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·Original Article·

Identification of autophagy signaling network that contributes to stroke in the ischemic rodent brain *via* gene expression

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ABSTRACT

Autophagy plays a vital role in cerebral ischemia and may be a potential target for developing novel therapy for stroke. In this study, we constructed an autophagy-related pathway network by analyzing the genes related to autophagy and ischemic stroke, and the risk genes were screened. Two autophagy-related modules were significantly up-regulated and clustered to influence cerebral ischemia. Besides, three key modular genes (NFKB1, RELA, and STAT3) were revealed. With 5-fold cross validation, the ROC curves of NFKB1, RELA, and STAT3 were 0.8256, 0.8462, and 0.8923. They formed a complex module and competitively mediated the activation of autophagy in cerebral ischemia. In conclusion, a module containing NFKB1, RELA, and STAT3 mediates autophagy, serving as a potential biomarker for the diagnosis and therapy of ischemic stroke.

Keywords: cerebral ischemia; autophagy; functional module; stroke; gene expression profile

INTRODUCTION

Ischemic stroke is one of the major causes of death and disability[1-5]. It is generally considered to be a heterogeneous and multifactorial disorder and caused by both conventional environmental risk factors and genetic factors[6, 7]. It can cause acute neuronal death *via* oxygen and nutrient depletion, and result in disruption of the blood-brain barrier[8]. Acute ischemic stroke resulting from intracranial vessel occlusion is associated with high morbidity and mortality[9]. Therefore, it is pivotal to explore the pathogenesis of ischemic stroke. Moreover, since most ischemic strokes (~80%) occur in the territory of middle cerebral artery (MCA), many animal stroke models of middle cerebral artery occlusion (MCAO) have been developed for studies of ischemic stroke[10].

Recent research has shown that ischemic insult activates autophagy, and an autophagic mechanism may contribute to ischemic neuronal injury[11]. Autophagy can be induced by pattern-recognition receptors and stresses such as nutrient depletion, closed head injury, or focal cerebral ischemia. Cerebral ischemia-induced microglial autophagy contributes to ischemic neuronal inflammation and injury. Autophagy is a process for the intracellular bulk degradation of cellular constituents that has multiple effects on immunity[12-14]. Blocking autophagy in epithelial cells enhances host cell death and finally leads to tissue destruction and inflammation[15]. Moreover, autophagy eliminates the abnormal protein aggregates and the damaged organelles in neurons after transient cerebral ischemia[16]. Besides, ischemic stroke increases autophagosomes and activates the autophagy-related pathways (ARPs)[17]. After neonatal hypoxia-ischemia, autophagy increases in neurons, indicating that over-activation of autophagic pathways is a potential protective mechanism in the early stage of brain injury[18]. It has been demonstrated that protective autophagy is induced and further promotes the neuroprotective effect on ischemic stroke by regulating mitogen-activated protein kinase

(MAPK) signals[19]. It has also been reported that global ischemia increases the autophagosomes *via* decreasing autophagosome degradation[16]. The activation of autophagic and lysosomal pathways has been implicated in neuronal injury in a rat model of permanent focal cerebral ischemia[11]. Furthermore, by regulating the TSC2-mTOR-S6K1 signaling pathway, autophagy is induced and further promotes neuronal survival during cerebral ischemia[20].

Based on the above findings, autophagy plays a vital role in cerebral ischemia, and may be a potential target for developing novel therapies for stroke. Thus, it is essential to explore the mechanism of autophagy in cerebral ischemia. In the current study, by using gene-expression and network information, we predicted autophagy-related genes that are highly correlated with ischemic stroke.

MATERIALS AND METHODS

Screening for Differentially-expressed Genes in Ischemic Brain

The National Center for Biotechnology Information (NCBI) GEO database[21] (http://www.ncbi.nlm.nih.gov/geo/) was screened to retrieve the gene expression profiles of the ischemic brain. Through searching for "ischemic brain" in the NCBI GEO database, 3007 results in 158 series were obtained from the DataSets database. Subsequently, we selected "series" for Entry type and "Expression profiling by array" for Study type. Consequently, 54 candidate series of *Mus musculus* and 40 candidate series of *Rattus norvegicus* were acquired. Then, several criteria were applied to screen the candidate series: (1) the tissue must be brain; (2) the study was not related to the reaction of ischemic brain; (3) eliminating series exposed to drug research; (4) eliminating series dealing with hypoxia-ischemia/reperfusion; (5) eliminating samples subjected to MCAO for >24 h. Finally, three series (GSE38037, GSE32529, and GSE58720) were screened for further study, and the samples with MCAO were the test groups. In GSE38037, there were eight samples (4 normal and 4 test samples)[22]. In GSE32529, there were 224 samples including 6 experimental conditions such as LPS treatment + ischemic challenge, and CpG treatment + ischemic challenge[23, 24]. In this study, the samples of "brain-unhandled" (i.e. non-treated) were selected as the normal control, and the brain-ischemia challenged (3 h and 24 h) samples were used as test groups. Finally, 6 normal and 8 test groups were obtained. In GSE58720, the sham and MCAO groups were measured at 24 h after reperfusion. Here, we considered the sham samples as the control group and the MCAO samples as the test groups. Finally, 3 test samples and 3 control groups were selected in GSE58720. The gene expression of each sample was estimated and normalized. Each probeset ID was mapped to gene symbol according to the corresponding platform. If multiple probsets were mapped to the same gene, the expression value for the gene was summarized as the arithmetic mean of the values of multiple probesets (on the log2 scale).

Gene Expression Significance Analysis

With the "MetaDE" package of R project, we merged the three expression profiles and screened the differentially-expressed genes (DEGs) by false discovery rate (FDR) <0.01. Then, Database for annotation, Visualization and Integrated Discovery 6.7 (DAVID, http://david.abcc.ncifcrf.gov/home.jsp) software was used to explore the functions of the DEGs, including biological processes and pathways. P <0.05 was set as the threshold used for enrichment analysis of the GO_BP and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

Construction of Autophagy-Related Pathway Network

There were 7 ARPs in KEGG (http://www.kegg.jp/kegg/pathway.html): Regulation of autophagy, Lysosome, FoxO signaling pathway, AMPK signaling pathway, Prion diseases, Shigellosis, and mTOR signaling pathways. All the autophagy-related pathways and pathway genes were extracted. To systematically analyze the role of autophagy genes in cerebral ischemia, the STRING v10 database (http://string-db.org/) was used to obtain a protein-protein interaction network of *Mus*. By integrating the protein interaction information of DEGs and autophagy-related pathway-gene information, we constructed an ARP[13] complex network. Four topological properties (degree, average shortest path length, closeness centrality, and clustering coefficient of the ARP network) were analyzed by the "network analyzer" plugin in Cytoscape software (www.cytoscape.org/).

Functional Module Analysis

The "MINE" plugin of Cytoscape was used to identify functional modules in the ARP networks, using a cutoff value for the connectivity degree of nodes (proteins in the network >2). Then, we set the module as a unit and achieved the module expression in different samples by calculating the average expression value of genes that were in the module.

$$M_{ik} = \frac{\sum_{j=1}^{n} S_{jk}}{n} \quad (1)$$

where M_{ik} is the expression value of module i in sample k. n is the number of genes in module i. S_{jk} is the expression value of module gene j in sample k.

For the "module expression profile", we further utilized the "limma" package of R project to screen the differentially-expressed modules between the disease and normal groups. Modules with P <0.05 were considered to be significantly expressed.

Identification of Potential Risk Genes

The important autophagy genes in ischemic stroke were screened based on the network topological properties in the ARP network, using Neighborhood scoring (NS) and Interconnectivity (ICN) optimization algorithms. The NS algorithm was based on the distribution of genes in the network and the expression level to screen the candidate genes[25]. Based on the NS, we weighted every node in the network by combining the fold change of the candidate node and its neighborhoods. If the node was differentially expressed and was linked directly to multiple nodes that were also DEGs, then we considered that the node and its neighbors were correlated. Thus, we inferred that the node was a potential target or diagnosis marker. We followed formula (2) to score the node.

$$Score(i) = \frac{1}{2} * FC_i + \frac{1}{2} * \frac{\sum_{j=1}^{N_i} FC_j}{|N_i|} \quad (2)$$

in which i is the candidate gene i in the network. FC_i represents the fold change of gene i between disease samples and normal samples. N_i is the number of neighbors for node i. j is the neighbor node of candidate gene i. Considering the properties of node self, if node i or its neighbors were not differentially expressed, then the $Score(i)$ was 0.

The ICN[26] mainly calculates the correlation between the node and all the DEGs, so we followed formula (3) to weight the genes.

$$Score(i) = \frac{1}{n} * \sum_{j=1}^{n} \frac{2 + N_{i,j}}{\sqrt{N_i * N_j}} \quad (3)$$

where n is the number of all DEGs. N_i is the number of interacting genes for gene i, and N_j is the number of interacting genes for gene j. $N_{i,j}$ is the number of genes shared by gene i and DEG j. In this algorithm, single nodes were rejected. All the genes were ranked in descending order, and a node with a higher score was considered to be more important.

The NS focuses on the expression variation of the node itself and the affected nodes. If more genes are affected and the fold change is more evident, then the gene is more important than other genes in disease development. The ICN focuses on the correlation between the candidate gene and DEGs. If the degree of correlation of a gene with all DEGs is higher, then that gene would be considered more important than others.

RESULTS

Differentially-Expressed Genes

By combining the three expression profiles (GSE38037, GSE32529 and GSE58720), we acquired 15 ischemic samples and 13 normal samples. With the "limma" package of R project, we finally acquired 337 common significant DEGs (P <0.05 and FDR <0.01) among GSE38037, GSE32529, and GSE58720 (Fig. 1A; Table S1). After functional enrichment, the common DEGs were involved in the Ribosome, Toll-like receptor signaling pathway, MAPK signaling pathway, and the Chemokine signaling pathway, among others (Fig. 1B). Besides, these DEGs played important roles in such processes as vasculature development, blood vessel development, and the regulation of angiogenesis (Fig. 1C).

ARP Network Analysis

Four hundred and ninety-two genes related to the autophagy-associated pathway were acquired from the KEGG database. By integrating the DEGs and autophagy-related genes, an ARP network was constructed that included 1356 nodes and 1983 edges (Fig. 2). We calculated the closeness centrality, average clustering

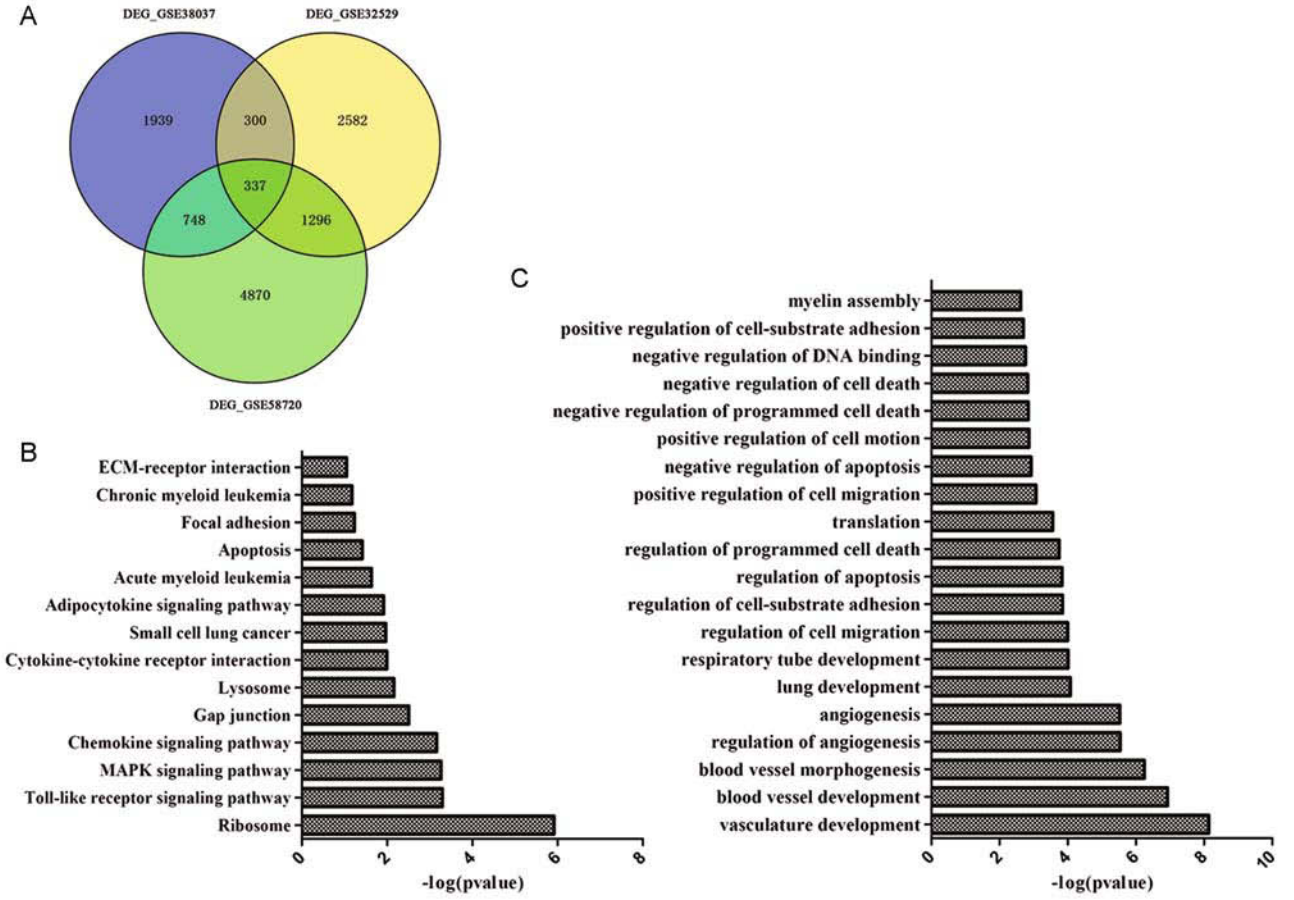

Fig. 1. Differentially-expressed genes (DEGs) acquired from different studies and function analysis of common DEGs. A, relationships of DEGs among GSE38037, GSE32529, and GSE58720; B, significantly enriched pathways of common DEGs; C, significant biological processes regulated by common DEGs. The larger –log (*P* value), the more important the function.

coefficient, degree distribution, and average shortest path length (Fig. 3). The degree of nodes in the ARP network followed a power-law distribution, and had small-world network characteristics such as a large average clustering coefficient and a short average shortest path length.

Module Analysis in ARP Network

The functional module expression was built for 15 functional modules (Fig. 4A), and two differentially-expressed modules were identified including modules 4 and 10 (Fig. 4B). Using the five-fold cross-validation test, we acquired the ROC curves of all modules (Table 1). Modules 4 and 10 had the highest classification accuracy, with ROC curves >0.85.

Key Autophagy Genes

We ranked the genes acquired by NS and ICN in descending order and screened the top 50 genes. Subsequently, we analyzed these 50 genes and obtained five common genes: NFKB1, RELA, STAT3, JAK2, and SHC1. Genes in the same module had similar or the same biological functions to affect the development of cerebral ischemia. Thus, it was likely that the three genes STAT3, NFKB1, and RELA in modules 4 and 10 affect autophagy in the development of ischemic stroke. Here, we focused on the analysis of STAT3, NFKB1 and RELA. At the gene-expression level, the three genes were up-regulated in the disease groups compared with the normal groups (Fig. 4). Five-fold cross validation showed that the ROC curves of all the risk genes were >0.80 (Fig. 4C). Therefore, these genes may be potential risk genes in the development of ischemic stroke, which was highly correlated with the

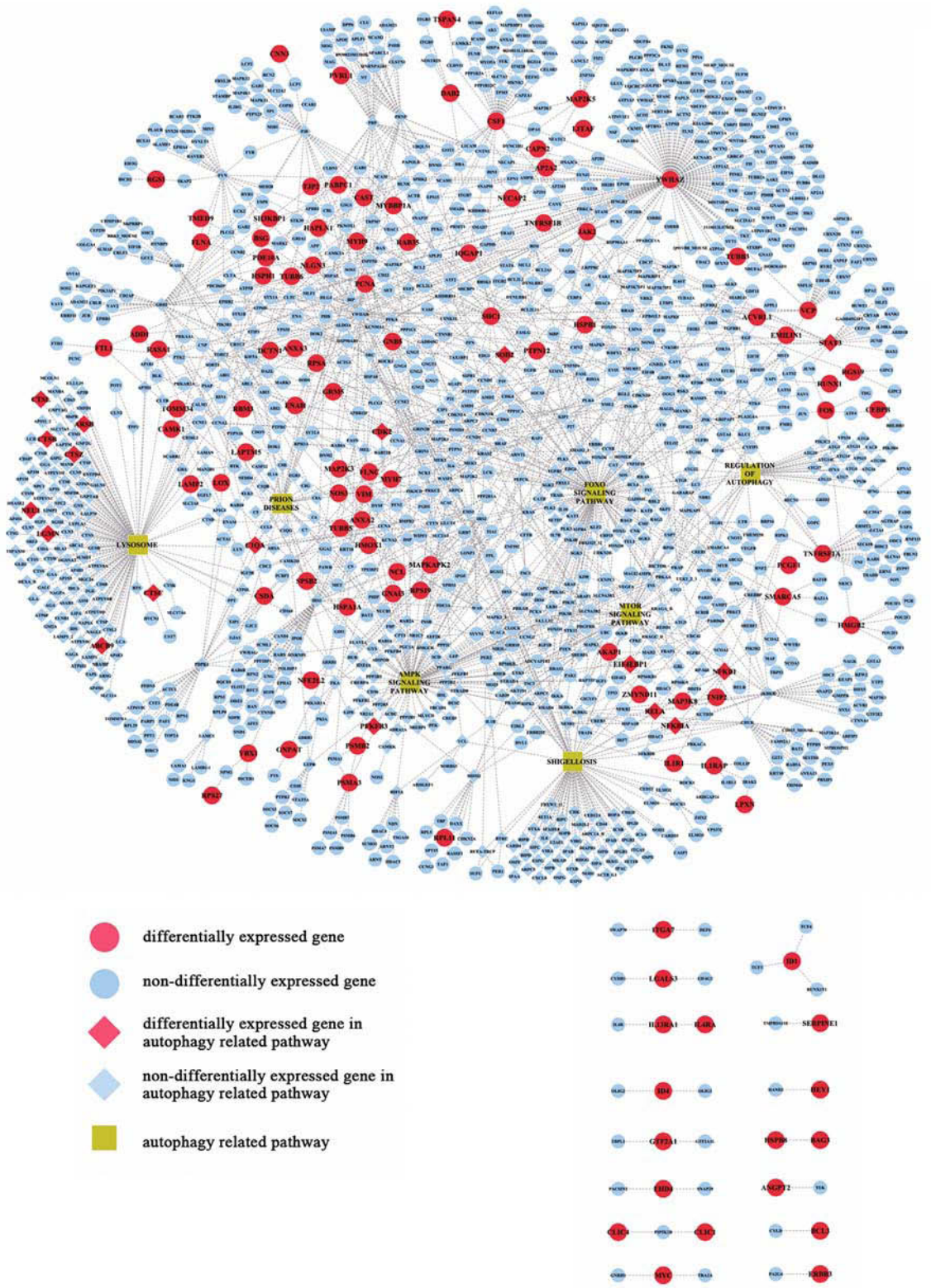

Fig. 2. The autophagy-related pathway network. Red circles, autophagy-related DEGs; blue circles, autophagy-related genes that were not differentially expressed between disease and normal groups; yellow squares, autophagy-related pathways.

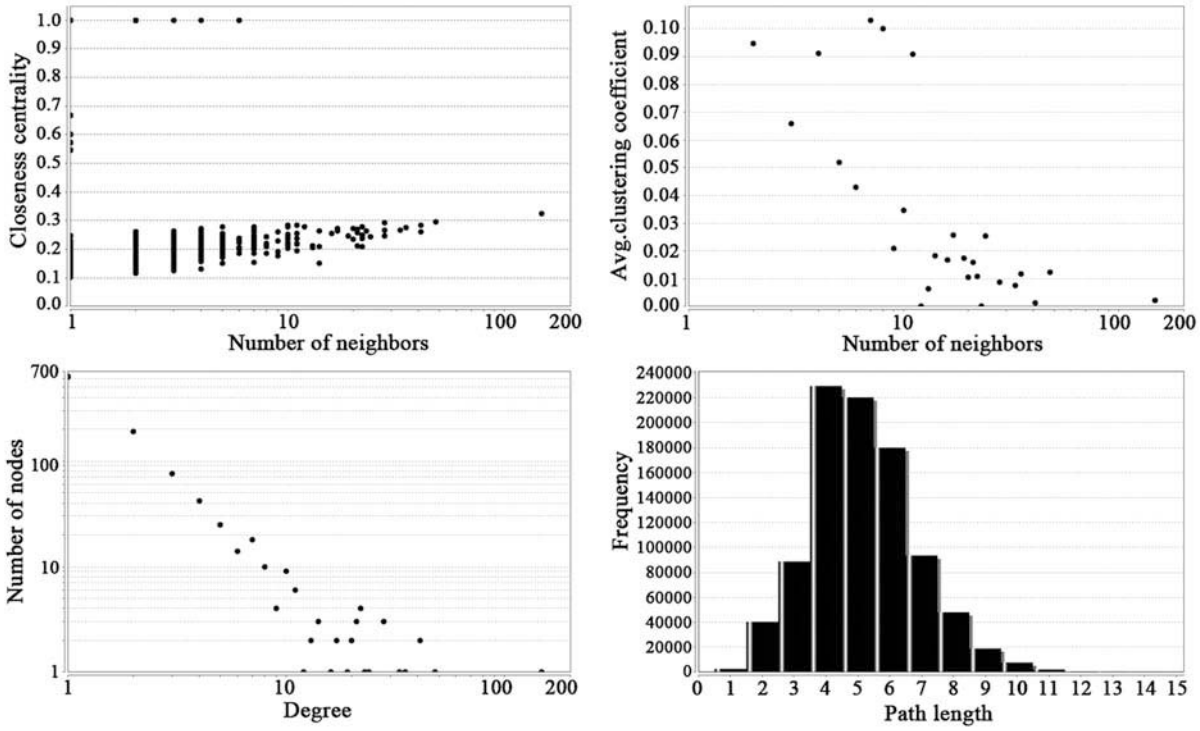

Fig. 3. Topological properties of the ARP network. Closeness centrality, average clustering coefficient, degree distribution, and average shortest path length.

Table 1. Properties of all modules in the ARP network

Modules	Nodes	Edges	P Value	ROC curve	Modular genes
module_1	5	8	0.0313	0.7282	TGFBR2, ACVRL1, CDH5, ENG, TGFBR1
module_2	6	9	0.0019	0.7641	INPP5D, CD22, SOS1, CBL, SHC1, GRB2
module_3	5	7	0.2016	0.5538	PRNP, HSPA5, APP, KCNMA1, PRP
module_4	**5**	**7**	**0.0006**	**0.8769**	**IL6ST, GHR, SHC1, JAK2, STAT3**
module_5	4	5	0.0851	0.6718	CCND1, CDKN1B, CDK4, P27
module_6	4	5	0.1274	0.7641	TNFSF6, FASL, PSTPIP1, PTPN12
module_7	4	5	0.1055	0.6872	PSMA1, PPP2R1A, PSMA3, PSMB2
module_8	4	5	0.1799	0.6256	AP2B1, DNAJC6, AP2A2, YWHAZ
module_9	4	5	0.1373	0.6718	LMNA, WDFY2, AKT, FOXO1
module_10	**3**	**3**	**0.0053**	**0.8513**	**HDAC1, RELA, NFKB1**
module_11	3	3	0.0044	0.7128	BLNK, GRB2, SH3KBP1
module_12	3	3	0.2375	0.6154	RICTOR, MAPKAP1, FRAP
module_13	3	3	0.1318	0.6769	AP1B1, CALM3, YWHAZ
module_14	3	3	0.2311	0.6564	CCNA2, ABL1, CDK2
module_15	3	3	0.0599	0.7231	CLTA, KCNMA1, YWHAZ

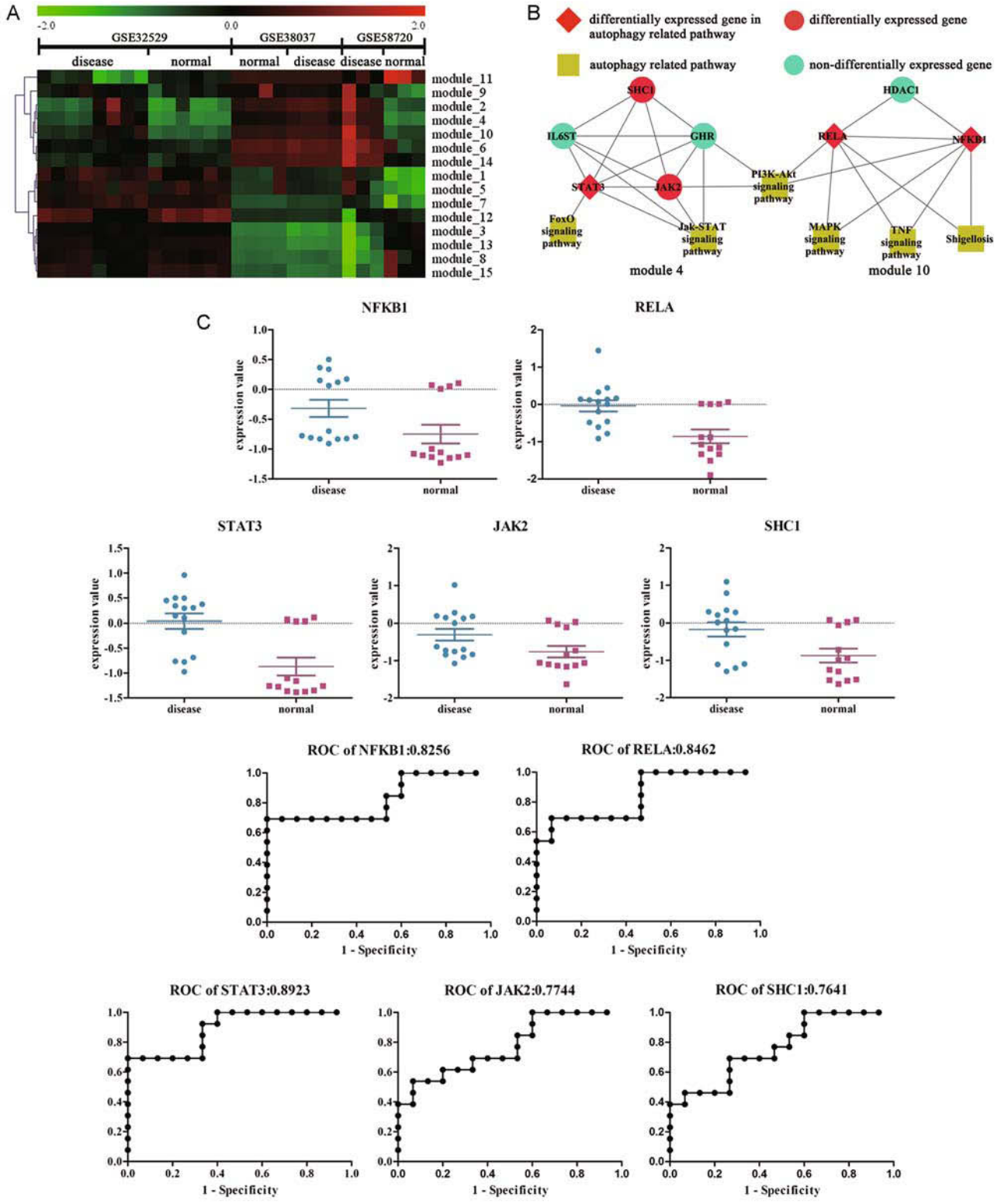

Fig. 4. Modules in the ARP network. A, heat map analysis of module expression. Green to red represents low to high expression. B, differentially-expressed modules in the ARP network. C, expression levels and ROC curves of NFKB1, RELA, STAT3, JAK2, and SHC1.

autophagy related pathways in the ARP network.

DISCUSSION

Autophagy is involved in neuronal death following cerebral ischemia, and plays a pivotal role in the course of ischemic stroke[27-29]. Moreover, autophagic pathways such as the mTOR pathway are closely related to the ischemic brain[30]. However, the regulatory network has not been fully explained. In this study, we used the gene expression profile of ischemic stroke from the GEO to explore possible pathogenesis associated with autophagy. By constructing a gene regulation network, we mined highly-correlated modules, and screened autophagy risk genes. Fifteen modules were obtained from the new network, among which modules 4 and 10 were significantly dysregulated, with ROC values >0.8. These two modules contained 5 DEGs, including RELA, NFKB1, and STAT3, and these three genes are closely associated with the autophagic pathway.

The autophagy-related modules were significantly up-regulated and clustered to influence cerebral ischemia. These modules are involved in several pathways including the FOXO signaling pathway and MAPK signaling pathway. The FOXO pathway was associated with most of the modules, and positive regulation of modules would promote the activation of FOXO pathways. Indeed, activation of FOXO pathways is neuroprotective against transient global cerebral ischemia[31, 32]. The MAPK signaling pathway plays a significant part in cerebral ischemia[33-35]. MAPK is an upstream regulator of mTORC1 and autophagy can be induced *via* the MAPK-mTOR signaling pathway[19]. In fact, the mTOR signaling pathway that induces autophagy has neuroprotective effects on ischemic stroke[19]. This protective autophagy is favorable in the treatment of stroke and avoids unfavorable side-effects[19]. In addition, our results indicated that genes in two modules interacted with each other and commonly stimulated the PI3K-Akt signaling pathway. Thus, our results verified previous studies showing that autophagy-related modules may play vital roles in cerebral ischemia.

Among all the modular genes in the autophagy-related pathway, three risk genes (NFKB1, RELA, and STAT3) that were differentially expressed in cerebral ischemia were extracted. NFKB1 (also known as NFKB or p50) and RELA form a complex in the NF-κB signaling pathway. Commonly, they form a dimer. IκBα, an inhibitor of NF-κB, combines with the dimer, sequestering this complex outside the nucleolus. Once IκBα is degraded, the dimer would be translocated into the nucleolus, initiating transcription[36] and regulating the expression of numerous genes involved in immunity, inflammation, proliferation, and apoptosis[37, 38]. Several studies have indicated that dysregulation of these three factors is significantly involved in brain diseases, while suppression of the NF-κB signaling pathway contributes to neuroprotection against cerebral ischemia[39-41]. NF-κB is activated after cerebral ischemia and can potentiate ischemic injury, activating many genes involved in the pathogenesis of cerebral ischemia, such as TNF-α, IL-1β, IL-6, ICAM-1, iNOS, and COX-2[42]. Results from immunohistological assay and western blot for NF-κB (p65) in the ischemic penumbra of rats showed that flurbiprofen inhibits NF-κB to protect against cerebral ischemia/reperfusion injury[40]. Brain tissue damage has also been correlated with the dysregulated NFKB1[43], blockade of which protects the brain against ischemic damage by regulating inflammatory responses[44, 45]. NFKB1 combines with RELA to form the NFKB1/ RELA complex that is a key molecule in the progression of ischemic stroke[46]. Notably, NFKB1/RELA induces pro-apoptotic transcription in acute brain ischemia and its activation is an important event in ischemic neuronal injury[47]. Blocking NF-κB activation or knocking out the p50 subunit of NF-κB can protect against infarct injury or can result in the development of a smaller infarct volume[48]. Moreover, IκBα is an inhibitor of NF-κB, and its dysregulation prevents brain damage after stroke. The activation of signal transducer and activator of transcription-3 (STAT3), a pivotal part of the JAK-STAT signaling pathway, is reportedly induced by cerebral ischemia[49-51]. Increased expression of the transcripts of IL-6 and JAK2, which are the essential components of STAT3 activation, is also related to the pathogenesis of cerebral ischemic damage[51]. All three risk genes were differentially expressed in cerebral ischemia. It is likely that their dysregulation modulates the development of cerebral ischemia and targeting the three genes would reduce post-ischemic brain injury.

Recent findings have demonstrated that NF-κB complex-related factors (NFKB1 and RELA) also affect the autophagy pathway in cerebral ischemia[52]. Previous studies have shown that RELA and NFKB1 modulate

canonical autophagy and their activation mediates the repression of autophagy[53-55]. RELA has been reported to modulate an increase of autophagy by BECN1[56]. However, in mice with focal cerebral ischemia and NFKB1 knockout, there are significantly more Beclin-1/TUNEL-positive cells than in wild type mice[52], indicating that cerebral ischemia-induced autophagy-like injury is regulated by the NF-κB pathway. It has also been found that a high-fat diet is cardioprotective against ischemia-reperfusion injury involving NF-κB dependent enhancement in autophagy and decreased apoptosis[57]. Moreover, variations within NFKB1 and IκBα can potentially influence the function of NF-κB[58] and in turn the autophagy process. As autophagy participates in the neuronal death and functional loss induced ischemia/reperfusion injury[59-61], genes involved in autophagy would be associated with cerebral ischemia. Both RELA and NFKB1 were up-regulated in cerebral ischemia according to our research and further activated the NF-κB pathway. It has been validated that the NF-κB pathway regulates the autophagy-like injury induced by cerebral ischemia[52]. Besides, inhibition of NF-κB *in vivo* prevents cerebral ischemic injury[62]. IκBα interacts with RELA and NFKB1 to form a complex and competitively regulates autophagy in the development of cerebral ischemia. Autophagy induced by the complex in cerebral ischemia would protect against neuronal injury.

In conclusion, Autophagy is a basic catabolic progress for cell survival under stress. Using a computational bioinformatics approach, we obtained two autophagy-related modules. In accordance with previous reports, our result also verified that the FOXO and MAPK signaling pathways are important in cerebral ischemia. Besides, NF-κB complex-related factors (NFKB1 and RELA) and STAT3 play a key role in inducing autophagy in the development of cerebral ischemia, and are potential targets of therapy.

ELECTRONIC SUPPLEMENTARY MATERIAL

Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s12264-015-1547-3.

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