

Investigation of Active Ingredients and Mechanism of Sanao Decoction in the Treatment of Chronic Cough by Network Pharmacology

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Research

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Abstract

Background: Chronic cough significantly affects human health and quality of life. Studies have shown that Sanao Decoction (SAD) can clinically treat chronic cough. To investigate its mechanisms, we used the method of network pharmacology to conduct research at the molecular level.

Methods: The active ingredients and their targets were screened by pharmacokinetics parameters from the traditional Chinese medicine system pharmacology analysis platform (TCMSP). The relevant targets of chronic cough were obtained from two databases: GeneCards and DrugBank. Take the intersection to get potential targets of SAD to treat chronic cough and establish the component-target regulatory network by CytoScape3.7.2 and protein-protein interaction (PPI) network by STRING 1.0. The function of the target gene and related pathways were analyzed by the Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) in the Database for Annotation, Visualization, and Integrated Discovery (DAVID). The significant pathways and their relevant targets were obtained and the target-pathway network was established by CytoScape3.7.2. Finally, molecular docking of the core active components and relevant targets was performed.

Results: A total of 98 active components, 113 targets were identified. The component-target and target-pathway network of SAD and PPI network were established. Enrichment analysis of DAVID indicated that 2062 terms were in biological processes, 77 in cellular components, 142 in molecular functions and 20 significant pathways. In addition, the molecular docking showed that quercetin and luteolin had a good combination with the corresponding targets.

Conclusions: It indicates that the active compounds of SAD, such as quercetin, luteolin, may act on AKT1, MAPK1, RELA, EGFR, BCL2 and regulate PI3K-Akt signaling pathway, AGE-RAGE signaling pathway in diabetic complications and Fluid shear stress and atherosclerosis pathway to exert the effects of anti-inflammatory, anti-airway remodeling, anti-oxidant stress and repair airway damage to treat chronic cough.

Background

Chronic cough is deemed to be a cough that lasts for more than 8 weeks and the chest X-ray has no evidence of lung disease, usually accompanied by airway inflammation. The etiology is complex and there are four common causes: eosinophilic bronchitis (EB, 22%), postnasal drip syndrome (PNDs, 17%), cough variant asthma (CVA, 14%) and gastroesophageal reflux cough (GERC, 12%) [1]. It is related to cough receptors, which may be more sensitive or overstimulated by physical or chemical factors in the long-term inflammatory environment [2]. In modern medicine, etiological or symptomatic treatment is mostly used. For example, if the cause is determined to be eosinophilic bronchitis, glucocorticoid treatment is considered. However, the clinical effect is not obvious or the symptoms are relieved but it is easy to resume. Symptomatic treatment often uses antitussives and expectorants, in which the

symptoms are improved but the cause has not meant eradicated. Therefore, for long-term use, it is not recommended [3].

Traditional Chinese medicine (TCM) has definite advantages in the treatment of chronic cough and it thinks that all the viscera make people cough, not only the lungs from Huangdi Neijing, a book about TCM more than two thousand years ago. SAD is composed of *Ephedra Herba* (Mahuang), *AmygdalusCommunis* Vas (Kuxingren) and *Glycyrrhiza Uralensis* Fisch (Gancao), which has been served in relieving cough and asthma for 1800 years [4]. Hua Li [5] divided 80 children with a chronic cough into a control group and a treatment group, which were addressed in conventional symptomatic treatment and SAD plus Liujunzi decoction, respectively. The results showed that the effective rate of the control group was 62.50% and the treatment group was 95.00%. Jin Lu [6] separated 124 patients with a chronic cough into the western medicine treatment group and TCM treatment group, treated with Pentovirine and SAD plus Zhisu decoction, respectively. The results showed that the effective rate of TCM treatment group (96.77%) was higher than that of the other group (77.42%) and the incidence of adverse reactions (1.61%) was lower than that in the Western medicine treatment group (17.74%). According to the clinical symptoms of atypical patients, other herbs are often added based on SAD to treat chronic cough. However, the active ingredients and mechanism of SAD for the treatment of chronic cough have not been elucidated due to its complexity. To enhance and expand the clinical application of SAD, the network pharmacology approach was utilized to elucidate its pharmacological basis.

First proposed by Professor Hopkins A.L in 2007, the network pharmacology, based on the theory of system biology, established the bimolecular network of drugs and systematically analyzed the interaction between drugs and organisms from multiple levels [7]. It is compatible with the theory of TCM about multi-components, multi-targets and multi-pathways in the treatment of diseases. In 2007, Shao Li used the method of network pharmacology to study the relationship between the molecular mechanism of TCM cold and heat syndrome and the neuroendocrine-immune network system, laying the foundation for the development and application of network pharmacology in TCM [8]. In recent years, network pharmacology has provided a new way of studying the pharmacological mechanism of TCM from the molecular level, and given a hint of the development of new drugs for TCM.

In the present study, the active components and their targets of SAD were obtained from TCMSp. The targets related to chronic cough were collected and compared with the above to screen the targets of SAD for chronic cough. The component-target regulatory network and protein-protein interaction network were established. Then the enrichment analysis of GO and KEGG pathways was performed to identify the significant pathways and targets. Molecular docking was used to further identify the relationship between the active components and potential targets (Fig. 1).

Methods

Active components and their targets screening

The chemical components of SAD can be consulted on TCMSP, (<http://lsp.nwu.edu.cn/tcmsp.php>) [9]. TCMSP can provide information, including molecular name, oral bioavailability (OB), blood-brain barrier (BBB), intestinal epithelial permeability, drug-likeness (DL) and lipid-water partition coefficient. Three herbs in this prescription were input: *Ephedra Herba*, *AmygdalusCommunis* Vas and *Glycyrrhiza Uralensis* Fisch, and the active components and their targets were further screened by setting the criteria of OB 30% and DL 0.18. OB reflects the speed and degree of absorption of the drug in human circulation by oral administration and is one of the pharmacokinetics important parameters. DL describes the similarity between the compound and the known drug. The average DL of drugs included in DrugBank is 0.18 [10].

Potential targets screening

Use “chronic cough” as the keyword, search the relevant targets in the GeneCards database [11] and find the targets of clinical medication for treating chronic cough in the Drugbank database [12] as a supplement. Then standardized processing of target information was performed in the Uniprot database [13]. Compare the targets of active components with the relevant targets of chronic cough to obtain the potential targets of SAD for the treatment of chronic cough, and use an online tool (<https://bioinfogp.cnb.csic.es/tools/venny/>) [14] to draw a Venny diagram.

Component-target and PPI network construction

Use CytoScape3.7.2[15] software to construct the component-target network of SAD. The potential targets of SAD for chronic cough were submitted to the STRING1.0 database [16] to construct a PPI network. Set biological type to “Homo sapiens”, the minimum interaction threshold to “highest confidence> 0.9”, and the rest is the default settings to establish the PPI network. The PPI network can be analyzed through the MCODE plugin in CytoScape3.7.2 to obtain hypothetical protein functional modules, and then the biological processes involved can be analyzed and their functions described.

Bioinformatics analysis

The DAVID [17] platform provides systematic and comprehensive biological function annotation information, which is used primarily for the enrichment analysis of differential gene function and pathway. Upload the potential targets of SAD for chronic cough, set FDR <0.05 and perform enrichment analysis of GO [18] and KEGG [19] pathway based on biological processes, cellular components and molecular functions.

Target-pathway network construction

Construct the target-pathway network of SAD by uploading the top 20 pathways and related targets obtained from the previous step to CytoScape3.7.2.

Molecular Docking

The components were sorted by degree in the component- target network and the top 2 were selected. The mol2 format file of components can be downloaded in ZINC (<http://zinc.docking.org/>) [20] and the PDB format file of targets in PDB database (<https://www.rcsb.org/>) [21]. Then perform the processing of targets to remove the water and ligands bound by Pymol software [22]. Finally, use AutoDock VINA for molecular docking, analyze the binding energy and hydrogen bonds and use Pymol for visual analysis [23].

Results

Screening of active components and their targets

92 active ingredients of *Glycyrrhiza Uralensis* Fisch, 18 of *Amygdalus Communis* Vas, and 21 of *EphedraHerba* were screened, including 98 active ingredients such as quercetin, luteolin, kaempferol, see details in Table 1. The active ingredients of *Glycyrrhiza Uralensis* Fisch had 1725 targets, *Amygdalus Communis* Vas 201 targets, and *EphedraHerba* 494 targets. After the merger, delete duplicate values and the targets that have not been experimentally verified. Finally, we obtained 237 targets.

Table 1 Information on active compounds

MOL_ID	MOL_Name	MOL_Mass	OB (%)	DL	source	degree
MOL000098	quercetin	302.25	46.43	0.28	multidrug	75
MOL000006	luteolin	286.25	36.16	0.25	<i>Ephedra Herba</i>	39
MOL000422	kaempferol	286.25	41.88	0.24	multidrug	29
MOL004328	naringenin	272.27	59.29	0.21	multidrug	20
MOL003896	7-Methoxy-2-methyl isoflavone	266.31	42.56	0.2	<i>Glycyrrhiza Uralensis</i> Fisch	17
MOL000497	licochalcone a	338.43	40.79	0.29	<i>Glycyrrhiza Uralensis</i> Fisch	17
MOL002565	Medicarpin	270.3	49.22	0.34	<i>Glycyrrhiza Uralensis</i> Fisch	15
MOL000392	formononetin	268.28	69.67	0.21	<i>Glycyrrhiza Uralensis</i> Fisch	15
MOL000358	beta-sitosterol	414.79	36.91	0.75	<i>Ephedra Herba</i>	15
MOL004891	shinpterocarpin	322.38	80.3	0.73	<i>Glycyrrhiza Uralensis</i> Fisch	14
MOL010921	estrone	270.4	53.56	0.32	<i>Amygdalus Communis</i> Vas	10
MOL012922	I-SPD	327.41	87.35	0.54	<i>Amygdalus Communis</i> Vas	10

Abbreviation: MOL: molecule, OB: oral bioavailability, DL: drug-likeness

Screening of potential targets

3734 targets of chronic cough were obtained from the GeneCards. Depending on experience, targets with a score greater than the median were set as potential targets of chronic cough. For example, the maximum score of targets obtained from the GeneCards database was 87.54, the minimum was 0.33, the median was 5.12, so the targets with score > 5.12 were the potential targets for chronic cough. Combined with the DRUGBANK database to supplement applicable targets, delete duplicate values after merging, and finally get 821 targets. Finally, obtain 113 targets of SAD to treat chronic cough through the intersection of the targets of the active components and the related target of chronic cough, and draw the Venny diagram (Fig. 2).

Construction of component-target and PPI network

Cytoscape3.7.2 was used to construct the component-target network of SAD (Fig. 3). There were 211 nodes (98 compounds and 113 targets) and 938 edges. After analysis, quercetin, luteolin, kaempferol, naringenin, 7-Methoxy-2-methyl isoflavone were highly connected and they had more targets than other components.

The 113 targets that were related to the treatment of chronic cough by SAD were uploaded to the STRING1.0 platform to obtain the PPI network by setting the combining score > 0.4. The PPI network (Fig. 4a) is an undirected graph about the interaction of proteins, in which the module was used to describe some areas with high protein density, which was considered to be a biologically meaningful collection that can represent protein complexes or proteins in the same pathway [24]. Therefore, it was necessary to further identify its internal module. The MCODE plugin in Cytoscape3.7.2 can be used to analyze the interaction using the complex molecular detection algorithm where a module with a score >3 was significant. Finally, 3 clusters were obtained. In Figure 4b, Module 1 contains 53 nodes and 1064 edges with a score of 40.923. In Figure 4c, Module 2 contains 16 nodes and 31 edges with a score of 4.133. In Figure 4d, Module 3 contains 4 nodes and 5 edges with a score of 3.500.

GO and KEGG enrichment analysis

The DAVID platform was utilized to perform GO and KEGG enrichment analysis. It indicated that many targets and pathways were closely related to chronic cough. GO analysis showed that 2281 GO terms were enriched in chronic cough: 2062 in biological processes, 77 in cellular components, and 142 in molecular functions. As shown in Fig. 5a, the main biological processes involved cellular response to oxidative stress, response to nutrient levels, response to molecule of bacterial origin, response to lipopolysaccharide. As shown in Fig. 5b, the functions of related targets to regulate chronic cough were mainly enriched in phosphatase binding, protein phosphatase binding, cytokine receptor binding and kinase regulator activity. According to KEGG analysis, 148 pathways were related to the treatment of chronic cough, including PI3K-Akt signaling pathway, AGE-RAGE signaling pathway in diabetic complications, Fluid shear stress and atherosclerosis and Kaposi sarcoma-associated herpesvirus infection, as shown in Fig. 5d.

Construction of the target-pathway network

Cytoscape3.7.2 was used to construct the target-pathway network of SAD (Fig. 6) and topological analysis of 20 pathways and 75 related targets was performed. It suggested that AKT1, MAPK1, MAPK3 and RELA were highly connected, and may be the principal targets of SAD in the treatment of chronic cough.

Verification of Molecular Docking

The quercetin and luteolin are respectively docked with the corresponding targets, setting the binding energy < -5 kcal/mol as the screening criterion, which is believed that the molecules meeting this condition may be connected to the target [25]. Generally, the lower the binding energy, the more stable the

conformation bound. It can be seen from Table 2 and Fig. 7 that quercetin and luteolin may bind to the targets to a certain extent.

Table 2 Information on molecular docking

NO.	Targets	PDB_ID	Compounds	Affinity(kcal/mol)
1	RELA	3GUT	Quercetin	-6.7
			Luteolin	-7.3
2	AKT1	1UNP	Quercetin	-5.8
			Luteolin	-6.1
3	MAPK1	4IZ5	Quercetin	-8.5
			Luteolin	-8.3
4	EGFR	4LL0	Luteolin	-9.2
5	BCL2	5AGW	Quercetin	-7.8
6	EGF	1IVO	Quercetin	-8.3
7	SOD1	4XCR	Quercetin	-7.4
8	CAV1	Q03135(SWISS-MODEL)	Quercetin	-5.7

Discussion

SAD is composed of three herbs that contains 98 compounds at least, which have been proven effective in treating cough for 1800 years. However, its complexity and unclear mechanism limit its clinical use only under the guidance of traditional Chinese medicine theory. After analysis by network pharmacology, we obtained its main active compounds, such as quercetin, luteolin, kaempferol, which had the most targets and existed in all the herbs. Quercetin and kaempferol are both flavonoids, and modern studies have demonstrated that they are both effective for cough and asthma [26]. 1977, quercetin tablets were used clinically for the treatment of chronic bronchitis, which had the effect of dispelling phlegm, relieving cough and asthma, with an effective rate of more than 90% [27]. Liang Huang [28] found that quercetin can inhibit the expression of inflammatory mediators such as TNF- α and IL-8 by reducing the activity of ERK1/2, p38MAPK and PKC, thereby inhibiting the excessive release of proinflammatory mediators. Another study reported that quercetin can inhibit the activation of neutrophils by lipopolysaccharide (LPS), mainly by affecting the expression of neutrophil adhesion molecules CD62L, CD11b / CD18 [29], and also inhibit LPS from delaying the spontaneous apoptosis of neutrophils, which could avoid the expansion of inflammatory response [30]. Hua Zou [31] showed that luteolin can reduce the expression level of IL-4 in lung tissue and up-regulate the IFN- γ expression level to improve airway inflammation in

asthmatic young mice by establishing a model of ovalbumin-sensitized asthmatic mice, which can be speculated that this may be related to the inhibition of irreducible nitric oxide synthase/nitric oxide signaling pathway.

Considering that there will be a continuous inflammatory response, some structural changes and damages in the airway during the process of chronic cough, we will explore the mechanisms of SAD in treating chronic cough from the following perspectives by analyzing the target-pathway network combining with the results of GO and KEGG enrichment analysis (Fig 8).

Anti-inflammatory

Enrichment analysis of the KEGG pathway showed that PI3K-Akt signaling pathway, AGE-RAGE signaling pathway in diabetic complications and Fluid shear stress and atherosclerosis pathway had the most enriched genes, and all three pathways were associated with inflammation. In the PI3K-Akt signaling pathway, if we chose selectively to activate NF- κ B, the permeability of the vascular wall can be enhanced by releasing VEGF, and the release of inflammatory mediators to the local trachea can be increased by releasing IL-6 and TNF- α , causing local mucosal edema, which thickened tracheal walls and restricted airflow [32]. BCL-2 is just an anti-apoptosis gene in the PI3K-Akt signaling pathway, which can inhibit eosinophil apoptosis to keep the airway in a long-term inflammatory and hyperreactive state. Herein, we can reduce inflammation by inhibiting the expression of BCL2 [33]. AGE-RAGE signaling pathway in diabetic complications and Fluid shear stress and atherosclerosis pathway both intersect with multiple signaling pathways, including the PI3K-Akt signaling pathway. Nuclear factor erythroid 2-related factor 2 in the Fluid shear stress and atherosclerosis pathway can regulate the expression of nitric oxide synthase (eNOS) that can catalyze the production of NO, which is an important cellular signal molecular that can regulate NF- κ B in the response to inflammation. It can be assumed that SAD can inhibit the release of inflammatory mediators by regulating the above signaling pathways, thereby inhibiting the occurrence of inflammation.

Anti-airway remodeling

Due to the long duration of chronic cough, the patient's trachea will undergo structural changes, that is, airway remodeling, such as metaplasia of epithelial cells to goblet cells which can lead the abnormal increase in goblet cells, the sensitivity of the epithelial cells to oxidative stress and the thickening of airway smooth muscle [34]. Studies showed that the expression of epidermal growth factor receptor (EGFR) and its ligand (HB-EGF) significantly increased in the lung tissues of asthmatic mice, and the area of superficial collagen and the number of goblet cells were positively correlated with it. Through the use of inhibitors to block the EGFR-mediated signaling pathway, it was found that the expression levels of EGFR and HB-EGF were significantly reduced, and the area of superficial collagen, the number of goblet cells, and the degree of epithelial thickening were also lower [35]. EGF is a mitogen of a variety of cells, which can activate the tyrosinase and MAPK cascade to transmit signals to the nucleus combined with the EGFR, thereby promoting the proliferation of airway smooth muscle cells and fibroblasts [36]. The results of GO analysis indicate that Molecular Function is mainly enriched in phosphatase binding and

kinase regulator activity, which can be inferred that SAD may block EGFR signal transduction by inhibiting the phosphorylation of signaling proteins by acting on EGFR receptors or phosphatase and kinase, and thereby inhibits the proliferation of airway-related cells and resists airway remodeling.

Antioxidant stress

Cough variant asthma is a form of chronic cough. Studies have demonstrated that asthma is a disease of oxidative stress [37], in which its oxidant level and antioxidant level are not balanced [38]. Inhibiting the occurrence of oxidative stress can effectively relieve asthma. It has been reported that glucocorticoids can alleviate the level of lung oxidative stress in asthmatic mice by regulating the Keap1-Nrf2 pathway and affecting the expression of downstream antioxidant response elements [39]. In Molecular Function results of GO analysis, response to oxidative stress involved the most genes.

By analyzing module 2 in the PPI network, it implicated that SOD1 and CAV1 acted on NCF1. CAV1 encodes the scaffolding protein that can activate the NADPH oxidase. NCF1 encodes a subunit of neutrophil NADPH oxidase which can be switched on to produce superoxide anion. SOD1 encodes superoxide dismutase 1 that catalyzes superoxide anion to molecular oxygen and hydrogen peroxide. Therefore, we can speculate that SAD may relieve asthma through the regulation of these targets to anti-oxidative stress.

Repair airway damage

Normal airway epithelial cells are composed of most cilia cells and a small number of goblet cells that secrete mucus. In the process of epithelial repair, the epithelial cells at the edge of the wound undergo an epithelial-mesenchymal transformation, and the fibroblasts in the thin sheath of fibroblasts proliferate and differentiate into myofibroblast, which synthetic fibrous matrix to provide a temporary protective barrier and support the expansion and migration of the epithelial surface. When the epithelial surface recovers, the epithelial cells will separate and first differentiate into mucus secretory cells with the apoptosis of myofibroblasts [40]. In the whole process, there are the proliferation, differentiation and apoptosis of cells. In the enrichment analysis, we found numerous genes that can regulate cell proliferation and differentiation, such as MAPK1, MAPK2, MAPK8, MAPK9, FOS, RAF1, etc. Studies have reported that vasoactive intestinal peptide promotes the repair of epithelial damage by regulating the MAPK signaling pathway [41].

Conclusions

In this study, the network pharmacology method was utilized to explain the possible mechanism of SAD in treating chronic cough from molecular level, and some active ingredients, targets and significant pathways were found, which could offer guidance for researches about how SAD treat chronic cough by some specific mechanisms and may inspire researchers to develop potential components as new drugs. However, there are some deficiencies, such as no experiments to verify, some essential chemical components such as ephedrine with bronchial smooth muscle expansion effect and glycyrrhizin with

glucocorticoid-like effect did not meet the criteria in the screening and failed to enter this study. Therefore, further research is needed to explain and supplement the results of this study.

Abbreviations

AKT1: RAC-alpha serine/threonine-protein kinase; BBB: blood-brain barrier ; BCL2: Apoptosis regulator Bcl-2; CAV1: Caveolin-1; CD11b/18/62L: Integrin alpha M; CVA: Cough variant asthma ; DAVID: Database for Annotation, Visualization, and Integrated Discovery; DL: Drug likeness; EB: Eosinophilic bronchitis; EGF: Epidermal growth factor; EGFR: Epidermal growth factor receptor; eNOS: the expression of nitric oxide synthase; ERK1/2: Mitogen-activated protein kinase 3/1; FOS: Proto-oncogene c-Fos; GERC: Gastroesophageal reflux cough ; GO: Gene Ontology; HB-EGF: Heparin-binding EGF-like growth factor; IFN- γ : Interferon gamma; IL-4/6/8: Interleukin-4/6/8; KEGG: Kyoto Encyclopedia of Genes and Genomes; LPS: Lipopolysaccharide ; MAPK1/2/3/8/9: Mitogen-activated protein kinase 1/2/3/8/9; MCODE: Molecular Complex Detection; NADPH: Nicotinamide adenine dinucleotide phosphate; NCF1: Neutrophil cytosol factor 1; NF- κ B: Nuclear factor kappa-light-chain-enhancer of activated B cells; NO: Nitric oxide; OB: Oral bioavailability; PKC: Protein kinase C; PNDs: Postnasal drip syndrome ; PPI: Protein-protein interaction; RAF1: RAF proto-oncogene serine/threonine-protein kinase; RELA: Transcription factor p65; SAD: Sanao Decoction; SOD1: Superoxide dismutase [Cu-Zn]; TCM: Traditional Chinese medicine; TCMSP: Traditional Chinese medicine system pharmacology analysis platform; TNF- α : Tumor necrosis factor alpha; VEGF: Vascular endothelial growth factor.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for application

Not applicable

Availability of data and materials

The data involved in this study are available from the corresponding author on reasonable request.

Competing interest

The authors declare that they have no competing interests.

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Authors' contributions

MS and XL conceived and designed this study. XL, MS, XW, and SQ participated in the data collection and the analysis of the results. YL, ZL, LL, JQ and XD aided in drafting and revising the manuscript. All authors read and approved the final manuscript.

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Figures

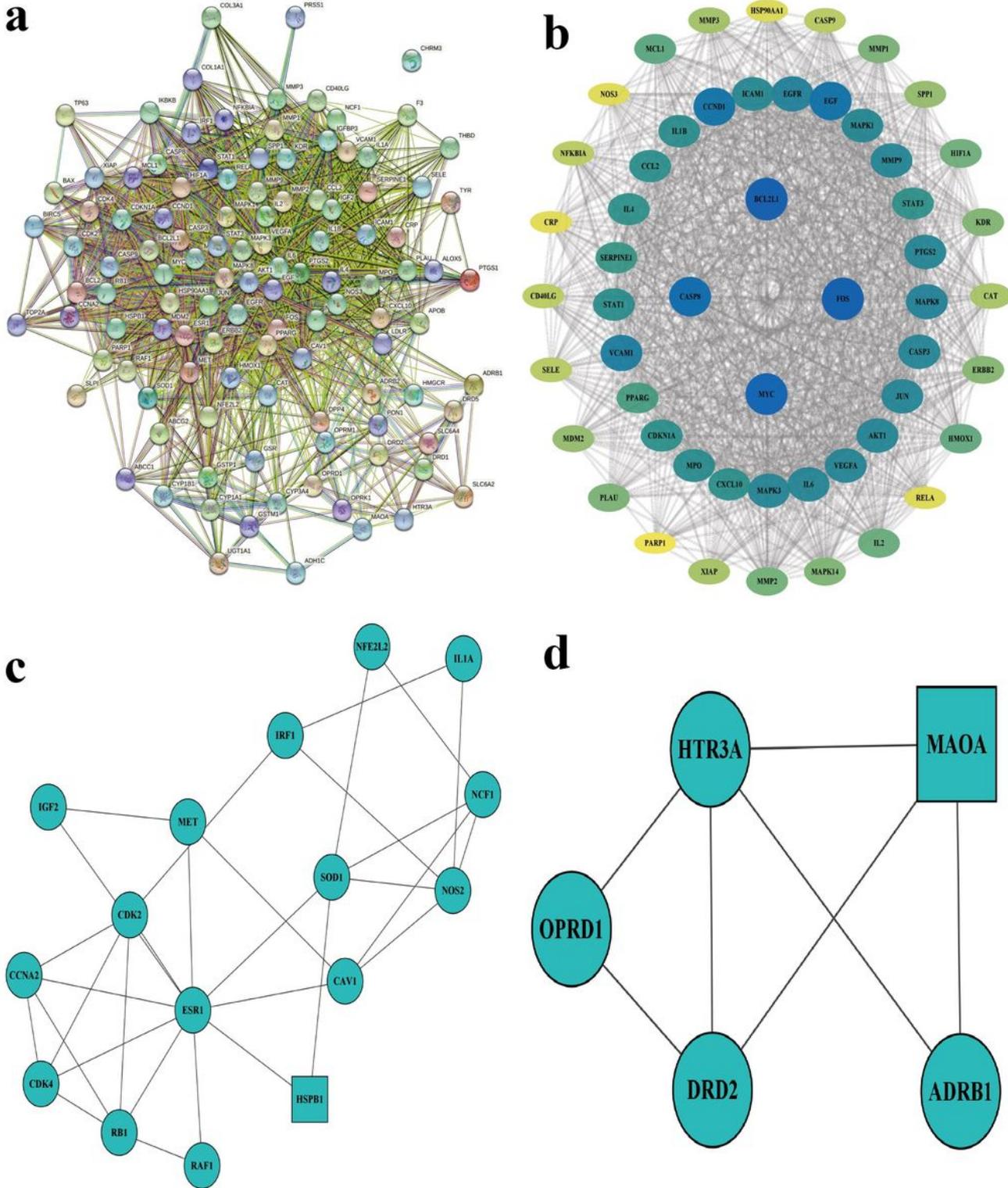


Figure 1

The network and clusters of PPI. Fig 4a represents the PPI network, Fig 4b-4d represent the cluster 1-3, respectively.

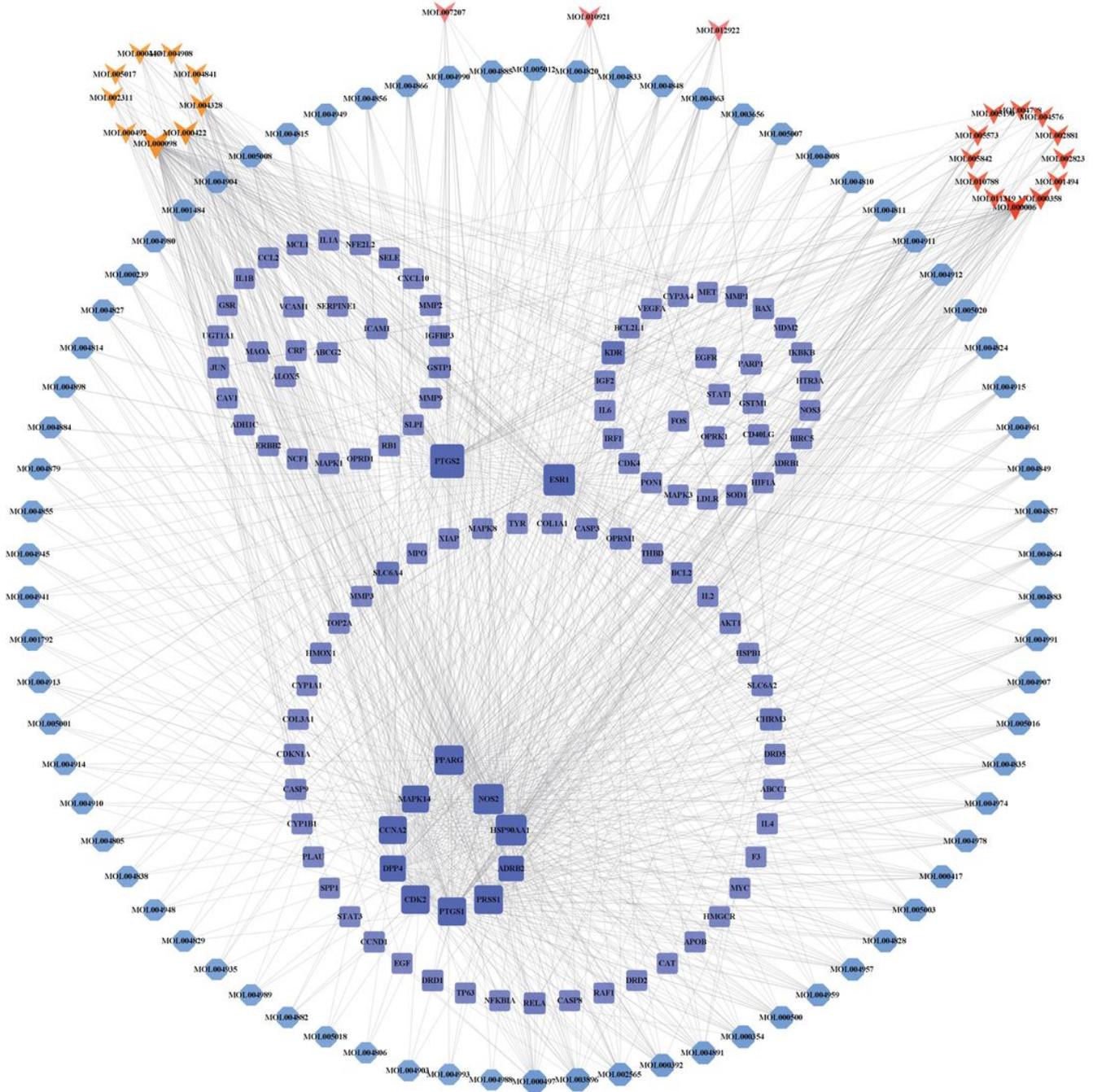


Figure 1

The component-target network of SAD. The octagon nodes represent *Glycyrrhiza Uralensis* Fisch, and the V shape represent others, of which the red is *Ephedra Herba*, the yellow is multidrug, and the pink is *Amygdalus Communis* Vas. The round rectangle nodes represent the targets, in which the target with more components is bigger in shape and darker in color.

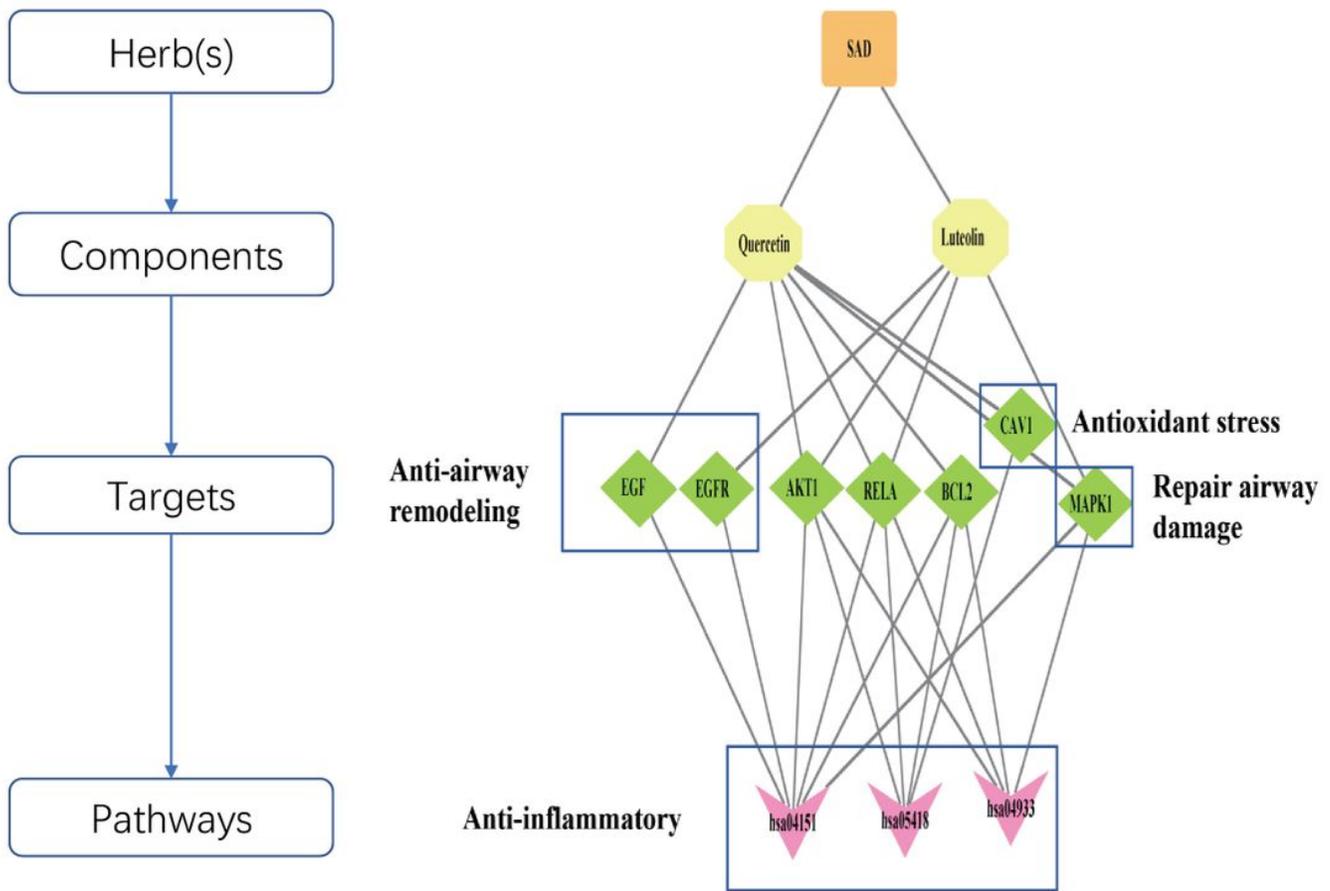


Figure 1

The graphical description of the design in the discussion.

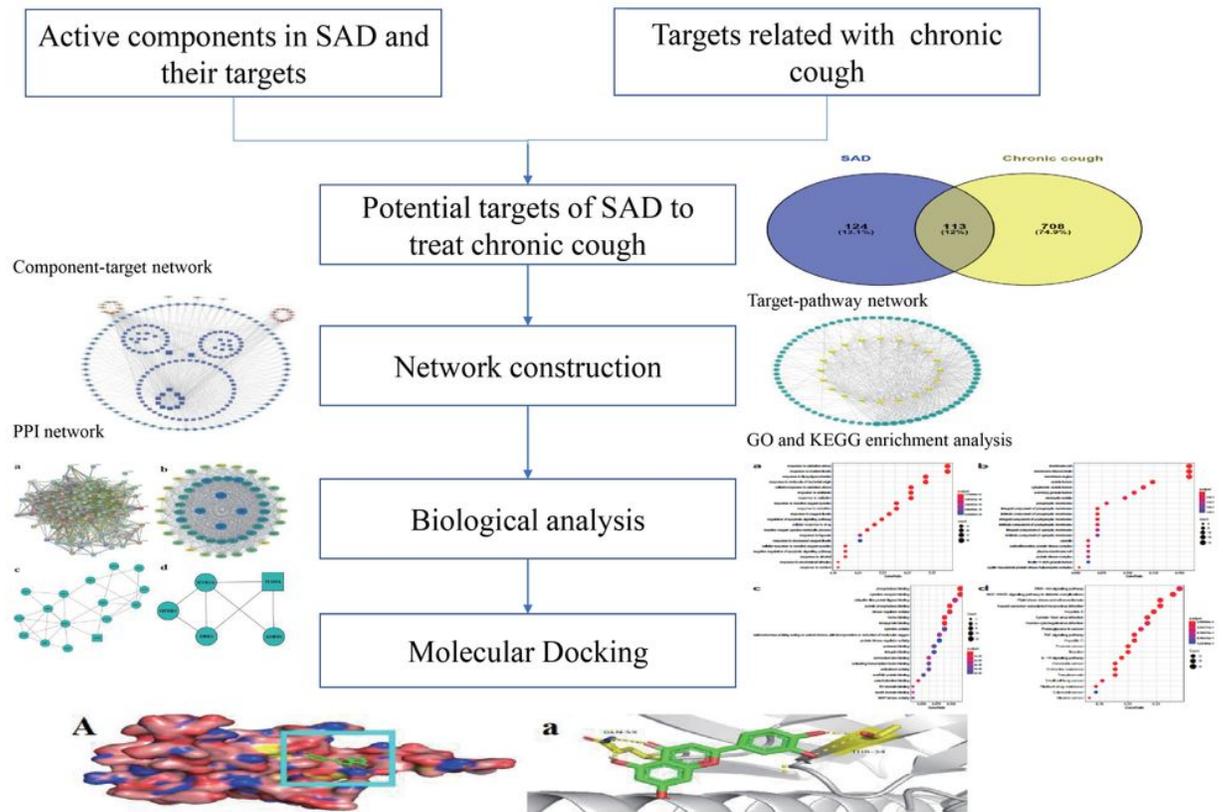


Figure 1

The workflow chart of exploring the mechanism of SAD to treat chronic cough by network pharmacology

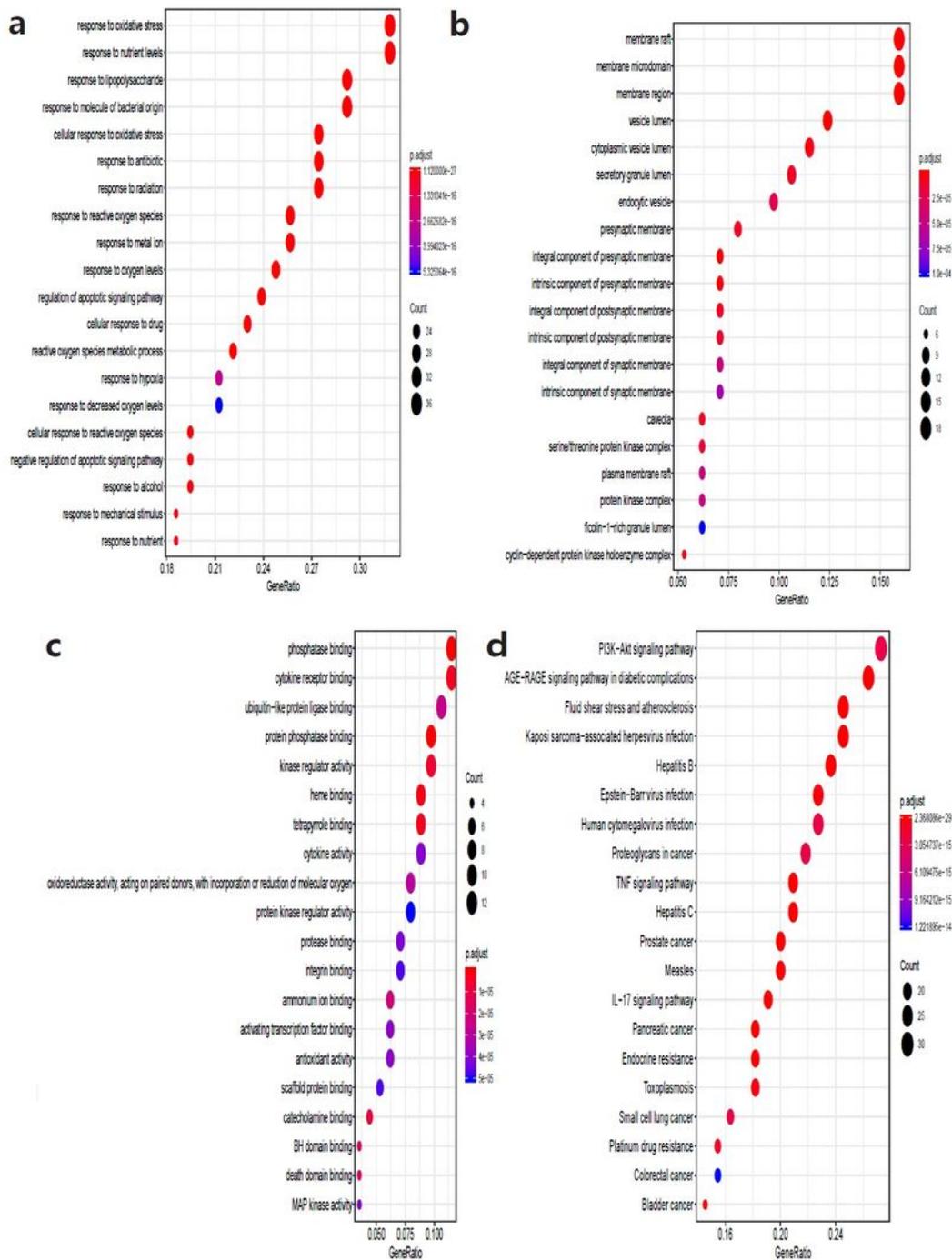


Figure 1

The enrichment analysis of GO terms and KEGG pathways. Figure 5a- 5c describe the biological processes, cellular components and molecule function, respectively. Figure 5d describes the KEGG pathways. The vertical axis represents related items, and the horizontal axis represents the percentage of genes contained in each item. The color of the dot represents the P-value, the redder the greater the P-

value is, the bluer the smaller. The size of the dot represents the number of genes involved, the larger the number, the more genes.

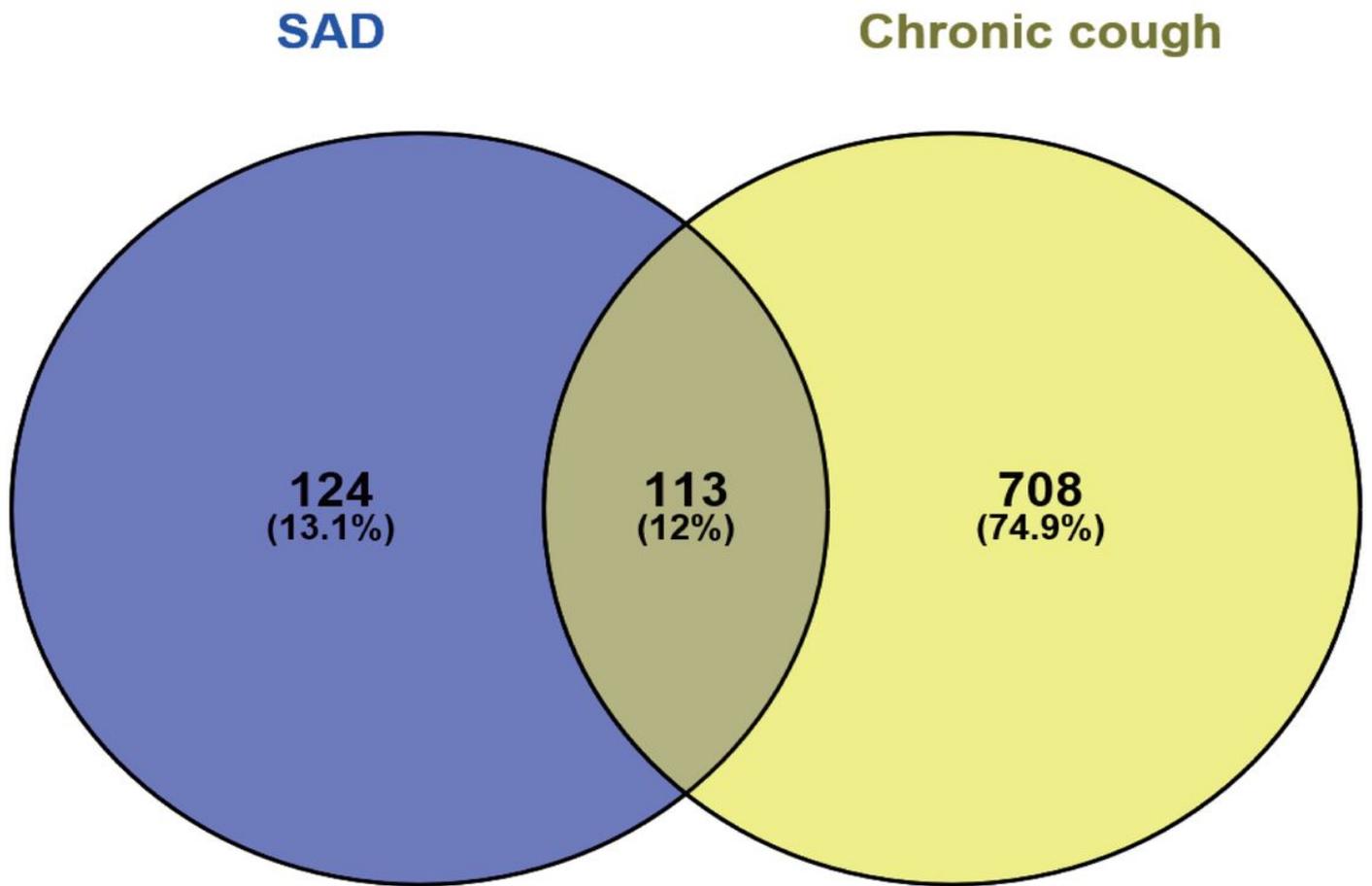


Figure 2

The Venny diagram of targets of SAD and chronic cough.

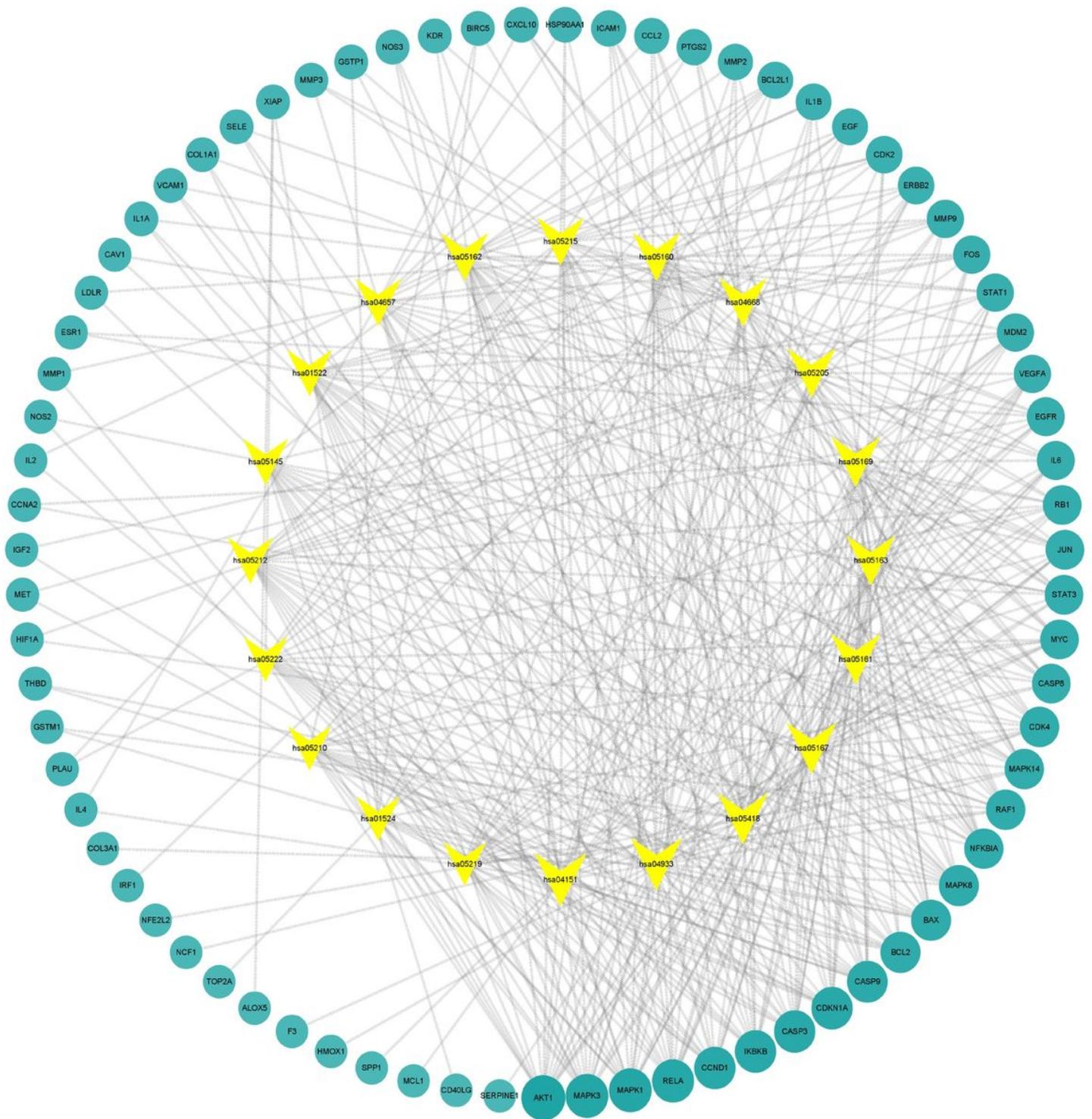


Figure 6

The target-pathway network of SAD. The blue nodes represent the targets and the yellow represent the pathway. The darker the color and the bigger the shape, the higher the degree, which refers that the more nodes it links.