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Bioinformatics analysis of gene expression profiling for identification of potential key genes among ischemic stroke

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Abstract

This study aimed to identify the key differentially expressed genes (DEGs) following ischemic stroke (IS).

The GSE22255 microarray dataset, which contains samples from peripheral blood mononuclear cells of 20 IS patients and 20 sex- and age-matched controls, was downloaded from the Gene Expression Omnibus. After data pre-processing, DEGs were identified using the Linear Models for Microarray Data package in R. The Search Tool for the Retrieval of Interacting Genes database was used to predict the interactions among the products of DEGs, and then Cytoscape software was used to visualize the protein–protein interaction (PPI) network. DEGs in the PPI network were then analyzed using the Database for Annotation, Visualization, and Integrated Discovery online software to predict their underlying functions through functional and pathway enrichment analyses.

A total of 144 DEGs were identified in IS samples compared with control samples, including 75 upregulated and 69 downregulated genes. Genes with higher degrees in the PPI network included *FOS* (degree = 26), *TP53* (degree = 22), *JUN* (degree = 20), *EGR1* (degree = 18), *JUNB* (degree = 16), and *ATF3* (degree = 15), and these genes may function in IS by interacting with each other (e.g., EGR1-JUN, EGR1-TP53, ATF3-FOS, and JUNB-FOS). Functional enrichment analysis indicated that the downregulated *TP53* gene was enriched in immune response and protein targeting categories.

A*TF3* and *EGR1* may have an important protective effect on IS, whereas *FOS*, *JUN,* and *JUNB* may be associated with the development of IS. In addition, *TP53* may function as an indicator of poor prognosis for IS through its association with the immune response and protein targeting.

Abbreviations: (*IL-1β*) = interleukin-1β, APC = activated protein C, ATF3 = activating transcription factor 3, BCL2 = B-cell leukemia/lymphoma 2, BDNF = brain-derived neurotrophic factor, COX-2 = cyclooxygenase-2, CREB = responsive element-binding, CTMP = carboxyl-terminal modulator protein, DAVID = Database for Annotation, Visualization, and Integrated Discovery, DEGs = differentially expressed genes, EGR1 = early growth response protein 1, GEO = Gene Expression Omnibus, HSP70 = heat shock protein 70, IS = ischemic stroke, JNK = Jun N-terminal kinase, KEGG = Kyoto Encyclopedia of Genes and Genomes, MCA = middle cerebral artery, mTOR = mechanistic target of rapamycin, NCBI = National Center of Biotechnology Information, NF-κB = nuclear factor-kappa B, OGD = oxygen and glucose deprivation, PBMCs = peripheral blood mononuclear cells, PPI = protein–protein interaction, STRING = Search Tool for the Retrieval of Interacting Genes, ZFHX3 = Zinc Finger Homeobox 3.

Keywords: differentially expressed genes, enrichment analysis, ischemic stroke, protein–protein interaction network

1. Introduction

Stroke (also called brain attack, cerebrovascular accident, or cerebrovascular insult) is the second most common cause of death worldwide, ranking only after heart disease.[1,2] Stroke is characterized by symptoms such as inability to move or feel one side of the body, inability to speak, or understand problems and dizziness.[2] Advanced age is one of the main risk factors for stroke. Approximately 95% of patients with stroke are over 45 years of age, and two-thirds of stroke cases happen in those older than 65 years of age.[3,4] Ischemic stroke (IS) induced by lack of blood flow is one of the main types of stroke.[5] In 2013, stroke accounts for 6.4 million deaths (12% of the total) worldwide, and approximately half of these deaths result from IS.[6]

Recent studies have shown that the deletion or downregulation of phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*) gene activates mechanistic target of rapamycin (mTOR) signaling[7] or the PTEN/v-Akt murine thymoma viral oncogene homolog (Akt) pathway[8] to protect against neuronal death after brain injury caused by oxygen and glucose deprivation (OGD) in IS patients. Kallikrein gene transfer has neuroprotective effects in IS patients by the promotion of glial cell migration and survival

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and suppression of apoptosis through the activation of the Akt–B-cell leukemia/lymphoma 2 (BCL2) signaling pathway and inhibition of oxidative stress.[9] The 511C/T interleukin-1β (*IL-1β*) polymorphism plays roles in IS and myocardial infarction among young individuals and in the inflammatory response of mononuclear cells.[10] The rs7193343-T sequence variant of the Zinc Finger Homeobox 3 (*ZFHX3*) gene on chromosome 16q22 is related to IS and cardioembolic stroke in an integrated analysis of 5 sets of stroke samples.[11] However, despite all of this research, the mechanism underlying IS remains unclear.

Atherosclerosis of the thoracic aorta is an independent risk factor for IS, and peripheral blood mononuclear cells (PBMCs) are of significance because blood-borne white blood cells, first polymorphonuclear cells and then mononuclear cells (i.e., PBMCs), respond to IS. Mononuclear cells can selectively migrate and infiltrate into ischemic brain tissue and then adhere to vascular endothelial cells, which is a main event in the development of atherosclerosis.[12,13] In addition, this early cellular inflammation and its consequences may evolve into cerebral infarct and reperfusion injury. In addition, the tail and damage DNA in the PBMCs of IS patients was significantly higher than that in those of convalescent patients.[14] Thus, PBMCs are frequently chosen to detect differentially expressed genes (DEGs) for the prediction of gene changes related to IS.

In 2012, Krug et al[15] performed microarray analysis in PBMCs from 20 IS patients and 20 matched controls and identified 580 DEGs. However, the interactions among gene products and the potential functions of these genes were not analyzed. In our study, the microarray data deposited by Krug et al[15] were studied to further screen DEGs and analyze the protein–protein interaction (PPI) network of their products. Moreover, functions of DEGs in the PPI network were predicted by functional and pathway enrichment analyses. This study may provide new insights into therapeutic targets for IS.

2. Methods

2.1. Microarray data

The gene expression microarray data (GSE22255) deposited by Krug et al[15] were downloaded from the National Center of Biotechnology Information (NCBI) Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/), which was based on the platform of the GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array. GSE22255 included data on PBMCs from 20 IS patients (10 females and 10 males, mean age at examination, 60.2 ± 10.6 years) and 20 sex- and age-matched controls (10 females and 10 males, mean age at examination, 58.7 ± 11.0 years). All of the participants were adult Caucasians, and IS patients had only 1 stroke episode at least 6 months before blood collection. The controls did not have a family history of stroke. Individuals with active allergies or severe anemia were also excluded. The research by Krug et al[15] was approved by the ethics committees of the participating institutions, and all subjects provided informed consent.

2.2. Data pre-processing and DEG screening

After the raw data were downloaded, the Affy package[16] in Bioconductor was used for data pre-processing. Pre-processing included background correction, quantile normalization, and probe ID to gene symbol transformation. Probes were mapped to NCBI entrez genes using Gene ID converter.[17] If there were multiple probes that corresponded to the same gene, their average value was taken as the final gene expression level. Then, the Linear Models for Microarray Data (limma) package in R was used to identify DEGs between IS and control samples. The cut-off criteria for DEGs were defined as a $P < .05$ and a $|\log_2$ fold change (FC)$| > .3$ because a $P < .05$ is commonly used for screening DEGs [18,19] and the $|\log_2 FC| > .3$ combined $P < .05$ was used to control the number of DEGs within a manageable but useful range, not too many or too few.

2.3. Construction of the PPI network

The Search Tool for the Retrieval of Interacting Genes (STRING),[20] a database that facilitates searching of both functional and physical interactions of proteins, was used to predict the interactions among the products of DEGs, with the default threshold of the combined score $> .4$ in STRING. Cytoscape software (http://www.cytoscape.org/), a standard tool for integrated analysis and visualization of biological networks, such as molecular interaction networks and biological pathways, with computer-based assistance, was used to visualize the PPI network.[21] Cytoscape Core are available for layout, scripting, file formatting, and linking the network to databases with functional annotations.[21] In addition, nodes and edges in the figures were used to represent large biological data in an easily interpretable manner. Nodes represent biological molecules and edges connect the nodes to indicate their relationship.[22] The pivotal nodes in the PPI network were identified based on their connectivity degrees.

2.4. Functional and pathway enrichment analyses

GO analysis is dedicated to the functional study of large-scale transcriptomic or genomic data.[23] The Kyoto Encyclopedia of Genes and Genomes (KEGG) database provides information on function of molecules or genes.[24] The genes with their products included in the PPI network were subjected to the Database for Annotation, Visualization, and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov)[25] algorithm to find significantly related biological processes and KEGG pathways. The thresholds were set at a $P < .05$ and an enriched gene count > 2.[26]

3. Results

3.1. Identification of DEGs

After pre-processing, a total of 144 DEGs were identified in IS samples compared with control samples, including 75 upregulated genes [e.g., *FOS*, *JUN*, *JUNB*, early growth response protein 1 (*EGR1*), and activating transcription factor 3 (*ATF3*)] and 69 downregulated genes [e.g., tumor-suppressor protein p53 (*TP53*)].

3.2. PPI network analysis

The PPI network for DEGs is shown in Fig. 1, which has 59 nodes and 212 interactions. The top 20 nodes with higher degrees are listed in Table 1, including FOS (degree = 26), TP53 (degree = 22), JUN (degree = 20), EGR1 (degree = 18), JUNB (degree = 16), and ATF3 (degree = 15). In particular, EGR1, JUNB, and ATF3 could interact with each other and also had interactions with other proteins (e.g., EGR1-JUN, EGR1-FOS, EGR1-TP53, ATF3-FOS, ATF3-JUN, JUNB-JUN, and JUNB-FOS).

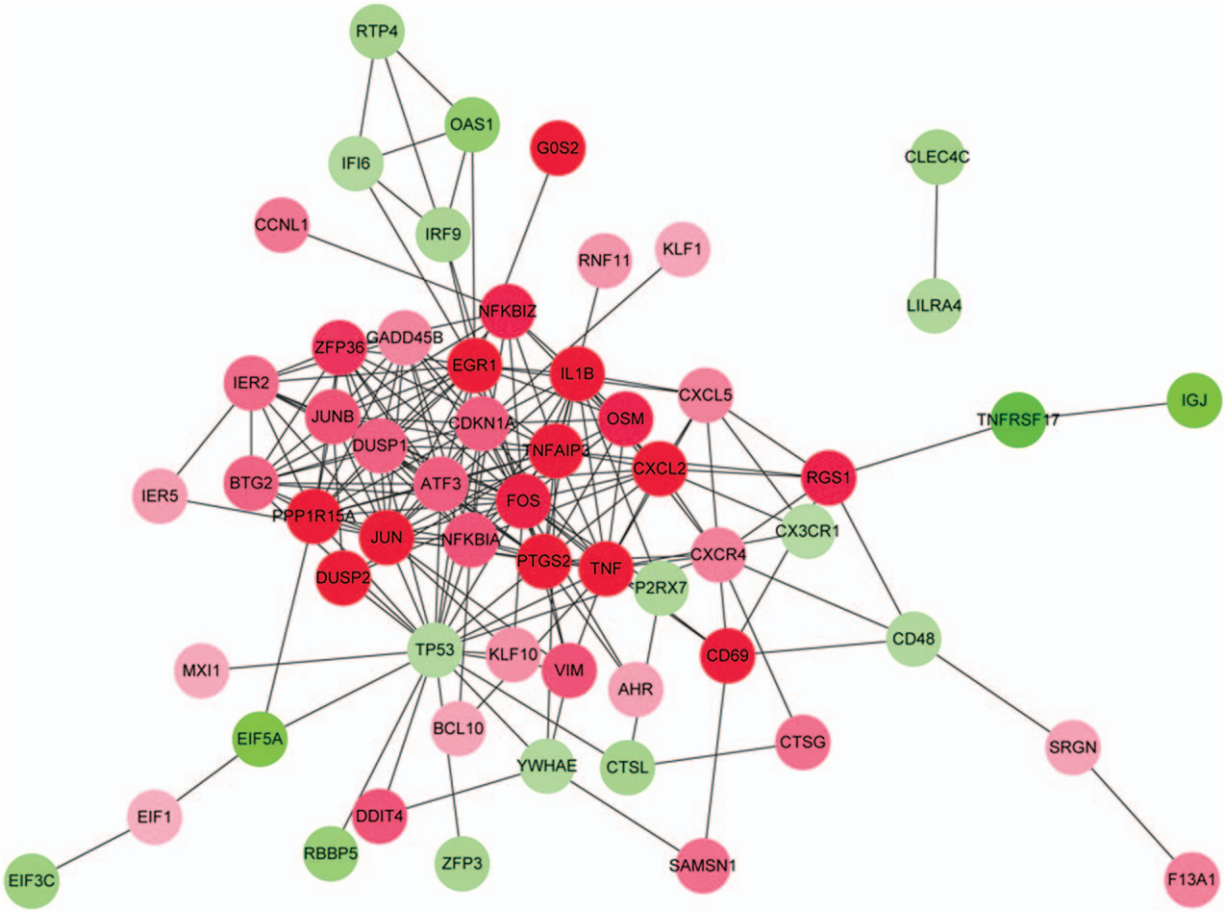

Figure 1. The protein–protein interaction (PPI) network for the differentially expressed genes (DEGs) in ischemic stroke (IS) samples. Red and green nodes represent upregulated genes and downregulated genes, respectively. The nodes with a deeper color had larger $|\log_2$ fold change (FC)| values.

3.3. Enrichment analysis for DEGs in the PPI network

DEGs in the PPI network were further subjected to DAVID software analysis to find significant biological processes and KEGG pathways. Functional enrichment analysis revealed that the upregulated genes were significantly enriched in response to the organic substance category ($P=1.86E-08$, which involved *EGR1*, *FOS*, *JUN*, and *JUNB*), apoptosis ($P=2.54E-08$, which involved *JUN*), and programmed cell death ($P=2.99E-08$, which involved *JUN*) (Table 2). Moreover, these genes were mainly involved in the MAPK signaling pathway ($P=3.01E-04$, which involved *FOS* and *JUN*), T cell receptor signaling pathway ($P=5.83E-04$, which involved *FOS* and *JUN*), and cancer pathways ($P=3.04E-02$, which involved *FOS* and *JUN*) (Table 3).

Furthermore, the downregulated genes were involved in immune responses ($P=5.80E-05$, which involved *TP53*), protein targeting ($P=1.56E-03$, which involved *TP53*) and mitochondrial depolarization regulation ($P=7.74E-03$) (Table 2). However, no pathway was significantly enriched for the downregulated genes.

4. Discussion

In this study, a total of 144 DEGs were identified in IS samples compared with control samples, including 75 upregulated genes and 69 downregulated genes. FOS (degree=26), TP53 (degree=22), JUN (degree=20), EGR1 (degree=18), JUNB (degree=16), and ATF3 (degree=15) had higher degrees in the PPI network of the DEGs. Importantly, EGR1, JUNB, and ATF3 could interact with each other and also had interaction with other proteins (e.g., EGR1-JUN, EGR1-FOS, EGR1-TP53, ATF3-FOS, ATF3-JUN, JUNB-JUN, and JUNB-FOS). Thus, these genes might function in IS through such interactions.

Moonlighting proteins are a subset of multifunctional proteins that have 2 or more different functions performed by 1 polypeptide chain.[27] Our predicted hub genes in the PPI network such as *ATF3*, *FOS*, and *JUN* are reported to be moonlighting proteins in the Moonprot database[28] and may be related to IS by regulating a variety of multifunctional mechanisms. *ATF3* encodes a member of the activation transcription factor/adenosine 3′, 5′-monophosphate (cAMP) responsive element-binding (CREB) protein family.[29] Because it exists in different isoforms and has many transcription factor binding sites, such as activation protein 1 (AP-1) and nuclear factor-kappa B (NF-κB), *ATF3* plays a crucial role in cellular stress responses, wound healing, cell adhesion, oncogenesis, and multiple signal transduction pathways.[30] Recently, *ATF3* is found to be significantly overexpressed in brain ischemia. Its knockdown worsens the brain injury and inflammatory responses through activating the NF-κB signaling pathway, indicating that *ATF3* may serve as a key protective regulator in ischemic injury.[31]*ATF3* inhibits carboxyl-terminal modulator protein (*CTMP*) expression through binding to the ATF/cAMP/

Table 1

The top 20 gene products with higher degrees in the protein–protein interaction network for the differentially expressed genes (DEGs).

Gene	Degree	Gene	Degree	Gene	Degree	Gene	Degree
FOS	26	*TNF*	16	*ATF3*	15	*CXCL2*	12
TP53	22	*TNFAIP3*	16	*DUSP1*	15	*BTG2*	11
JUN	20	*NFKBIA*	16	*IL1B*	14	*IER2*	11
EGR1	18	*JUNB*	16	*CDKN1A*	14	*GADD45B*	11
PTGS2	17	*PPP1R15A*	15	*ZFP36*	13	*CXCR4*	10

CREB site and *CTMP* siRNA treatment maintains *ATF3* levels. Furthermore, the ATF3 to CTMP signaling cascade may be endogenously neuroprotective and assist in repair of ischemic brain injury.[32]*ATF3* affects the pathways of caspase-dependent neuronal apoptotic signal transduction that are induced by reperfusion injury and focal cerebral ischemia.[33]*ATF3* expression and synaptic activity promote resistance of hippocampal neurons to loss of synapses and acute dendrotoxicity, which contributes to functional restoration of neuronal networks after excitotoxic insults.[34] Therefore, *ATF3* may have an important protective effect in IS.

FOS (including c-Fos, FosB, Fra1, and Fra2) and JUN (including c-Jun, JunB, and JunD), members of the AP-1 family, can dimerize in various combinations via a leucine zipper region.[35] They have multiple biochemical functions, including cell proliferation, transformation, and apoptosis[36], as well as responding to DNA damage.[37] Pathway enrichment suggested that the upregulated *FOS* and *JUN* were enriched in the MAPK signaling pathway, the T cell receptor signaling pathway, and cancer pathways. It has been demonstrated that the MAPK-c-Jun N-terminal kinase (JNK) signaling pathway is involved in injury, and blocking this pathway prevents apoptosis in areas of aminoglycoside damage.[38] Reportedly, the protease-resistant peptide of JNK can function as a neuroprotective agent for stroke through preventing c-Fos transcription and c-Jun activation.[39] In the rat brain, after middle cerebral artery occlusion and a 2-hour reperfusion, the expression of *FOS*, brain-derived neurotrophic factor (*BDNF*), cyclooxygenase-2 (*COX-2*), and heat shock protein 70 (*HSP70*) are inhibited by magnesium sulphate, which has therapeutic potential.[40,41] Furthermore, the mRNA expression of *FOS* and *HSP70* is significantly repressed by hypothermia, which is associated with decreased lesion expansion in the process of acute focal ischemia.[42,43] Together, these findings indicate that *FOS*, *JUN*, and *JUNB* might be associated with the development of IS.

Functional enrichment analysis showed that upregulated *EGR1* was enriched in response to the organic substances category. Previous studies have shown that *EGR1* can regulate the transcriptional network in gliosis caused by ischemic injury.[44]*EGR1* expression may protect against neuronal damage as well as ameliorate thrombosis and inflammation in stroke.[45,46]*EGR1* may act as an important protective

Table 2

The top 10 biological processes for both upregulated differentially expressed genes (DEGs) and downregulated DEGs in the protein–protein interaction (PPI) network.

DEGs	Term	Name	Count	Gene symbol	*P*
Upregulated genes	GO:0010033	Response to organic substance	14	*EGR1, BCL10, TNF, PTGS2, KLF10, NFKBIA, JUNB, FOS, CDKN1A, BTG2, DUSP1, JUN, IL1B, PPP1R15A*	1.86E-08
	GO:0006915	Apoptosis	13	*BCL10, TNF, NFKBIA, AHR, DDIT4, OSM, CXCR4, JUN, IL1B, GADD45B, TNFAIP3, PPP1R15A, SRGN*	2.54E-08
	GO:0012501	Programmed cell death	13	*BCL10, TNF, NFKBIA, AHR, DDIT4, OSM, CXCR4, JUN, IL1B, GADD45B, TNFAIP3, PPP1R15A, SRGN*	2.99E-08
	GO:0051384	Response to glucocorticoid stimulus	7	*FOS, CDKN1A, TNF, PTGS2, DUSP1, IL1B, JUNB*	5.13E-08
	GO:0031960	Response to corticosteroid stimulus	7	*FOS, CDKN1A, TNF, PTGS2, DUSP1, IL1B, JUNB*	8.62E-08
	GO:0008219	Cell death	13	*BCL10, TNF, NFKBIA, AHR, DDIT4, OSM, CXCR4, JUN, IL1B, GADD45B, TNFAIP3, PPP1R15A, SRGN*	1.78E-07
	GO:0016265	Death	13	*BCL10, TNF, NFKBIA, AHR, DDIT4, OSM, CXCR4, JUN, IL1B, GADD45B, TNFAIP3, PPP1R15A, SRGN*	1.92E-07
	GO:0009617	Response to bacterium	8	*BCL10, FOS, TNF, PTGS2, JUN, NFKBIA, IL1B, CTSG*	6.35E-07
	GO:0014070	Response to organic cyclic substance	7	*FOS, CDKN1A, PTGS2, BTG2, JUN, IL1B, JUNB*	7.07E-07
	GO:0051051	Negative regulation of transport	7	*OSM, TNF, PTGS2, NFKBIA, IL1B, MXI1, SRGN*	1.35E-06
Downregulated genes	GO:0006955	Immune response	7	*LILRA4, IGJ, TP53, TNFRSF17, CLEC4C, OAS1, IFI6*	5.80E-05
	GO:0006605	Protein targeting	4	*RTP4, TP53, EIF5A, YWHAE*	1.56E-03
	GO:0006886	Intracellular protein transport	4	*RTP4, TP53, EIF5A, YWHAE*	7.45E-03
	GO:0051900	Regulation of mitochondrial depolarization	2	*P2RX7, IFI6*	7.74E-03
	GO:0042110	T cell activation	3	*CD48, P2RX7, TP53*	8.35E-03
	GO:0034613	Cellular protein localization	4	*RTP4, TP53, EIF5A, YWHAE*	9.65E-03
	GO:0070727	Cellular macromolecule localization	4	*RTP4, TP53, EIF5A, YWHAE*	9.84E-03
	GO:0007005	Mitochondrion organization	3	*P2RX7, TP53, IFI6*	9.94E-03
	GO:0042981	Regulation of apoptosis	5	*P2RX7, TP53, EIF5A, YWHAE, IFI6*	9.98E-03
	GO:0043067	Regulation of programmed cell death	5	*P2RX7, TP53, EIF5A, YWHAE, IFI6*	1.03E-02

Table 3

The pathways involved the upregulated genes in the protein–protein interaction (PPI) network.

DEGs	Term	Name	Count	Gene symbol	*P*
Upregulated genes	hsa04621	NOD-like receptor signaling pathway	5	*TNF*, *CXCL2*, *NFKBIA*, *IL1B*, *TNFAIP3*	6.77E-05
	hsa04010	MAPK signaling pathway	7	*FOS*, *TNF*, *DUSP2*, *DUSP1*, *JUN*, *IL1B*, *GADD45B*	3.01E-04
	hsa04620	Toll-like receptor signaling pathway	5	*FOS*, *TNF*, *JUN*, *NFKBIA*, *IL1B*	4.52E-04
	hsa04660	T cell receptor signaling pathway	5	*BCL10*, *FOS*, *TNF*, *JUN*, *NFKBIA*	5.83E-04
	hsa04060	Cytokine-cytokine receptor interaction	6	*OSM*, *TNF*, *CXCL5*, *CXCR4*, *CXCL2*, *IL1B*	2.24E-03
	hsa04662	B cell receptor signaling pathway	4	*BCL10*, *FOS*, *JUN*, *NFKBIA*	2.52E-03
	hsa05200	Pathways in cancer	5	*FOS*, *CDKN1A*, *PTGS2*, *JUN*, *NFKBIA*	3.04E-02
	hsa04062	Chemokine signaling pathway	4	*CXCL5*, *CXCR4*, *CXCL2*, *NFKBIA*	3.07E-02
	hsa04210	Apoptosis	3	*TNF*, *NFKBIA*, *IL1B*	4.10E-02

regulator in IS through its induction in response to organic substances. Functional enrichment analysis indicated that downregulated *TP53* was enriched in the immune response and protein targeting categories. The Arg/Arg genotype of *TP53* controls neuronal vulnerability to apoptosis and is a predictor of poor prognosis for stroke.[47] Through preventing p53-mediated apoptosis in brain cells, activated protein C (*APC*) functions as a protector of the brain after ischemic injury.[48–50] Thus, we suggest that similarly, *TP53* functions as a predictor of poor prognosis for IS, through functions involving immune responses and protein targeting.

In addition, it is worth noting that although we used the same GSE22255 dataset as that used in a study by Bi et al,[51] we used different cut-off criteria for screening DEGs. Our threshold value was less restrictive than theirs to avoid a small number of DEGs being identified, which could limit the ensuing bioinformatics analysis, because only 27 DEGs were screened in their study. Moreover, we have constructed the PPI network to analyze the interrelationship among DEGs. However, there were still some limitations in the present study. First, although the PPI network and enrichment analyses for DEGs were performed, the prediction of transcription factors and miRNAs that regulate DEGs as well as a coexpression network analysis are still needed to potentially offer a more comprehensive insight into IS. Second, although the cut-off criteria of a $P < .05$ and $|\log_2 (FC)| > .3$ were appropriate and common for screening DEGs, a stricter threshold value is needed to screen for highly significant genes. However, with the above criteria, we only identified 144 DEGs, which is relatively few, and thus, we decided against using a stricter threshold value. Lastly, although we speculated that several genes, such as *ATF3*, *EGR1*, *FOS*, *JUN*, *JUNB*, and *TP53*, were closely associated with IS; further experiments should be performed to validate this.

In conclusion, through bioinformatics analysis, a total of 144 DEGs were identified in IS samples compared with control samples. *ATF3* and *EGR1* might have an important protective effect on IS, whereas *FOS*, *JUN*, and *JUNB* might be associated with the development of the IS response. In addition, *TP53* may function as a predictor of poor prognosis for IS through immune responses and protein targeting. However, these results should be confirmed by further experimental research.

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