosis pathway in brain ischemia by bioinformatics methods. Yang *et al* (42) point that pathological nerve pain may be related to immune dysfunctions. Here, our results showed that *RAC2* gene and FcγR-mediated phagocytosis pathway may have vital effect on the progress of pathological nerve pain in nervous system. Therefore, RAC2 may play an important role in the development of

Table III. Enriched pathways for the nodes in module A and B.

Pathway ID	Pathway name	Count	P-value	Genes
MEA				
rno04080	Neuroactive ligand-receptor interaction	4	$9.62x10^{-4}$	*P2RY6, P2RY2, LPAR4, HTR2A*
rno04750	Inflammatory mediator regulation of TRP channels	3	$3.13x10^{-3}$	*P2RY2, PLCB1, HTR2A*
MEB				
rno04666	FcγR-mediated phagocytosis	5	$1.60x10^{-6}$	*PTPRC, RAC2, FCGR2B, HCK, VAV2*
rno04662	B cell receptor signaling pathway	4	$5.40x10^{-5}$	*PTPN6, RAC2, FCGR2B, VAV2*
rno04650	Natural killer cell mediated cytotoxicity	4	$1.54x10^{-4}$	*PTPN6, RAC2, ITGB2, VAV2*
rno05150	Staphylococcus aureus infection	3	$1.65x10^{-3}$	*C5AR1, FCGR2B, ITGB2*
rno04660	T cell receptor signaling pathway	3	$6.45x10^{-3}$	*PTPN6, PTPRC, VAV2*
rno04670	Leukocyte transendothelial migration	3	$7.91x10^{-3}$	*RAC2, ITGB2, VAV2*
rno04062	Chemokine signaling pathway	3	$1.67x10^{-2}$	*RAC2, HCK, VAV2*
rno04810	Regulation of actin cytoskeleton	3	$2.47x10^{-2}$	*RAC2, ITGB2, VAV2*

Table IV. Degree of top 10 miRNAs in the miRNAs-target regulatory network.

miRNA	Degree
miR-29A	15
miR-29B	15
miR-29C	15
miR-27A	12
miR-27B	12
miR-26A	9
miR-26B	9
miR-377	8
miR-202	8
miR-520G	8

miRNA, microRNA.

brain ischemia by mediating FcγR-mediated phagocytosis pathway.

In recent years, Kriegel *et al* reveal that the miR-29 family, including *miR-29A*, *miR-29B-1*, *miR-29B-2* and *miR-29C* (43), is found to be enriched in astrocytes (44). Previous study also shows that miR-29 family is downregulated in cortex (45), but is upregulated in hippocampus after focal ischemia (46). *miR-29A/B-1* is reported in Alzheimer's disease (47). Ouyang *et al* uncover that *miR-29A* is significantly upregulated in astrocytes, and regulates ischemic injury by BH3-only protein PUMA (22). The study shows that *miR-29B* loss at the infarct site is an important contributor to stroke lesion by 12-lipoxygenase pathway (48). Downregulated *miR-29C* promotes ischemic brain damage by its target gene DNMT3a. REST, an upstream transcriptional controller of *miR-29C*, can impede *miR-29C* downregulation and ischemic neuronal death by reducing REST induction (49). In this study, the degrees of *miR-29A*, *miR-29B* and *miR-29C* were higher than other miRNAs in the miRNA-target regulatory network. These results suggest that miR-29A/B/C may be novel biomarkers for the protection of brain ischemia injury. Intriguingly, these results were also in accordance with previous studies (22,48,49). In addition, our results indicated that miR-29A/B/C regulated the upregulated target gene *Igf1* in brain ischemia. Nicholas *et al* find that *Igf1* and *Il1RAP* are direct targets of miR-29 in biliary atresia (50). Therefore, we speculated that *Igf1* targeted by miR-29A/B/C may have significant effect in brain ischemia. However, the detailed regulatory relationship between *Igf1* and *miR-29A/B/C* in brain ischemia is not validated.

In conclusion, this study indicated that RAC2 may function in brain ischemia through the FcγR-mediated phagocytosis pathway. Meanwhile, *miR-29A/B/C* and their target gene *Igf1* may have critical roles in brain ischemia. This study provides new insights into the molecular mechanisms for the progression of brain ischemia and suggests directions for future study. However, it is essential for verifying these results by the experiment in the future.

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Availability of data and materials

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

Authors' contributions

JH and YG conceived and designed the research. YZ acquired the data. WP, GW and XL analyzed and interpreted the data. JH and HY performed the statistical analysis. YG obtained the

funding. JH drafted the manuscript. YG, GW and XL revised the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The animal experiments were conducted in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study of Lai *et al* was approved by the Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

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