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Research paper

Network pharmacology-based study on the active substances and mechanism of Nao An Capsule in treatment of ischemic stroke

Chen Chen[a], Hui-Ling Li[b], Yuan Yi[a], Hua-Jun Fan[a,**], Chao Chen[a,b,c,*]

[a] School of Pharmacy, Guangdong Pharmaceutical University, Guangzhou, 510006, China
[b] School of Traditional Chinese Medicine, Guangdong Pharmaceutical University, Guangzhou, 510006, China
[c] The Key Unit of Chinese Medicine Digitalization Quality Evaluation of SATCM, Guangzhou, 510006, China

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ABSTRACT

Introduction: In clinical practice, Nao An Capsule (NAC), a traditional Chinese medicine (TCM) prescription, is regarded as having good therapeutic effect on ischemic stroke (IS). A network pharmacology based method was used to uncover the active substances and mechanism of NAC in treatment of IS.

Methods: The components of NAC, in addition to their interacting targets, were searched from the TCMSP database. The components were filtered by the metrics of oral bioavailability and drug likeness, and the targets were compared to the disease associated ones searched from the CTD and TTD databases. After that, the components and targets were fed into the Cytoscape software to build a component-target-disease network. Afterwards, the protein-protein interactions among the identical targets were visualized by the STRING database, and the GO and pathway enrichment analysis were performed by the Metascape database. Finally, the potential active substances and targets were validated by molecular docking simulation.

Results: 51 compounds, 325 interacting targets in addition to 54 disease-related targets were obtained, in which 16 identical targets were observed, and analyzed to be enriched in 18 pathways. 13 of those identical targets had compact relationships with each other in STRING analysis. The compounds interacting with the 13 targets had stronger affinities than the native ligands in molecular docking simulation.

Conclusion: The present method did not only preliminarily reveal the active substances and mechanism of NAC in treatment of IS from the perspective of network pharmacology, but show its potential application for research and development of other TCM prescriptions.

1. Introduction

Ischemic stroke (IS) is one of the three most lethal diseases in the world, and has high incidence, high morbidity and high mortality. IS is a multifactorially induced disease, and always accompanies many other diseases, such as hypertension, atherosclerosis, cerebral hemorrhage, inflammatory responses, cerebrovascular diseases, hematological diseases, metabolic diseases, etc [1,2]. As one of the main neurological diseases, IS has a clinical pathology of hypoxia, ischemia and necrosis of the regional brain tissue, which can cause the patients to become disabled, often for many years [3]. Its pathological manifestations contain brain energy metabolism, excitatory amino acid toxicity, oxidative/nitrative stress damage, inflammatory responsing, apoptosis, autophagy, etc [4]. At present, anti-IS drugs on the market can be mainly divided into anti-platelet aggregation drugs, anticoagulants, thrombolytic drugs, brain protective agents, neuroprotective drugs, vasodilation agents and inflammatory responsing inhibitors, etc. [5]. However, single-target treatment drugs are often difficult to achieve satisfying therapeutic effects for complex diseases like IS. TCM is a broad range of natural products, which has been used to treat various diseases in China and other eastern Asia countries for thousands of years. Different from the “one ingredient, one target” characteristics of most chemical drugs, TCM has multi-components, multi-targets, multi-channels, and synergistic effects. Chen et al summarized the research progress of TCM in IS treatments, and found that TCMs have significant efficacies in many cases [6].

Nao An capsule (NAC) is an oral Chinese patent drug, which is composed of Chuanxiong (*Ligusticum striatum* DC), Honghua (*Carthamus tinctorius* L.), Danggui (*Angelica sinensis* (Oliv.) Diels), Renshen (*Panax ginseng* C.A.Mey.), and Bingpian (*Borneolum Syntheticum*). It is clinically

* Corresponding author at: School of School of Traditional Chinese Medicine, Guangdong Pharmaceutical University, Guangzhou, 510006, China.
** Corresponding author. School of Pharmacy, Guangdong Pharmaceutical University, Guangzhou 510006,China.
E-mail addresses: junhuafan@126.com (H.-J. Fan), gdpuchenchao@163.com (C. Chen).

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used in acute phase of cerebral thrombosis, and recovering period with syndrome of Qi deficiency and blood stasis. According to the theories of TCM, Chuanxiong can activate blood circulation, Honghua removes blood stasis, Danggui enriches the blood, Renshen promotes Qi, and Bingpian induces resuscitation. These five herbs have very common and traditional uses in clinical practice. For example, Chuanxiong is believed to possess ideal therapeutic effects on cardiovascular and cerebrovascular diseases. Chen et al. reported a systematic review [7] and summarized the information about Chuanxiong on the ethnobotany, ethnopharmacological uses, phytochemicals, pharmacological activities, toxicology. Honghua is also regarded as an important herb to promote blood circulation, eliminate blood stasis, and alleviate pains, and is traditionally applied for cardiovascular, cerebrovascular and gynecological complications [8]. Readers can refer to [9–11] to know more about other three herbs. Under the traditional "King, Vassal, Assistant and Delevery servant" rule, all these five herbs are combined together to produce NAC. As reported [12], NAC has functions of activating blood circulation to remove blood stasis, and promoting Qi to clear collaterals, and has good theraputic effects on the treatment of IS. However, the main active substances and mechanism are not clear yet.

In this study, we proposed a network pharmacology based method in aiming at uncovering the active substances and mechanism of NAC in the treatment of IS. Firstly, the components from the five herbs, in addition to their interacting targets, were searched from the TCMSP database. The components were filtered by the metrics of oral bioavailability and drug likeness, and the targets were compared to the disease associated ones searched from the CTD and TTD databases. After that, the components and targets were fed into the Cytoscape software to build a component-target-disease network. Secondly, the protein-protein interactions among the identical targets were predicted by the STRING database, and the GO and pathway enrichment analysis were performed by the Metascape database. Lastly, the potential active substances and mechanism were concluded, and further validated by the molecular docking simulation and the literature searching.

2. Materials and methods

2.1. Collection of NAC compounds and their interacting targets

As to collect the NAC compounds, one can search in the TCM databases [13], analyze the patent medicine by HPLC [14], or analyze the serum of rats administered with the medicine by HPLC-MS [15]. In this work, we adopted the first method that is the easiest and most popular. Therefore, according to the prescription of NAC, the compounds from the five herbs were searched by the traditional Chinese medicine systems pharmacology database (TCMSP, http://lsp.nwu.edu.cn/tcmsp.php). TCMSP is a platform that includes chemicals, targets as well as pharmacokinetic properties for natural compounds involving oral bioavailability (OB), drug-likeness (DL), aqueous solubility and etc [16]. In this study, all the compounds were filtered by the metrics of OB $\geq$ 30% and DL $\geq$ 0.18. The targets interacting with the remained compounds were then searched. Subsequently, in order to obtain the unique names, all the targets were input to the Uniprot database, and the mapped gene symbols were yielded and deposited as TargetSet 1.

2.2. Acquisition of disease-associated targets

By the key words of "Ischemic Stroke" the disease-associated targets were searched from the Comparative Toxicogenomics Database (CTD, http://ctdbase.org/), the Therapeutic Target Database (TTD, http://bidd.nus.edu.sg/group/cjttd/), and the Drugbank Database (https://www.drugbank.ca/). It is noted that CTD contains curated and inferred target-disease associations. In this study, in order to reduce the false positives, only the curated targets with direct evidence that associates with the disease mechanism were considered. Finally, all the targets were mapped to obtain the gene symbols, removed the duplicates and recorded as TargetSet 2.

2.3. Construction of compounds-targets-Disease network

The NAC compounds and their targets were input to the Cytoscape 3.2.1 software to construct and visualize the Compounds-Targets-Disease network. In the network, the nodes represent the compounds, targets or disease, while the edges represent the associations between the nodes.

2.4. Gene enrichment and its interaction network analysis

The identical genes in TargetSet 1 and 2 were speculated to be important for uncovering the active substances and mechanism of NAC in treatment of IS. Thus, those identical genes were input to the Metascape database to perform the gene enrichment, functional annotation and pathway analysis. Metascape database (http://metascape.org/) integrates the resources from authoritative databases such as Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), UniProt, DrugBank, etc.

Those identical genes were also input to the STRING 11.0 database to score and visualize the protein-protein associations. STRING database (https://string-db.org/) aims to collect and integrate all publicly available sources of protein-protein interaction information, and to complement these with computational predictions.

2.5. Molecular docking simulation

The binding affinities of the potential active substances and their interacting targets in the enriched pathways were confirmed by molecular docking simulation. Here, the systems Dock web server (http://systemsdock.unit.oist.jp/iddp/home/index) was adopted for the simulation [17]. It has two unique features. One is that it allows screening of a large number of proteins with ease, and does not need stepwise methods for molecule preparation, parameter specification and result inspection. The other is that it incorporates an elaborately designed scoring function called docK-IN, which is a negative logarithm of the experimental dissociation / inhibition constant (pK_d / pKi), usually ranging from 0 to 10 (i.e. from weak to strong binding). A docking score of 5.52 (pK_d) is equal to a K_d of 3 μM, which is conventionally used to classify ligand binding activity. In this study, the test compounds' activities were compared to the ones of the corresponding native ligands.

3. Results

3.1. NAC compounds and their interacting targets

After the searching, filteration by pharmacokinetic properties, and removal of the duplicates, there remained 51 compounds in total, among which 7 compounds were originated from Chuanxiong, 22 compounds from Honghua, 2 compounds from Danggui, 21compounds from Renshen, and 3 compounds from Bingpian. It was noted that several compounds, e.g. kaempferol, were from both Honghua and Renshen. Meanwhile, the targets interacting with these compounds were also searched from the TCMSP database. By inputing these targets to the UniProt database, the unique gene symbols were obtained. After removal of the duplicates, 325 genes in total were remained and denoted as TargetSet 1.

3.2. Disease-associated genes

After removal of the duplicates, 54 disease-associated genes were obtained and denoted as TargetSet 2, in which 39 genes were from the CTD database, 8 from the Drugbank, and 7 from the TTD.

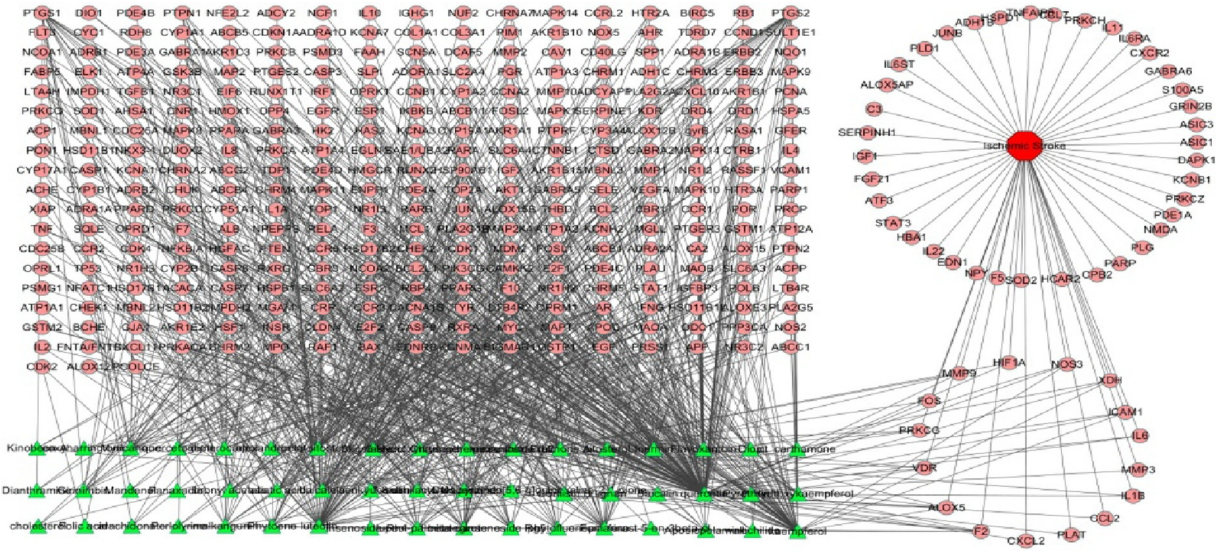


Fig. 1. The "Compound-Target-Disease" interaction network.

3.3. Network construction and analysis

All the above mentioned compounds and targets were fed into the Cytoscape software to visualize the compounds-targets-disease network. In Fig. 1, there were 415 nodes and 867 edges in total. On average, one compound interacted with 15.94 targets, while one target interacted with 2.5 compounds. Apparently, one can easily find that NAC had complex characteristics of multi-components, multi-targets and synergistic effects. In addition, it was observed that there were 16 identical genes both in TargetSet 1 and 2. As located in the bottom right circle in Fig. 1, these identical genes contained IL6, IL1B, NOS3, MMP9, ICAM1, F2, CXCL2, FOS, VDR, ALOX5, XDH, PLAT, CCL2, HIF1A, PRKCG and MMP3, which were speculated to have important roles for NAC in treatment of IS, and thus were in need for further analysis.

3.4. Visualization of gene enrichment and its interaction network

The 16 identical genes obtained in subsection 3.3 were imported into the Metascape database, where the species were selected as "Homo sapiens" and the "Express Analysis" was carried out. These genes were found to be enriched in top 18 pathways ($\boldsymbol{P} < 0.01$), which were shown in Fig. 2.

As is well known, the bionetwork often has characteristics of dominant modules, where a set of genes or proteins share similar molecular functions and have tight interactions with each other. In general, the functional modules have significant properties that help reveal the mechanism of the molecular network. Thus, in order to visualize the associations among the 16 identical genes they were also imported to the STRING database, where the minimum required interaction score was set at 0.7, and other parameters were at default. As shown in Fig. 3, except for 3 isolated nodes, the other 13 nodes constituted an interaction network, which indicated that these 13 targets might have similar molecular functions and be closely related to the mechanism of IS. Subsequently, the Fig. 2 was looked back to obtain the NAC compounds interacting with these 13 genes. The detailed information about the 13 targets, NAC compounds, structures and their herb origins were all listed in Tables 1 and 2.

3.5. Molecular docking result and analysis

To verify the 13 targets and their interacting compounds, the molecular docking simulation were carried out by the systems Dock method. The binding affinities of the testing compounds were compared to the ones of the corresponding native ligands. As the heat map in Fig. 4 illustrated, most compounds except for perlolyrine had stronger interactions than the native ligands. Perlolyrine is a hydroxyl-containing alkaloid with a high polarity and rapid metabolic conversion *in vivo*, and the resulted metabolite is more polar and more water-soluble which can be easily and rapidly excreted from the body [18]. This may be the reason for the poor performance in its docking simulation. Nevertheless, as for other compounds approximately half of the interactions yielded the docking scores (pK_d) of bigger than 7, which is equal to a K_d of 0.1 μM. Such results confirmed that most of these potential active compounds had very strong binding affinities with the key targets, and that the present network pharmacology method was to a great extent reasonable.

The compounds and targets in Fig. 4 were speculated to be the potential active compounds and the interacting targets of NAC in treatment of IS. As shown in Fig. 2, these targets were enriched in 18 pathways, which can be roughly classified into several categories, including inflammation-related, oxygen-related, ion transport regulation-related, tissue remodeling and others.

4. Discussion

As was reported about the Interleukin-4 (IL-4) and Interleukin-13 (IL-13) signaling pathway (Reactome: R-HSA-6785807), IS patients with brain tissue necrosis can activate autoimmune response mechanism and signal transduction of astrocytes, and then glial cells release IL-1B, IL-6, and other inflammatory factors, and trigger inflammation, which may cause irreversible death of neurons [19]. In human peripheral blood, IL-4 and IL-13 significantly downregulate the expression of proinflammatory signal transducers, while IL-13 shares many functional properties with IL-4 [20]. STAT3-upregulated plasma membrane proteins pathway is a sub-pathway of IL-4 and IL-13 signaling pathway, which include genes like MMP-9, MMP-3, ICAM-1, and etc. In the aged rats after reversible embolic occulsion of the middle cerebral artery (MCAO), the activation of STAT3 and the signaling mechanism of regulating astrocyte reactivity was truncated [21]. Besides, it was shown that increase in ICAM-1 expression can be through the STAT-3 signaling pathway, and ICAM-1 antibody drug can reduce leukocyte adhesion and infarct size in experimental stroke studies [22,23]. Also, STAT-3 can upregulate extracellular protein genes such as MMP-3 [24]. As reported in, the expression of MMP-9 increased in cerebral ischemia, so a developing new therapeutics was suggested to directly inhibit the activity or decrease MMP-9 expression by blocking

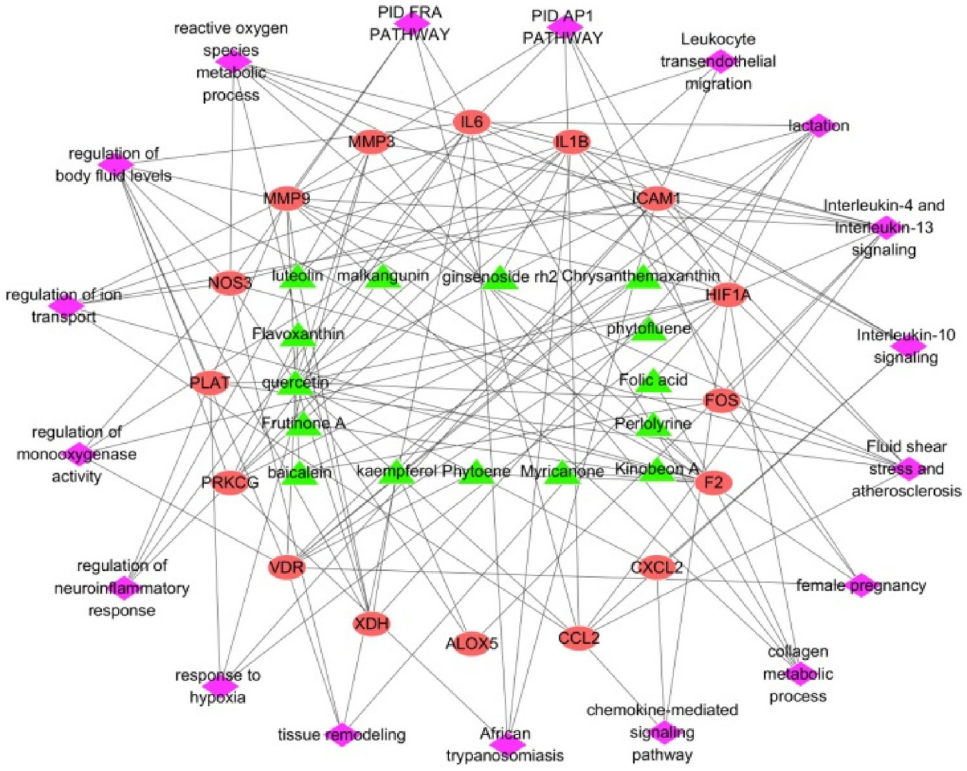


Fig. 2. The "Compound-Target-Pathway" interaction network.

signal pathways [25].

In acute cerebral ischemia, oxygen metabolism and nervous function are chaotic, and there exists an imbalance between oxygen tissue consumption and delivery [26]. Excessive reactive oxygen species (ROS) in ischemic tissue can oxidate lipids and proteins, causing peroxidation and irreversible effect to nucleic acids and sugars [27]. For IS, there is increasing evidence for the potential pathophysiological role of genes, among which the endothelial nitric oxide synthase gene (NOS3)

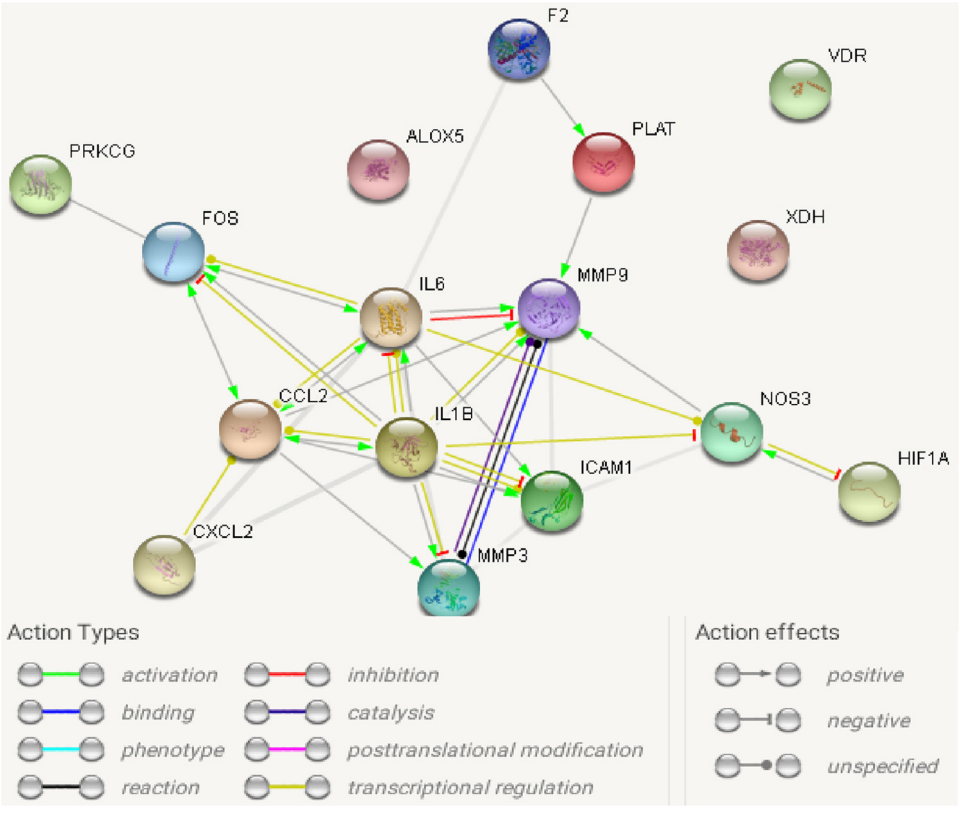
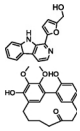
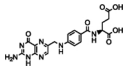
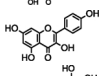
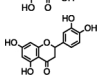
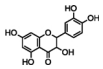
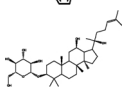
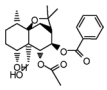
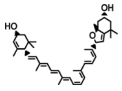


Fig. 3. The PPI interaction network of 16 identical genes by STRING.

Table 1
The 13 targets and the counts of their interacting compounds.

NO	Uniprot ID	Gene symbol	Target name	Edge
1.	P05231	IL6	Interleukin-6	9
2.	P01584	IL1B	Interleukin-1 beta	8
3.	P13500	CCL2	C-C motif chemokine 2	7
4.	P14780	MMP9	Matrix metalloproteinase-9	7
5.	P05362	ICAM1	Intercellular adhesion molecule 1	6
6.	P08254	MMP3	Stromelysin-1	5
7.	P29474	NOS3	Nitric oxide synthase	5
8.	P01100	FOS	Proto-oncogene c-Fos	4
9.	P19875	CXCL2	C-X-C motif chemokine 2	3
10.	P00734	F2	Prothrombin	2
11.	P00750	PLAT	Tissue-type plasminogen activator	2
12.	P05129	PRKCG	Protein kinase C gamma type	1
13.	Q16665	HIF1A	Hypoxia-inducible factor 1-alpha	1

Table 2
The compounds and their properties, structures and herbal origins.

Molecule Name	OB%	DL	Structure	Origin
Perlolyrine	65.95	0.27		Chuanxiong
Myricanone	40.60	0.51		Chuanxiong
FA(Folic acid)	68.96	0.71		Chuanxiong
Flavoxanthin	60.41	0.56		Honghua
Kinobeon A	48.47	0.36		Honghua
Baicalin	40.12	0.75		Honghua
Kaempferol	41.88	0.24		Honghua、Renshen
Luteolin	36.16	0.25		Honghua
Quercetin	46.43	0.28		Honghua
Frutinone A	65.90	0.34		Renshen
Ginsenoside rh2	36.32	0.56		Renshen
Malkangunin	57.71	0.63		Renshen
Chrysanthemaxanthin	38.72	0.58		Renshen

is reported as a genetic risk factor [28]. It was found that deficiency of NOS3 exerted a sustained beneficial effect on post-ischemic myocardium two days after reperfusion with preserved mitochondrial function, which was presumed that it can reduce inducible NOS induction, limit the hypoxia state, and thus reduce the damage caused by cardiac ischemia [29]. As one can see, NOS3 plays an important role in reactive oxygen species metabolic process pathway (GO:0072593). Amino acid toxicity, as an important pathogenesis factor of IS, is often associated with calcium ion transport [30]. As the amino acid promote Ca^{2+} influxing, it results in Ca^{2+} toxicity accumulation, and causes the decrease of intracellular pH. In the regulation of ion transport pathway (GO:0043269), C-C motif chemokine 2 (CCL2) belongs to positive

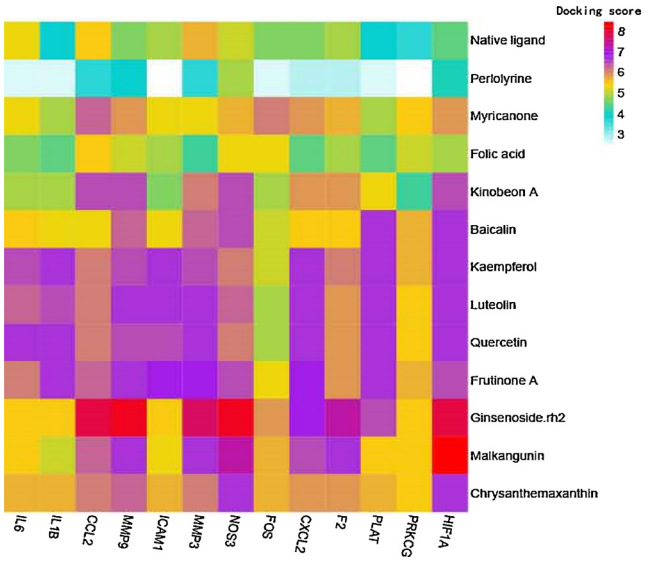


Fig. 4. The heat map of the docking score.

regulation of calcium ion import, and is of great importance for the transport of calcium ion. In the pathogenesis of stroke damage, CCL2 levels were elevated by increasing the influx of monocytes and inflammatory cells [31]. What is more, calcium has significant effects in both apoptosis and necrosis, while many calcium channels are affected by reactive oxygen or reactive nitrogen species [32].

As the potential active compounds in NAC listed in Table 2 are concerned, they belong to flavonoids, alkaloids, organic acids and others. Luteolin is a flavonoid, which can not only significantly reduce inflammatory response of activated macrophages, but also down-regulate the expression of proinflammatory cytokines, such as IL-6, IL-1B, etc [33]. Quercetin, also being a flavonoid, is always found in many medicinal plants and is often used in the treatment of cardiovascular and cerebrovascular diseases. Experiments showed that quercetin can improve the learning and memory ability of rats with chronic cerebral ischemia, reverse the damage caused by ischemia and inhibit the voltage-dependent Na^{+} channel [34]. Besides, quercetin is also known for its neuroprotective effect, reversibly inhibits homomeric rat acid-sensing ion channels (ASICs), while ASICs are always associated with many pathophysiological processes, such as neuronal death during the period of ischemic stroke [35]. In the same way, hyperhomocysteinemia is a risk factor of IS, so a new thought for stroke prevention is supplementing folic acid to reduce plasma homocysteine level [36]. Ginsenosides are the major pharmacologically active ingredients of ginseng, and appear pharmacological activities including vasorelaxation, antioxidation, anti-inflammation and anti-cancer, etc [37]. Shao et al found that Ginsenoside rh2 may protect against myocardial ischemia-reperfusion (I/R) injury in rats with high-fat diet by improving oxidative stress in high-fat rats, and ginsenoside also can reduce the level of IL-6 in the damaged myocardium of high-fat rats [38].

In Fig. 2 the targets were also enriched in the pathway of fluid shear stress and atherosclerosis. As known, hemodynamic shear stress is an important determinant of endothelial function and phenotype. Low shear stress is prevalent at atherosclerosis-prone sites, and stimulates an atherogenic phenotype. When a blood clot forms on an atherosclerotic plaque within a blood vessel in the brain and blocks blood flow to that part of the brain, the stroke will occur. By the determination of the cerebral vascular hemodynamic index (CVHI), one clinical study showed that NAC could improve cerebral vascular function and reduce the incidence of IS [39]. Another reported that NAC had a good clinical effect in the intervention of high-risk stroke patients, and was conducive to cognitive function and upper limb function recovery [40].

The above mentioned targets and pathways were typical and

consistent with the mainstream pharmacological recognition on the occurrence and development of IS. Frankly speaking, those results were not very innovative. Nevertheless, they showed the feasibility and accuracy of the present work, which also has potential application for research and development of other TCM prescriptions

5. Conclusion

In this paper, a network pharmacology based study was performed to reveal the active substances and mechanism of NAC against IS. 51 compounds and 325 targets in addition to 54 disease-related targets were obtained, and 16 identical targets were observed to be enriched in 18 pathways. In the STRING analysis, 13 targets and their interacting compounds were yielded, which had very strong affinities in the molecular docking simulation.

However, the current method still has room to improve. The present results were only based on the computational methods, and still required to confirm by further research. The pharmacological tests in vitro and in vivo are ongoing, which help understand and evaluate the pathway analysis results in this work. Moreover, as to take the dose dependence factor into the network analysis, we aims to construct a weighted heterogeneous network in our future work, by considering the compounds concentrations, the binding force between the compounds and the target proteins, the microenvironment of the interaction, etc. to define the weights.

Declaration of Competing Interest

Authors declare no conflict of interests

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