doi: 10.4149/gpb_2024023

Bioinformatics screening and verification of ischemic stroke-related key genes and drug prediction

Shuai Jiang[1], Zhanhui Liu[1], Xian Zhang[1], Rui Zhang[1], Bo Sun[1], Chao Gao[1], Pengfei Hou[1] and Meirong Sun[2]

[1] *Department of Neurosurgery, The Ninth Hospital of Xi'an, Xi'an, China*
[2] *Department of Gynaecology and Obstetrics, The Central Hospital of Xi'an, Xi'an, China*

Abstract. Stroke is one of the major causes of disability and death worldwide. The lack of effective medical treatment for stroke heightens the need for new therapeutic targets. In this study, we obtained two microarray data sets from the Gene Expression Omnibus (GEO) database and identified differential genes (DEGs) between MCAO and control groups. Then, enrichment analysis of the DEGs was performed using DAVID and Metascape. The results show 27 DEGs shared between the two datasets. The functional enrichment analysis showed that these genes are mainly enriched in immune response, complement and coagulation cascades, apoptotic processes. The four hub genes (C1qc, Fcgr2b, C1qb, and Cd14) were screened out using the Cytoscape. Next, real-time PCR and Western blot analysis showed that expression of C1q and CD14 increased at 14 days after tMCAO. Furthermore, we took eight small molecule compounds with the lowest score using Cmap and studied their background characteristics. These results are built on a meta-analysis of data, which are generally accessible from the online space. Finally, we evaluated the protective effect of the rolipram through behavior tests after tMCAO, and results showed that the rolipram significantly attenuated neurobehavioral dysfunction at 14 days after brain ischemia. The present results provide novel insights into the biological process and potential therapeutic drugs involved in stroke.

Key words: Bioinformatics analysis — Ischemic stroke — C1q — CD14 — Rolipram

Introduction

Stroke is the second leading cause of death globally, posing a significant threat to human well-being. Approximately 5.5 million individuals die from stroke annually worldwide (Campbell et al. 2019). The remaining physical disability and neurological dysfunction after onset bring great mental pressure and economic burden to the patients and their families. The risk of stroke increases with age, and 87% of stroke patients are ischemic stroke patients (Benjamin et al. 2019; Zhou M et al. 2019).

Correspondence to: Meirong Sun, Department of Gynaecology and Obstetrics, The Central Hospital of Xi'an, 161 Xiwu Road, Xi'an, 710004, China
E-mail: mr_sun2023@163.com

Ischemia has induced nerve cell damage, and its pathological process is complex. It involves various mechanisms, including inflammatory response, excitatory amino acid toxicity, intracellular calcium overload, energy metabolism disorders, free radical damage, and apoptosis (Langhauser et al. 2012; Zhao et al. 2018; Brenna et al. 2020). After cerebral ischemia-reperfusion injury, the inflammatory cascade is activated, and the production of reactive oxygen species and free radicals in brain cells increases (Dodson et al. 2017; Zhou F et al. 2019). Moreover, studies have shown that innate immune responses accompanying ischemic stroke involve various immune cells and various pathways, and contribute significantly to stroke pathophysiology (Kurisu et al. 2019). Altogether, immune responses and inflammation have emerged as important elements in the pathological progres-

sion of stroke. However, these mechanisms are intricate and have not been fully elucidated.

At present, the main clinical treatment of cerebral ischemia includes cerebral vascular recanalization and neuroprotection. Antiplatelet aggregation drugs can be used to treat cerebral ischemia, but studies have pointed out that the long-term combined use of antiplatelet aggregation drugs increases the risk of hemorrhage and causes cerebral hemorrhage, resulting in serious consequences (Savarese et al. 2016; Signorelli et al. 2020). Utilizing a growing understanding of stroke pathophysiological pathways, many small molecule inhibitors (SMIs) have been developed to inhibit thrombosis, reduce ischemia-induced excitotoxicity, ameliorate mitochondrial injury, and attenuate the inflammatory response (Flores et al. 2014). However, further study is still needed to confirm their effectiveness.

Bioinformatics analysis might be a valuable tool for stroke research. To find more effective therapeutic targets and potential drugs, many scientists look for genes closely related to cerebral ischemia using bioinformatics methods (Zhu et al. 2019). Therefore, we used bioinformatics technology to analyze public databases and identify ischemic stroke key genes, and performed enrichment analysis to identify pathways playing an important role in ischemic stroke. Then, the key genes' mRNA and protein expression levels were verified in experimental mice. Lastly, we attempted to predict potential stroke treatment drugs and investigated the usefulness of these drugs by evaluating their protective effect in experimental mice. We expect to find potential therapeutic drugs and provide insights for the development of ischemic stroke treatments.

Methods

Microarray data

To obtained more comprehensive and accurate results, the data expression profiles of stroke were searched in the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/), and two independent datasets of research on MCAO (middle cerebral artery occlusion), GSE97537 and GSE52001, were included in our study. Then, screened differential genes (DEGs) of two datasets were used Venn Diagram to construct the co-expression DEGs (Co-DEGs).

The GSE97537 data set contained 5 sham samples and 7 ischemic samples. GSE52001 contained 3 sham samples and 3 ischemic samples. The data type was expression profiling by array, and the species was Rattus norvegicus. Subsequently, these datasets were extracted from the total RNA of brain tissues for mRNA gene expression profile analysis (Table 1).

Identification of DEGs

We applied an interactive web tool GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r) which uses the GEO query and Limma R packages from the Bioconductor project to perform comparisons against the processed data tables provided by the original submitter. The cut-off criteria were $|\log_2 \text{fold change (FC)}| > 1$ and adjust p-value < 0.05. Volcano plots were also obtained by GEO2R.

Co-DEGs annotation

Database for Annotation, Visualization and Integrated Discovery (DAVID) (https:// david.ncifcrf.gov/home.jsp) (Huang et al. 2007) and Metascape (http://metascape.org/gp/index.html) (Zhou Y et al. 2019) are two common enrichment tools. The KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analysis was used by David v6.8. We also uploaded the Co-DEGs and performed annotation of biological processes in Metascape. The difference was statistically significant at $p < 0.05$.

Hub genes identification and expression

The Search Tool for the Retrieval of Interacting Genes (STRING) (http://string-db.org) (Szklarczyk et al. 2019) can convert Co-DEGs into expressed proteins and structure the PPI (protein-protein interaction) network. We got a PPI network of Co-DEGs through STRING and visualized it by Cytoscape (version 3.8.0) (Shannon et al. 2003).

Molecular Complex Detection tool (MCODE) (version 1.6.1), an open plug-in of Cytoscape, was performed to identify system modules from the PPI network. The criteria were that the MCODE scores > 5, maximum depth = 100, cut-of = 2, k-score = 2, and node score cut-off = 0.2. In addition, CytoHubb was also used to screen out hub genes and sequenced them by the arithmetic of MCC (maximal clique centrality). The Degree of each node was calculated and the

Table 1. Basic information about the datasets

GEO	Platforms	Number of samples		Organism	Tissue	References
		Sham	Ischemic			
GSE97537	GPL1355	5	7	Rattus norvegicus	Brain	Takuma et al. (2017)
GSE52001	GPL14746	3	3	Rattus norvegicus	Brain	Lai et al. (2015)

network composed of the top 10 genes with Degree score was regarded as the core sub-network. Finally, the candidate genes with the highest MCODE score module and the top 10 genes with a degree score of MCC were integrated, and then the intersection was defined as hub genes.

Drug prediction of ischemic stroke

The Connectivity Map (Cmap) database can be used to compare the similarity between drug-induced gene profiles and gene expression and obtain a connectivity score ranging from −100 to +100. A score of less than 0 indicates that the compound may have therapeutic effects on the disease (Jiang et al. 2021). This paper uploaded the differentially up-regulated Co-DEGs to the Cmap online database (https://clue.io/) for drug prediction. The small molecule compounds with the lowest connectivity score were included as the result of this prediction. The main mechanism of these small molecule compounds was summarized by a literature search.

Animals and experimental design

Wild-type C57BL/6J mice (10–12-week-old, male) were purchased from Vital River Laboratory Animal Technology (Beijing, China). All mice were kept on a standard 12-h light/dark cycle with *ad libitum* access to food and water. All animal procedures were approved by the Animal Research Ethics Committee of Xi'an Ninth Hospital Animal Laboratory. These experiments were carried out in accordance with the National Institutes of Health Guide for the Care and use of Laboratory Animals (National Institutes of Health Publication No. 80-23, revised in 1996). In total, 60 mice were used for the experiments. Mice were euthanized at 14 days after transient middle cerebral artery occlusion (tMCAO) for real-time PCR and Western blot analysis. PCR and Western-blot analyzes were performed in samples obtained from 2 experimental groups: simulated and model groups. Each experimental group included 6 mice. Behavior tests were performed at 0, 1, 3, 7, and 14 days after tMCAO. For behavior tests, animals were assigned into the following four groups: sham, model, rolipram (low dose), and rolipram (high dose). Each experimental group included 12 mice.

Animal surgery and drug administration

tMCAO was performed as previously described (Jin et al. 2011). Briefly, mice were anesthetized with 1.5% isoflurane using a Rodent Anesthesia Machine (RWD Life Science, Shenzhen, China). Then, the right common carotid artery, internal carotid artery (ICA), and external carotid artery (ECA) were surgically exposed. A silicone-coated surgical nylon filament was inserted into the right ICA through the ECA stump to block the origin of the middle cerebral artery. After 1 h of occlusion, the filament was gently removed for the reperfusion. The sham group mice underwent the same procedures except for filament insertion. Warming pads were used to maintain the body temperatures at 37.0 ± 0.5°C during surgery. Two hours after the induction of tMCAO, the rolipram was administered intraperitoneal injection once daily for one week at the same time points. Two doses of rolipram were applied in the present study: a low dose of 1.25 mg/kg and a high dose of 2.5 mg/kg.

Real-time PCR

At 14 days after tMCAO, brain samples were collected from the peri-infarct cortex. Total RNA was isolated from tissues using TRIzol Reagent (Vazyme, China) to manufacture protocol. First, total RNA was used for the reverse transcription reaction. Then, the pre-amplified cDNA samples were mixed with One-Step SYBR PrimeScript PLUS RT-PCR Kit (Vazyme, China). Finally, Quantitative PCR was conducted on an ABI7000 Real-time PCR system (Applied Biosystems, Inc., University Park, IL, USA).

GAPDH was used as a positive control. Primer sequences are shown as follows in Table 2.

Western blot

At 14 days after tMCAO, brain samples were collected from the periinfarct cortex. Total proteins were extracted using RIPA buffer (Beyotime Biotechnology, Haimen, China) with cOmplete™ protease inhibitor cocktail (Roche, Indianapolis, IN, USA). The protein concentrations were measured using a BCA protein assay kit (Beyotime Biotechnology). Next, 30 μg *per* lane proteins were separated on SDS polyacrylamide gels, and transferred to polyvinyl-difluoride membranes (Millipore, USA). The membranes were blocked with 5% (w/v) bovine serum albumin (BSA) (Sigma, USA) containing 1/1000 Tween 20 for 1 h and probed with primary antibodies overnight at 4°C. The primary antibodies:

Table 2. Primer sequences for polymerase chain reaction amplification

Gene	Sequence
CD14	F: (5'-3'): TGG GCG AGA AAG GAC TGA T
	R: (5'-3'): AGG AGC AAA GCC AAA GTT CC
C1qc	F: (5'-3'): AAG GAR GGG TAC GAC GGA CT
	R: (5'-3'): GTA AGC CGG GTT CTC CCT TC
GAPDH	F: (5'-3'): ACC ACA GTC CAT GCC ATC AC
	R: (5'-3'): TCC ACC ACC CTG TTG CTG TA

F, forward; R, reverse.

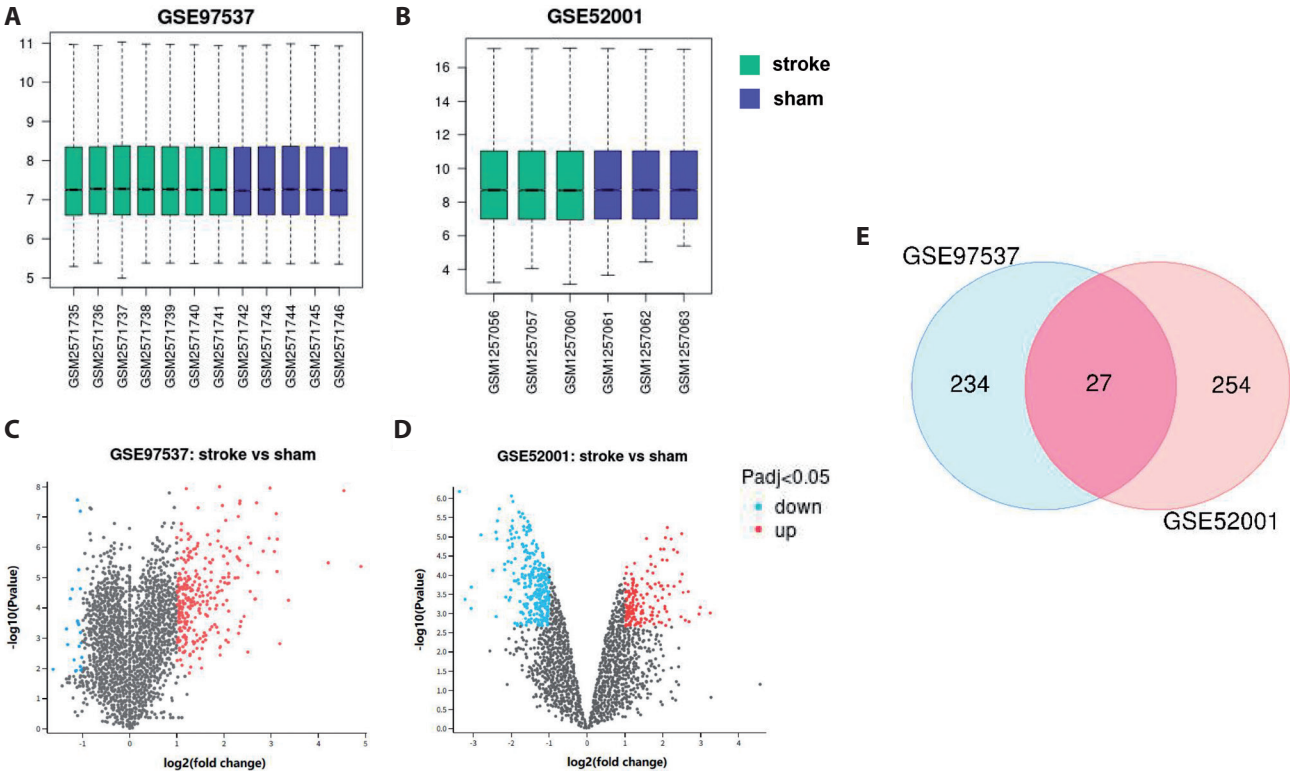

Figure 1. The DEGs information about the GSE97537 and GSE52001 datasets. **A.** Boxplot of GSE97537 gene chip. **B.** Boxplot of GSE52001 gene chip. **C.** The volcano plots present DEGs in the GSE97537. **D.** The volcano plots present DEGs in the GSE52001. **E.** The Venn diagram figured out 27 Co-DEGs between the GSE97537 and GSE52001 datasets.

anti-CD14 (Proteintech, 17000-1-AP, 1:500), anti-C1qc (Proteintech, 16889-1-AP, 1:500) and anti-β-actin (Proteintech; 20,536-1-AP). The membranes were washed three times with TBST (7 min each) and then incubated with corresponding secondary antibody (goat anti-rabbit IgG (H+L) secondary antibody, 1:5000), at room temperature for 1 h. After rinsing with TBST buffer three times, the labeled proteins were detected using the Tanon 5200 Chemiluminescent Imaging System (Tanon Science & Technology. China). β-actin was used as an internal loading control.

Behavior test

(1) Beam-walking test
The motor performance and coordination of mice were evaluated with the beam-walking test. The beam-walking test was performed as previously described (Qiao et al. 2019). Briefly, the beam-walking unit consisted of a 1 m beam with a smooth and flat surface (2.8 cm width) raised 50 cm above the floor. The mice were motivated to cross the beam by placing their home cage at the opposite end.
(2) Corner test
The corner test was performed as previously described with slight modification (Qiao et al. 2019). The mouse was placed between two boards with an angle of 30° facing the corner. The mouse was placed facing the corner halfway between the corner and the open ends of the boards. The non-ischemic rats turned non-selectively on both sides, but the tMCAO rats preferentially turned toward the non-impaired side. There was a 10 s rest between each trail and the turns toward the non-impaired side were recorded.
(3) The grip strength test
The strength grip test was used to test the motor coordination of the mouse. During the test, the grip strength test was performed using the grip strength meter (BSBIOGS3, Panlab, Harvard Apparatus). Each mouse was allowed to hold a metal grid with forelimbs and then pulled backward in the horizontal plane until they could no longer hold the grid. Grip strength was scored as grams (g) unit.

Statistical analysis

The GraphPad Prism Software 8 (La Jolla, CA, USA) was used for statistical analysis and the results were presented as the means of ±SEM. For experiments with more than two groups and one factor of the parameters, results were compared using a one-way analysis of variance (ANOVA) followed by Bonferroni's test. For experiments with more

than two groups and two factors of the non-parameters, results were compared using two-way ANOVA followed by Bonferroni's or Tukey's test. All tests were considered statistically significant difference at $p < 0.05$.

Results

Identification of Co-DEGs

After normalization, all samples had fundamentally the same gene expression mean values (Fig. 1A,B), indicating that the sample data sources were reliable. The volcano plot presents the DEGs between the ischemic and sham samples of the GSE97537, and GSE52001 datasets, respectively (Fig. 1C,D). While the Venn diagram shows the 27 DEGs shared between the two datasets (Fig. 1E), including 22 upregulated genes and 2 downregulated genes, the other three genes showed opposite expression trends in the two datasets (Table 2).

Co-DEGs annotation

To analyze the biological classification of the Co-DEGs, we performed functional and pathway enrichment analyses using Metascape and DAVID. First, Co-DEGs were uploaded to Metascape, and it was shown that the biological processes were remarkably enriched in response to wounding, negative regulation of immune response, regulation of tumor necrosis factor production, and gliogenesis (Fig. 2A,B). DAVID was utilized to analyze the KEGG functional of Co-DEGs. The KEGG pathway analysis demonstrated that the Co-DEGs were mainly enriched in pertussis, complement and coagulation cascades, staphylococcus aureus infection, and Chagas disease (Fig. 2C).

Module analysis of the key genes

We determined the interactions between the 27 DEGs using the STRING online database (version: 10.5) to identify PPIs underlying ischemic stroke (Fig. 3A). Next, we analyzed the

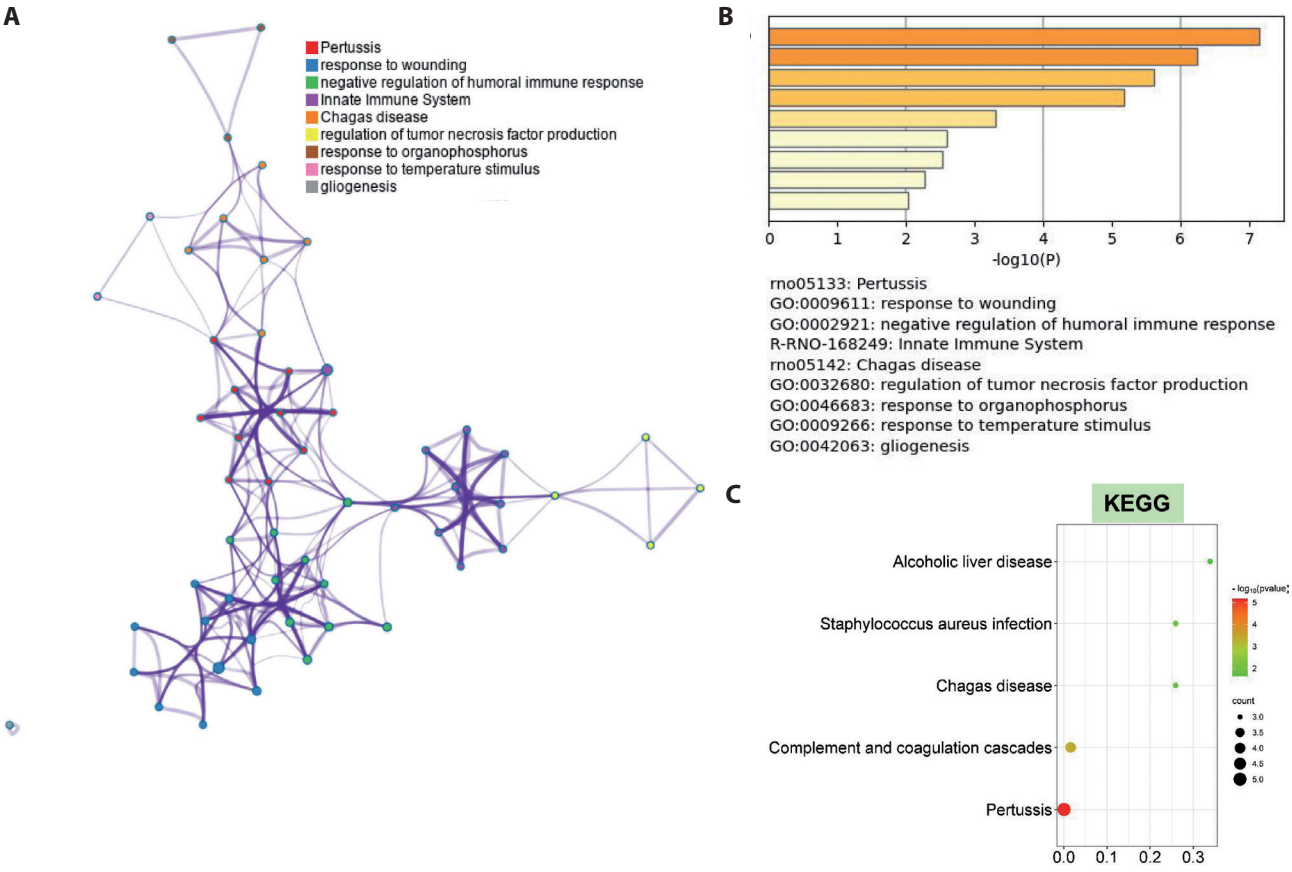

Figure 2. Enrichment analysis for the DEGs by Metascape. **A.** Network of enriched terms colored by cluster identity, where nodes that share the same cluster identity are typically close. **B.** Heatmap of enriched terms across input differently expressed gene lists, colored by *p*-values, *via* the Metascape. **C.** The KEGG (Kyoto encyclopedia of genes and genomes) functional annotation for the DEGs based on the DAVID (Database for annotation, visualization and integrated discovery).

PPI data using the MCODE app of Cytoscape. The hub genes of the sub-networks in the MCODE analysis were *C1qc, Fcgr2b, C1qb, and Cd14* (Fig. 3B). Moreover, we screened the top 10 genes using cytoHubb algorithms (MCC) to find hub genes. The screening hub genes are *C1qb, Fcgr2b, C1qc, Cd14, A2m, Serping1, Cd44, Plp2, S100a6, Timp1* (Fig. 3C,D), and those genes contains results on MCODE analysis. Ultimately, the four genes were *C1qc, Fcgr2b, C1qb, and Cd14,* which are defined as key genes.

Results of Cmap drug prediction

We downloaded the predicted drug outcomes from Cmap and ranked them based on their connectivity score. The top 8 small molecule compounds with the lowest connectivity score were verapamil, buspirone, flumazenil, rolipram, phenytoin, cholic-acid, 4,5-dianilinophthalimide (DAPH) and myricetin (Fig. 4, Table 3). The main mechanism of these small molecule compounds was summarized by a literature search (Table 4). Most of those small molecule compounds are clinically used in the treatment of various diseases except rolipram, myricetin and DAPH, which are still in the stage of laboratory research. The neuroprotective effect of rolipram has been reported in focal cerebral ischemia, but there are no clear data on whether the rolipram has the functional recovery effect. We aimed to determine if treatment with rolipram can improve motor dysfunction in the animal models of cerebral ischemia.

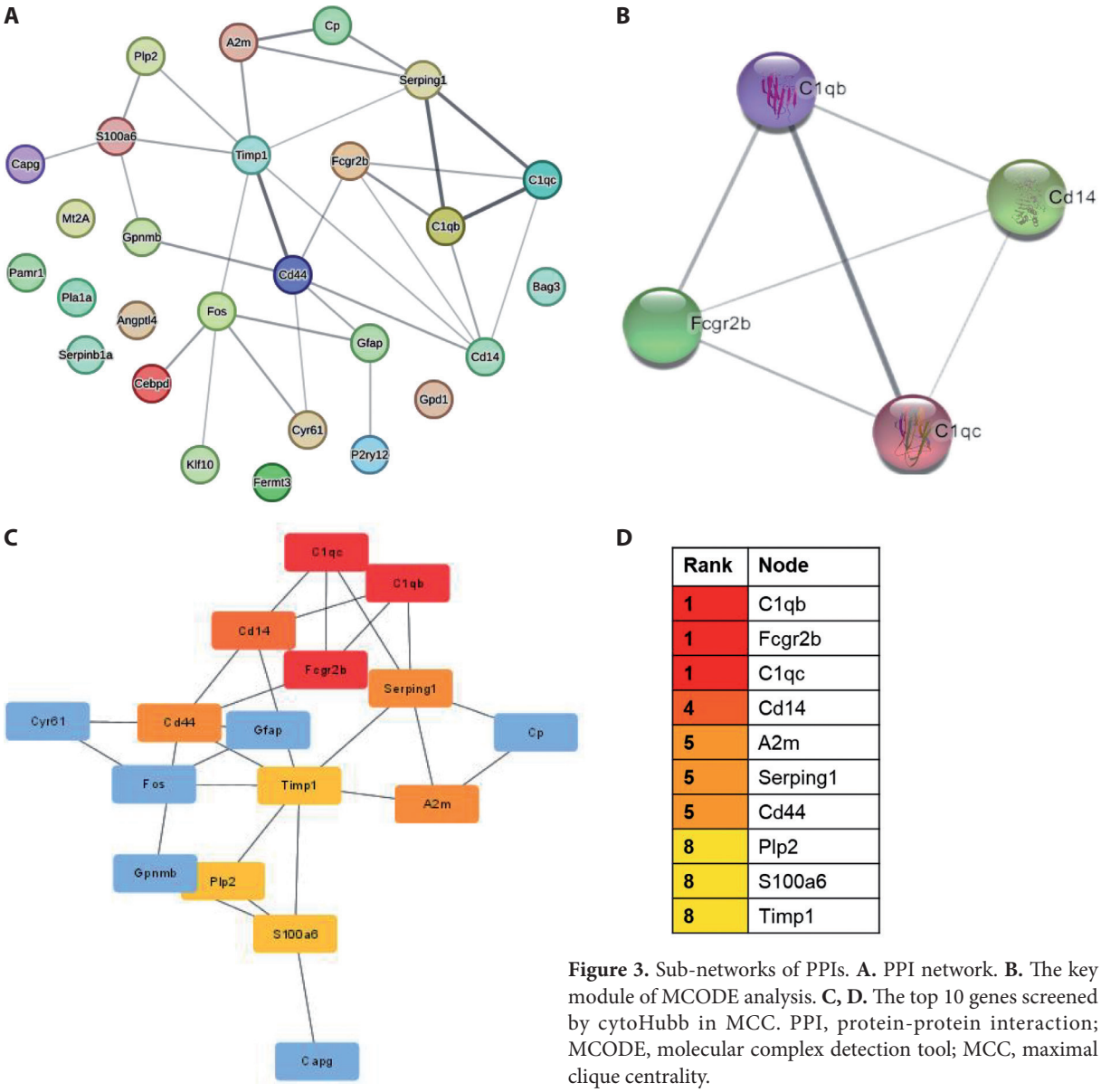

Figure 3. Sub-networks of PPIs. **A.** PPI network. **B.** The key module of MCODE analysis. **C, D.** The top 10 genes screened by cytoHubb in MCC. PPI, protein-protein interaction; MCODE, molecular complex detection tool; MCC, maximal clique centrality.

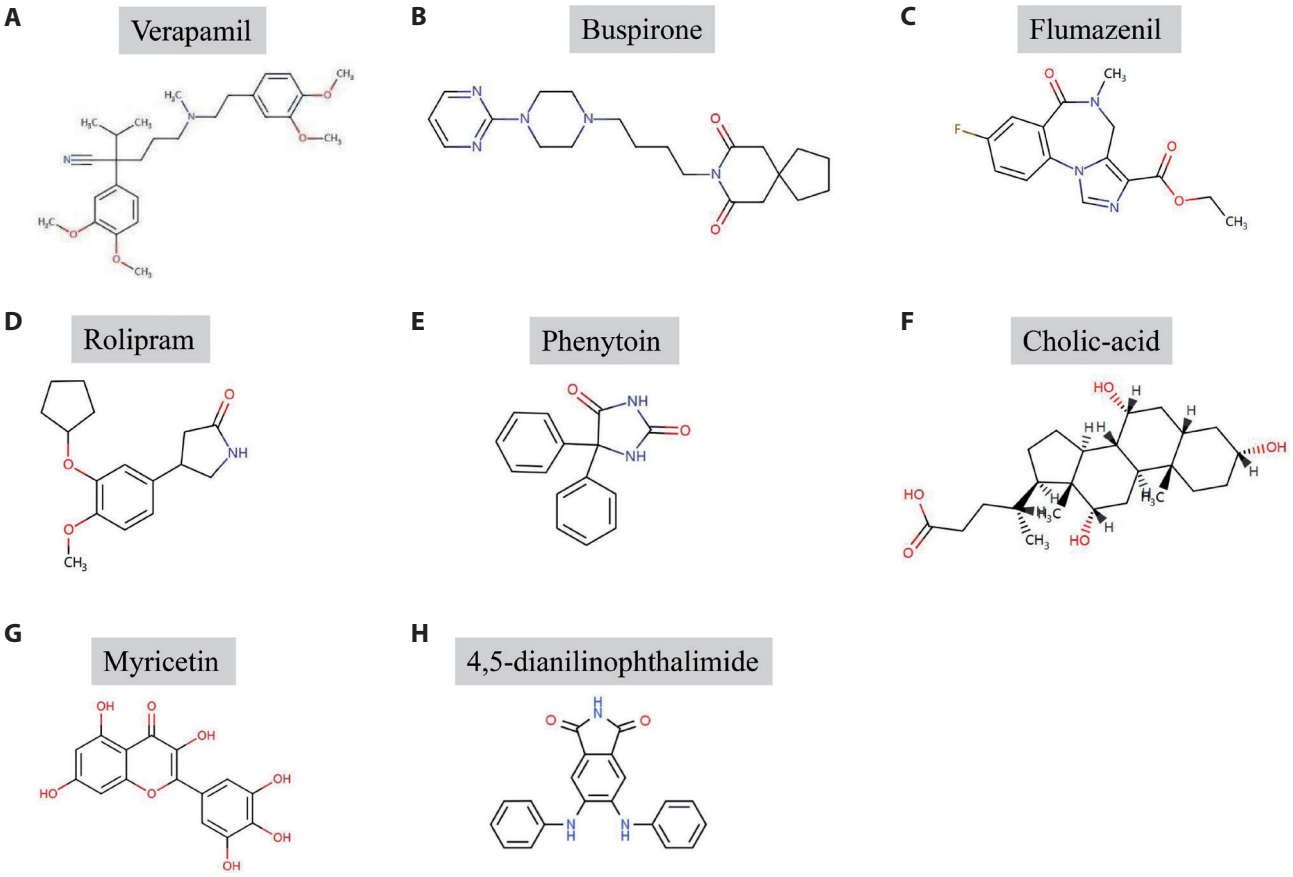

Figure 4. Structure of 8 potential drugs.

Expression of CD14 and C1qc was increased at 14 days after tMCAO

To validate the key differential genes expression revealed by bioinformatic analyses, the Real-time PCR and Western blot techniques were used to verify two key genes (*CD14, C1qc*) mRNA and protein expression levels. The Real-time PCR results showed that *CD14* mRNA and *C1qc* mRNA levels were increased in the periinfarct cortex after tMCAO (Fig. 5A,B). Western blot results showed that the expression of CD14 and C1qc was also increased markedly on day 14 post-injury (Fig. 5C,D).

Effects of rolipram on sensorimotor function after tMCAO

To evaluate the effects of rolipram on sensorimotor functional recovery after tMCAO, we designed the following three evaluation behavioral testing. During a beam-walking test, mice in the model group could not cross the beam at 0, 1, 3, 7, or 14 days. These mice fell off the beam earlier than those in the sham group. Rolipram (high dose) markedly improved beam crossing performance at 7 and 14 days after tMCAO compared with the model group (Fig. 6A). In the corner test, the model group mice performed more right turns than those in the sham group; this effect was improved by rolipram (high dose) treatment at 7 and 14 days after tMCAO (Fig. 6B). The grip strength test results shown that a progressive decline in grip strength as the model group mice. Treatment of rolipram (high dose) significantly ameliorated grip strength values at 7 and 14 days after tMCAO (Fig. 6C). In the behavioral test, rolipram (low dose) did not exhibit much benefit after tMCAO. The above experimental results suggest that treatment with high dose rolipram improves sensorimotor function in mice after stroke.

Discussion

At present, the pathogenesis of cerebral ischemia-reperfusion injury includes inflammation, excitatory amino acid toxicity, intracellular calcium overload, free radical damage, energy metabolism disorder, cell apoptosis, and many other mechanisms with complex interactions (Dirnagl et al. 1999; Gelderblom et al. 2009; George and Steinberg 2015). In this study, we screened hub genes of cerebral ischemia and predicted potential drugs based on a bioinformatics approach.

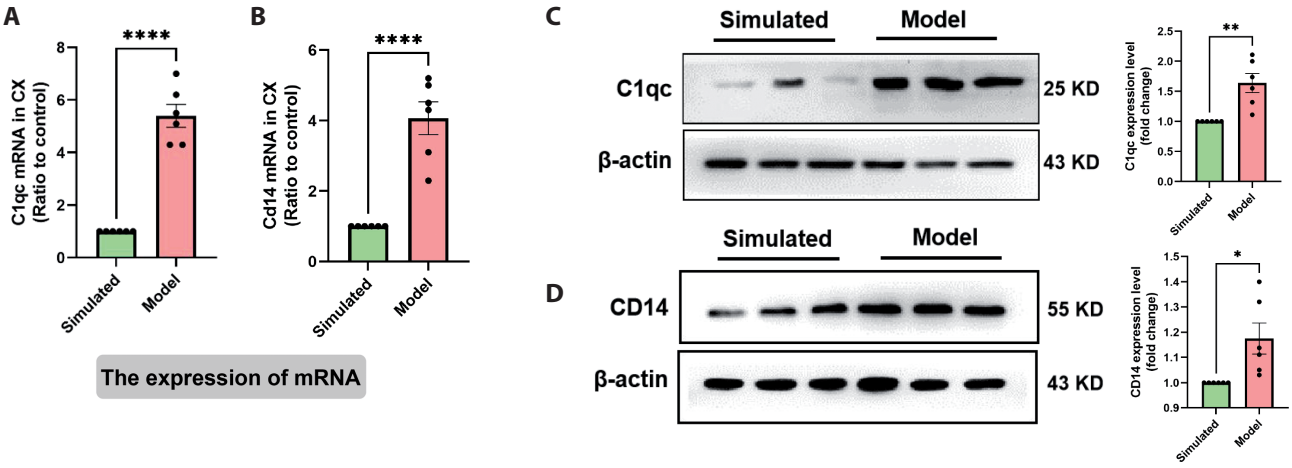

Figure 5. Expression of CD14 and C1qc was increased at 14 days after tMCAO. *C1q* mRNA level (**A**) and *CD14* mRNA level (**B**) were measured. Western blotting for C1qc (**C**) and CD14 (**D**) in periinfarct cortex on days 14 after tMCAO. Data are means ± SEM; $n = 6$; * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$. tMCAO, transient middle cerebral artery occlusion.

Table 3. Venn diagram co-expressed DEGs in GSE97537 and GSE52001

Gene symbol	Gene_name	DOWN/UP
Fos	FBJ osteosarcoma oncogene	DOWN
Pamr1	peptidase domain containing associated with muscle regeneration 1	DOWN
A2m	alpha-2-macroglobulin	UP
Angptl4	angiopoietin-like 4	UP
Bag3	Bcl2-associated athanogene 3	UP
C1qb	complement component 1, q subcomponent, B chain	UP
C1qc	complement component 1, q subcomponent, C chain	UP
Capg	capping protein (actin filament), gelsolin-like	UP
Cd14	CD14 molecule	UP
Cd44	Cd44 molecule	UP
Cebpd	CCAAT/enhancer binding protein (C/EBP), delta	UP
Cp	ceruloplasmin	UP
Fcgr2b	Fc fragment of IgG, low-affinity IIb, receptor (CD32)	UP
Fermt3	ferritin family member 3	UP
Gfap	glial fibrillary acidic protein	UP
Gpd1	glycerol-3-phosphate dehydrogenase 1 (soluble)	UP
Gpnmb	glycoprotein (transmembrane) nmb	UP
Mt2A	metallothionein 2A	UP
Pla1a	phospholipase A1 member A	UP
Plp2	proteolipid protein 2 (colonic epithelium-enriched)	UP
S100a6	S100 calcium binding protein A6	UP
Serpinb1a	serine (or cysteine) proteinase inhibitor, clade B, member 1a	UP
Serping1	serine (or cysteine) peptidase inhibitor, clade G, member 1	UP
Timp1	TIMP metallopeptidase inhibitor 1	UP
P2ry12	purinergic receptor P2Y, G-protein coupled, 12	DOWN (GSE92537) UP (GSE52001)
Cyr61	cysteine-rich, angiogenic inducer, 61	DOWN (GSE52001) UP (GSE92537)
Klf10	Kruppel-like factor 10	DOWN (GSE52001) UP (GSE92537)

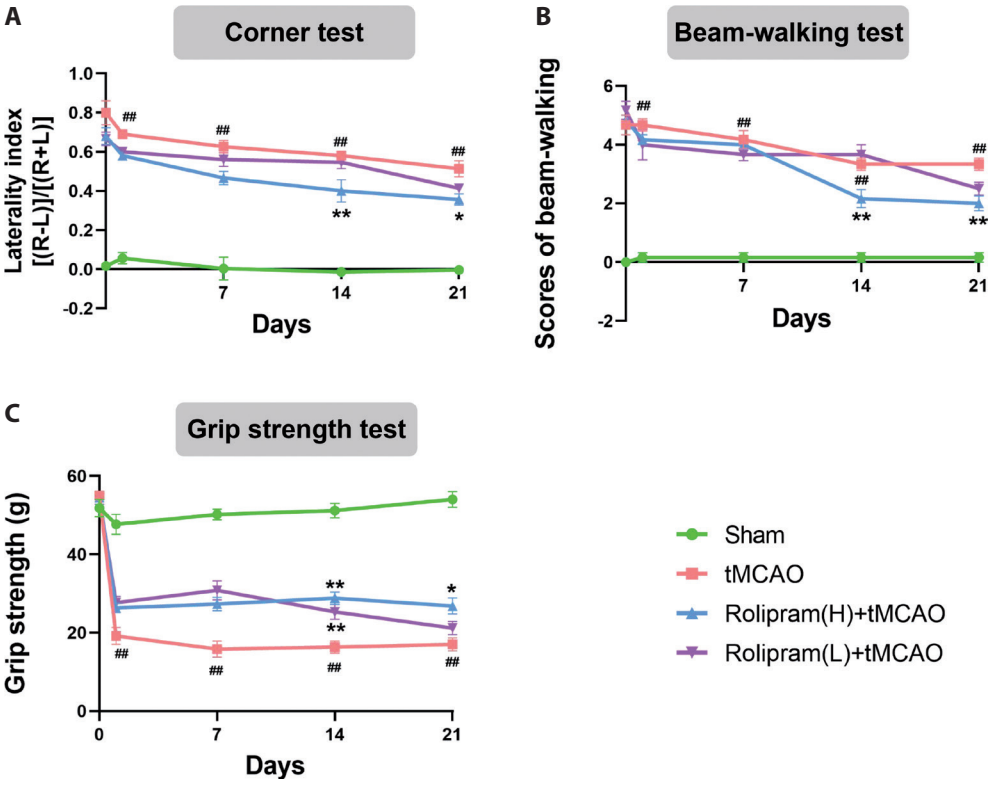

Figure 6. Sensorimotor recovery was promoted by the treatment of rolipram after the stroke. Rolipram reduced sensorimotor deficits as shown in the corner test (**A**), beam-walking test (**B**), and grip strength test (**C**). All data are represented as means ± SEM, $^{\#\#}$ $p < 0.01$ *vs.* Sham; * $p < 0.05$, ** $p < 0.01$ *vs.* tMCAO. tMCAO, transient middle cerebral artery occlusion; Rolipram (H), rolipram (high dose) group (2.5 mg/kg); Rolipram (L), rolipram (low dose) group (1.25 mg/kg).

Using a control and ischemic dataset, we identified 27 DEGs, which enriched in response to wounding, negative regulation of immune response, regulation of tumor necrosis factor production and gliogenesis. Moreover, the KEGG analysis revealed that the DEGs were mainly enriched in the complement and coagulation cascades, staphylococcus aureus infection, and chagas disease. The results from our bioinformatics analysis suggest that immune response and complement cascade play a very important role in cerebral ischemic injury. It has been revealed that after ischemic stroke, the damaged cells could cause systemic immune response by releasing the specific signals that activate immunodepression (Zhang et al. 2022). Further immune response strongly contributes to ischemia-elicited tissue damage in stroke, especially causing a second insult during reperfusion (Li et al. 2023). The complement cascade is associated with various neurological and non-neurological diseases (Garred et al. 2021). It is a part of the innate immune system; its primary physiological function is to promote the phagocytosis and digestion of target cells. Under the stimulation of external factors, the activation of the complement system can produce inflammatory reactions and affect the coagulation and plasminase system, leading to the injury of normal tissue cells in the body (Schafer et al. 2020). However, despite recent advances, the participation of the complement system in the relevant molecular mechanisms of stroke remains unclear.

In the present study, the MCODE score module and CytoHubb analysis revealed *C1qc, Fcgr2b, C1qb,* and *Cd14* as key genes which enriched in complement and coagulation cascades. The results of the analysis indicated these genes play an important role in cerebral ischemic injury.

C1q is the recognition component of the classical complement pathway. As a starting factor in the classical pathway of complement initiation, complement C1q is involved in the occurrence and development of many autoimmune diseases. However, the relationship between C1q and stroke has been less studied. Recently, it has been shown that patients with acute ischemic stroke and transient ischemic attack have high serum levels of C1q, which is the core gene

Table 4. Prediction results from Connectivity Map (Top 8 small molecule compounds)

Name	Description	Score
Verapamil	Calcium channel blocker	−99.93
Buspirone	Serotonin receptor agonist	−99.93
Flumazenil	Benzodiazepine receptor antagonist	−99.93
Rolipram	Phosphodiesterase inhibitor	−99.93
Phenytoin	Hydantoin antiepileptic	−99.93
Cholic-acid	Bile acid	−99.93
Myricetin	Androgen receptor agonist	−99.93
DAPH	EGFR inhibitor	−99.93

DAPH, 4,5-dianilinophthalimide.

Table 5. Summary of mechanism and application of negatively related small molecule compounds

Name	Pharmacology	Targets	Adaptation disease	Clinical trials (stroke)
Verapamil	L-type calcium channel blocker	Voltage-dependent L-type calcium channels	Vasopastic angina, unstable angina, and chronic stable angina	Ischemic stroke Phase I (completed)
Buspirone	Serotonin 5-HT1A receptor agonist	5-hydroxytryptamine receptor Dopamine receptor	Anxiety disorders	Acute ischemic stroke Phase III (terminated)
Flumazenil	Benzodiazepine antagonist	Gamma-aminobutyric acid receptor	Induced general anesthesia	No available
Rolipram	Phosphodiesterase inhibitor	c-AMP-specific 3',5'-cyclic phosphodiesterase 4D	(Investigational)	No available
Phenytoin	Non-specific sodium channel blocker	Sodium channel protein	Seizures	No available
Cholic-acid	Facilitates fat absorption and cholesterol excretion	Bile acid receptor Cytochrome-c	Bile acid synthesis disorders	No available
Myricetin	Antioxidant, anticancer and anti-inflammatory activities	Phosphatidylinositol 4,5-bisphosphate 3-kinase Tyrosine-protein kinase JAK1	(Experimental)	No available
DAPH	EGFR inhibitor	JAK/STAT signal path Protein tyrosine kinase	(Experimental)	No available

DAPH, 4,5-dianilinophthalimide.

selected in this paper (Sun et al. 2021). Moreover, this gene is positively correlated with the severity of ischemic stroke (Pedersen et al. 2009) showed that complement C1q could inhibit apoptosis caused by macrophage phagocytosis, thus delaying the formation of atherosclerosis. The formation of atherosclerotic plaque is an important mechanism of ischemic stroke. These results confirm that complement C1q plays an important role in the neurological impairment of ischemic stroke. CD14 is a glycosylphosphatidylinositol-anchored glycoprotein highly expressed and secreted by monocytes/macrophages. It can mediate cytokine secretion and inflammatory response (Wu et al. 2019). Some studies have confirmed that cerebral ischemia patients had high serum CD14 levels, and others have confirmed that it is positively correlated with the inflammatory injury process of cerebral ischemia (Roedig et al. 2019; Leskela et al. 2020). Importantly, the results of our experiments indicate that CD14 and C1q expression increases at protein and mRNA levels at 14 days after tMCAO. C1q and CD14 may be closely related to the occurrence and development of ischemic stroke and may become potential biomarkers or therapeutic targets for clinical diagnosis. However, its role in the pathophysiological process of cerebral ischemia also needs to be further explored. In this study, we used Cmap to screen out some important new drug candidates for stroke. These predicted drugs that were screened out may turn out to be effective against ischemic stroke.

Rolipram is a type IV-specific phosphodiesterase (PDE4) inhibitor that increases intracellular cyclic adenosine monophosphate (cAMP) levels in the central nervous system (Montana and Dyke 2002). Initially developed as an antidepressant, rolipram produces anxiolytic- and antidepressant-like effects and improves cognition in rodents (Li et al. 2009; Heckman et al. 2015). Beneficial effects of rolipram have also been described after experimental models of brain injury in rodents, such as encephalomyelitis (EAE), traumatic brain injury or spinal cord injury (Sommer et al. 1995; Atkins et al. 2007; Costa et al. 2013). In particular, rolipram exerts blood-brain barrier (BBB) stabilizing and anti-inflammatory effects in models of cerebral ischemia (Kraft et al. 2013). Furthermore, a lot of research has demonstrated that rolipram reduced the volume of cerebral infarction and improved memory deficits by decreasing cell death and enhancing the survival of newborn neurons in cerebral ischemia (Block et al. 1997; Sasaki et al. 2007; Li et al. 2011). Although the above studies showed the neuroprotective effects of rolipram in animal models of cerebral ischemia, it is unknown whether it can improve motor dysfunction in cerebral ischemia mice. Our behavior experimental results confirmed that treatment high dose rolipram improves sensorimotor function in mice after stroke. Nevertheless, the specific molecular mechanism still needs to be elucidated thoroughly in the future.

In summary, using two available datasets, we effectively identified ischemic stroke-related DEGs. We used two methods to identify four key genes related to the pathogenesis of cerebral ischemia. Moreover, Real-time PCR and Western blot analysis showed that expression of C1q and CD14 increased at 14 days in tMCAO mice. Finally, rolipram which is one of the drug candidates for stroke improved sensorimotor function in tMCAO mice. However, to elucidate whether rolipram might regulate expression of C1q and CD14 to exert its function, additional studies will

be needed. In conclusion, the present results provide novel insights into the biological process and potential therapeutic drugs involved in stroke.

Competing interests. The authors have no relevant financial or non-financial interests to disclose.

Author contributions. SJ and MS conceived this project and the design of the study. SJ explored differentially expressed genes from the GEO database and performed most experiments and interpreted the data. ZL and XZ performed functional enrichment analysis and some of the *in vitro* experiments. RZ and BS screened out key genes related to ischemic stroke. CG and PH found potential therapeutic agents for stroke. MS conceived this project. SJ wrote the manuscript. MS revised the manuscript.

Ethics approval. Approval was obtained from the ethics committee of Xi'an ninth hospital animal laboratory. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

Funding. This work was supported by Clinical research project of Xi'an Ninth Hospital (No. 2022-13).

Data availability. 1. Microarray Data were deposited into the Gene Expression Omnibus database under accession number GSE97537 and are available at the following URL: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE97537. 2. Microarray Data were deposited into the Gene Expression Omnibus database under accession number GSE52001 and are available at the following URL: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE52001. 3. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

References

Atkins CM, Oliva AA, Jr., Alonso OF, Pearse DD, Bramlett HM, Dietrich WD (2007): Modulation of the cAMP signaling pathway after traumatic brain injury. Exp. Neurol. **208,** 145-158
https://doi.org/10.1016/j.expneurol.2007.08.011

Benjamin EJ, Muntner P, Alonso A, Bittencourt MS, Callaway CW, Carson AP, Chamberlain AM, Chang AR, Cheng S, Das SR, et al. (2019): Heart disease and stroke statistics-2019 update: A report from the american heart association. Circulation **139,** e56-e528
https://doi.org/10.1161/CIR.0000000000000659

Block F, Tondar A, Schmidt W, Schwarz M (1997): Delayed treatment with rolipram protects against neuronal damage following global ischemia in rats. Neuropharmacology **8,** 3829
https://doi.org/10.1097/00001756-199712010-00033

Brenna S, Altmeppen HC, Mohammadi B, Rissiek B, Schlink F, Ludewig P, Krisp C, Schluter H, Failla AV, Schneider C, et al. (2020): Characterization of brain-derived extracellular vesicles reveals changes in cellular origin after stroke and enrichment of the prion protein with a potential role in cellular uptake. J. Extracell. Vesicles **9,** 1809065
https://doi.org/10.1080/20013078.2020.1809065

Campbell BCV, De Silva DA, Macleod MR, Coutts SB, Schwamm LH, Davis SM, Donnan GA (2019): Ischaemic stroke. Nat. Rev. Dis. Primers **5,** 70
https://doi.org/10.1038/s41572-019-0118-8

Costa LM, Pereira JE, Filipe VM, Magalhaes LG, Couto PA, Gonzalo-Orden JM, Raimondo S, Geuna S, Mauricio AC, Nikulina E, et al. (2013): Rolipram promotes functional recovery after contusive thoracic spinal cord injury in rats. Behav. Brain Res. **243,** 66-73
https://doi.org/10.1016/j.bbr.2012.12.056

Dirnagl U, Iadecola C, Moskowitz MA (1999): Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci. **22,** 391-397
https://doi.org/10.1016/S0166-2236(99)01401-0

Dodson M, Wani WY, Redmann M, Benavides GA, Johnson MS, Ouyang X, Cofield SS, Mitra K, Darley-Usmar V, Zhang J (2017): Regulation of autophagy, mitochondrial dynamics, and cellular bioenergetics by 4-hydroxynonenal in primary neurons. Autophagy **13,** 1828-1840
https://doi.org/10.1080/15548627.2017.1356948

Flores JJ, Zhang Y, Klebe DW, Lekic T, Fu W, Zhang JH (2014): Small molecule inhibitors in the treatment of cerebral ischemia. Expert Opin. Pharmacother. **15,** 659-680
https://doi.org/10.1517/14656566.2014.884560

Garred P, Tenner AJ, Mollnes TE (2021): Therapeutic targeting of the complement system: from rare diseases to pandemics. Pharmacol. Rev. **73,** 792-827
https://doi.org/10.1124/pharmrev.120.000072

Gelderblom M, Leypoldt F, Steinbach K, Behrens D, Choe CU, Siler DA, Arumugam TV, Orthey E, Gerloff C, Tolosa E, et al. (2009): Temporal and spatial dynamics of cerebral immune cell accumulation in stroke. Stroke **40,** 1849-1857
https://doi.org/10.1161/STROKEAHA.108.534503

George PM, Steinberg GK (2015): Novel stroke therapeutics: unraveling stroke pathophysiology and its impact on clinical treatments. Neuron **87,** 297-309
https://doi.org/10.1016/j.neuron.2015.05.041

Heckman PRA, Wouters C, Prickaerts J (2015): Phosphodiesterase inhibitors as a target for cognition enhancement in aging and Alzheimer's disease: A translational overview. Curr. Pharm. Des. **21,** 317-331
https://doi.org/10.2174/1381612820666140826114601

Huang DW, Sherman BT, Tan Q, Kir J, Liu D, Bryant D, Guo Y, Stephens R, Baseler MW, Lane HC, et al. (2007): DAVID bioinformatics resources: expanded annotation database and novel algorithms to better extract biology from large gene lists. Nucleic Acids Res. **35,** W169-175
https://doi.org/10.1093/nar/gkm415

Jiang H, Hu C, Chen M (2021): The advantages of connectivity map applied in traditional chinese medicine. Front. Pharmacol. **12,** 474267
https://doi.org/10.3389/fphar.2021.474267

Jin R, Song Z, Yu S, Piazza A, Nanda A, Penninger JM, Granger DN, Li G (2011): Phosphatidylinositol-3-kinase gamma plays a central role in blood-brain barrier dysfunction in acute experimental stroke. Stroke **42,** 2033-2044
https://doi.org/10.1161/STROKEAHA.110.601369

Kraft P, Schwarz T, Gob E, Heydenreich N, Brede M, Meuth SG, Kleinschnitz C (2013): The phosphodiesterase-4 inhibitor rolipram protects from ischemic stroke in mice by reducing blood-brain-barrier damage, inflammation and thrombosis. Exp. Neurol. **247,** 80-90 https://doi.org/10.1016/j.expneurol.2013.03.026

Kurisu K, Zheng Z, Kim JY, Shi J, Kanoke A, Liu J, Hsieh CL, Yenari MA (2019): Triggering receptor expressed on myeloid cells-2 expression in the brain is required for maximal phagocytic activity and improved neurological outcomes following experimental stroke. J. Cereb. Blood Flow Metab. **39,** 1906-1918 https://doi.org/10.1177/0271678X18817282

Lai W, Zheng Z, Zhang X, Wei Y, Chu K, Brown J, Hong G, Chen L (2015): Salidroside-mediated neuroprotection is associated with induction of early growth response genes (Egrs) across a wide therapeutic window. Neurotox. Res. **28,** 108–121 https://doi.org/10.1007/s12640-015-9529-9

Langhauser F, Gob E, Kraft P, Geis C, Schmitt J, Brede M, Gobel K, Helluy X, Pham M, Bendszus M, et al. (2012): Kininogen deficiency protects from ischemic neurodegeneration in mice by reducing thrombosis, blood-brain barrier damage, and inflammation. Blood **120,** 4082-4092 https://doi.org/10.1182/blood-2012-06-440057

Leskela J, Pietiainen M, Safer A, Lehto M, Metso J, Malle E, Buggle F, Becher H, Sundvall J, Grau AJ, et al. (2020): Serum lipopolysaccharide neutralizing capacity in ischemic stroke. PLoS One **15,** e0228806 https://doi.org/10.1371/journal.pone.0228806

Li L, Han Z, Wang R, Fan J, Zheng Y, Huang Y, Yang Z, Yan F, Liu P, Zhao H, et al. (2023): Association of admission neutrophil serine proteinases levels with the outcomes of acute ischemic stroke: a prospective cohort study. J. Neuroinflamm. **20,** 70 https://doi.org/10.1186/s12974-023-02758-1

Li LX, Cheng YF, Lin HB, Wang C, Xu JP, Zhang HT (2011): Prevention of cerebral ischemia-induced memory deficits by inhibition of phosphodiesterase-4 in rats. Metab. Brain Dis. **26,** 37-47 https://doi.org/10.1007/s11011-011-9235-0

Li Y-F, Huang Y, Amsdell SL, Xiao L, O'Donnell JM, Zhang H-T (2009): Antidepressant- and anxiolytic-like effects of the phosphodiesterase-4 inhibitor rolipram on behavior depend on cyclic AMP response element binding protein-mediated neurogenesis in the hippocampus. Neuropsychopharmacology **34,** 2404-2419 https://doi.org/10.1038/npp.2009.66

Montana JG, Dyke HJ (2002): Update on the therapeutic potential of PDE4 inhibitors. Expert Opin. Investig. Drugs **11,** 1-13 https://doi.org/10.1517/13543784.11.1.1

Pedersen ED, Loberg EM, Vege E, Daha MR, Maehlen J, Mollnes TE (2009): In situ deposition of complement in human acute brain ischaemia. Scand. J. Immunol. **69,** 555-562 https://doi.org/10.1111/j.1365-3083.2009.02253.x

Qiao Y, Ma Q, Zhai H, Li Y, Tang M (2019): Exposure to female estrous is beneficial for male mice against transient ischemic stroke. Neurol. Res. **41,** 536-543 https://doi.org/10.1080/01616412.2019.1580461

Roedig H, Nastase MV, Frey H, Moreth K, Zeng-Brouwers J, Poluzzi C, Hsieh LT, Brandts C, Fulda S, Wygrecka M, et al. (2019): Biglycan is a new high-affinity ligand for CD14 in macrophages. Matrix Biol. **77,** 4-22 https://doi.org/10.1016/j.matbio.2018.05.006

Sasaki T, Kitagawa K, Omura-Matsuoka E, Todo K, Terasaki Y, Sugiura S, Hatazawa J, Yagita Y, Hori M (2007): The phosphodiesterase inhibitor rolipram promotes survival of newborn hippocampal neurons after ischemia. Stroke **38,** 1597-1605 https://doi.org/10.1161/STROKEAHA.106.476754

Savarese G, Savonitto S, Lund LH, Paolillo S, Marciano C, Dellegrottaglie S, Parente A, Trimarco B, Luscher TF, Perrone-Filardi P (2016): Efficacy and safety of prolonged dual antiplatelet therapy: a meta-analysis of 15 randomized trials enrolling 85 265 patients. Eur. Heart J. Cardiovasc. Pharmacother. **2,** 218-228 https://doi.org/10.1093/ehjcvp/pvw011

Schafer N, Wolf HN, Enzbrenner A, Schikora J, Reichenthaler M, Enzmann V, Pauly D (2020): Properdin modulates complement component production in stressed human primary retinal pigment epithelium cells. Antioxidants (Basel) **9,** 793 https://doi.org/10.3390/antiox9090793

Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T (2003): Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. **13,** 2498-2504 https://doi.org/10.1101/gr.1239303

Signorelli SS, Platania I, Tomasello SD, Mangiafico M, Barcellona G, Di Raimondo D, Gaudio A (2020): Insights from experiences on antiplatelet drugs in stroke prevention: a review. Int. J. Environ. Res. Public Health **17,** 5840 https://doi.org/10.3390/ijerph17165840

Sommer N, Löschmann P-A, Northoff GH, Weller M, Steinbrecher A, Steinbach JP, Lichtenfels R, Meyermann R, Riethmüller A, Fontana A, et al. (1995): The antidepressant rolipram suppresses cytokine production and prevents autoimmune encephalomyelitis. Nature Medicine **1,** 244 https://doi.org/10.1038/nm0395-244

Sun T, Fu R, Li L, Wang Y, Li N (2021): Analysis of early predictive value of serum levels of complement C1q, tumor necrosis factor-related Protein 3 and Interleukin-6 in the progression of ischemic stroke. Minerva Med. 2021 https://doi.org/10.23736/S0026-4806.21.07513-3

Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, Simonovic M, Doncheva NT, Morris JH, Bork P, et al. (2019): STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res. **47,** D607-D613 https://doi.org/10.1093/nar/gky1131

Takuma A, Abe A, Saito Y, Nito C, Ueda M, Ishimaru Y, Harada H, Abe K, Kimura K, Asakura T (2017): Gene expression analysis of the effect of ischemic infarction in whole blood. Int. J. Mol. Sci. **18,** 2335 https://doi.org/10.3390/ijms18112335

Wu Z, Zhang Z, Lei Z, Lei P (2019): CD14: Biology and role in the pathogenesis of disease. Cytokine Growth Factor Rev. 48 24-31 https://doi.org/10.1016/j.cytogfr.2019.06.003

Zhang C, Wang L, Guo Y, Feng W (2022): Systematic analysis of brain and skull ischemic injury expression profiles reveals

associations of the tumor immune microenvironment and cell death with ischemic stroke. Front. Immunol. **13,** 1082546 https://doi.org/10.3389/fimmu.2022.1082546

Zhao Q-S, Li W, Li D, Liu T, Wang J-H, Gao Y, Yi L, Zhao RK (2018): Clinical treatment efficiency of mechanical thrombectomy combined with rhPro-UK thrombolysis for acute moderate/severe cerebral infarction. Eur. Rev. Med. Pharmacol. Sci. **22,** 5740-5746

Zhou F, Wang M, Ju J, Wang Y, Liu Z, Zhao X, Yan Y, Yan S, Luo X, Fang Y (2019): Schizandrin A protects against cerebral ischemia-reperfusion injury by suppressing inflammation and oxidative stress and regulating the AMPK/Nrf2 pathway regulation. Am. J. Transl. Res. **11,** 199-209

Zhou M, Wang H, Zeng X, Yin P, Zhu J, Chen W, Li X, Wang L, Wang L, Liu Y, et al. (2019): Mortality, morbidity, and risk factors in China and its provinces, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet **394,** 1145-1158 https://doi.org/10.1016/S0140-6736(19)30427-1

Zhou Y, Zhou B, Pache L, Chang M, Khodabakhshi AH, Tanaseichuk O, Benner C, Chanda SK (2019): Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. Nat. Commun. **10,** 1523 https://doi.org/10.1038/s41467-019-09234-6

Zhu W, Nan Y, Wang S, Liu W (2019): Bioinformatics analysis of gene expression profiles of sex differences in ischemic stroke. Biomed. Res. Int. **2019,** 2478453 https://doi.org/10.1155/2019/2478453

Received: November 24, 2023
Final version accepted: April 9, 2024