European Review for Medical and Pharmacological Sciences 2020; 24: 3360-3384

Utilizing integrating network pharmacological approaches to investigate the potential mechanism of *Ma Xing Shi Gan Decoction* in treating COVID-19

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Abstract. – Beginning in December 2019, coronavirus disease 2019 (COVID-19), due to 2019-nCoV infection, emerged in Wuhan and spread rapidly throughout China and even worldwide. Employing combined therapy of modern medicine and traditional Chinese medicine has been proposed, in which *Ma Xing Shi Gan Decoction* (MXSGD) was recommended as a basic prescription and applied widely in the clinical treatment of COVID-19. We investigated the underlying mechanism of MXSGD in treating COVID-19 utilizing the approaches of integrating network pharmacology. A total of 97 active ingredients of MXSGD were screened out, and 169 targets were predicted. The protein-protein interaction network exhibited hub targets of MXSGD, such as Heat shock protein 90, RAC-alpha serine/threonine-protein kinase, Transcription factor AP-1, Mitogen-activated protein kinase 1, Cellular tumor antigen p53, Vascular endothelial growth factor A, and Tumour necrosis factor. Gene Ontology functional enrichment analysis demonstrated that the biological processes altered within the body after taking MXSGD were closely related to the regulation of such processes as the acute inflammatory response, chemokine production, vascular permeability, response to oxygen radicals, oxidative stress-induced apoptosis, T cell differentiation involved in the immune response, immunoglobulin secretion, and extracellular matrix disassembly. KEGG enrichment analysis indicated that the targets of MXSGD were significantly enriched in inflammation-related pathways, immunomodulation-related pathways, and viral infection-related pathways. The therapeutic mechanisms of MXSGD on COVID-19 may primarily involve the following effects: reducing inflammation, suppressing cytokine storm, protecting the pulmonary alveolar-capillary barrier, alleviating pulmonary edema, regulating the immune response, and decreasing fever.

Key Words:
COVID-19, 2019 n-CoV, *Ma Xing Shi Gan Decoction*, Traditional Chinese medicine, Integrating network pharmacology.

Introduction

Since December 2019, an outbreak of pneumonia cases of the unrevealed cause was first detected

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in Wuhan city in China[1]. An exponential rate of confirmed cases mounted in Wuhan and swiftly ramped up to affect the whole country and the virus subsequently jumped to several other countries, such as Japan, South Korea, Italy, and the USA[2,3]. Statistics updated on March 17, 2020 showed that the epidemic situation has emerged as a pandemic in China, involving a total of 81,116 confirmed cases with 2,830 patients still in critical condition and 3,231 deaths. In addition, over 90,000 cases were substantiated in more than 100 countries and regions besides China[4]. Response measures taken by the Chinese Government to control outbreaks involved preliminary actions to stop travel in and out of Wuhan city and then all of Hubei Province[5]. Due to the risks of the epidemic situation, the World Health Organization (WHO) announced this outbreak as a public health emergency of international concern on January 30, 2020[3].

Chinese scientists isolated and verified a novel type of coronavirus named 2019-nCoV by WHO and Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by the International Committee on Taxonomy of Viruses[2]. The 2019-nCoV is classified as the seventh member of the coronavirus family and is known as the third coronavirus that induces fatal human illness after SARS-CoV and Middle East Respiratory Syndrome Coronavirus (MERS-CoV)[6]. The illness generated by the novel coronavirus was named coronavirus disease 2019 (COVID-19) by WHO[7]. The clinical signs and symptoms of COVID-19 were described primarily as viral pneumonia with fever and dyspnea, several cases even developed Acute Respiratory Distress Syndrome (ARDS), and chest radiographs showed bilateral pulmonary infiltration[1,8-10].

Because there are no explicit antiviral therapies for COVID-19 and the primary treatments are supportive at present, the *Proposed Diagnosis and Treatment* issued by China's National Health Commission integrated the therapies of traditional Chinese medicine (TCM) and modern medicine[11]. Recently, a growing number of patients who tested negative for viral nucleic acid have been discharged from the hospital after adopting TCM treatment. TCM intervention has been formally specified as an integrated therapeutic strategy in China for treating patients who developed COVID-19 symptoms. Among the *proposed diagnoses and treatments*, a classical TCM formula called *Ma Xing Shi Gan Decoction* (MXSGD) was recommended as one of the basic prescriptions for the treatment of COVID-19[12-14]. The application of MXSGD exhibited a prominent curative effect on COVID-19[15]. Meanwhile, MXSGD was the fundamental part of "*Qing Fei Pai Du Decoction*" issued by China's National Health Commission and has been applied widely in China for treating COVID-19 patients, achieving pleasing clinical results.

MXSGD, a notable classic formula recorded in *Treatise on Febrile Diseases* (Shang Han Lun in Chinese) edited by Zhang Zhongjing in the Han Dynasty, consists of *Herba Ephedra*, *Semen Armeniacae Amarum*, *Radix Glycyrrhizae*, and *Gypsum Fibrosum*. MXSGD has been widely applied for the treatment of colds, influenza, acute bronchitis, bronchial asthma, and even acute exacerbation of the chronic obstructive pulmonary disease[16-19]. Clinical trials have shown that MXSGD treatment shortened the time for fever resolution in patients with H1N1 influenza virus infection[20], and its antivirus activity and the ability to relieve virus-associated lung injury have attracted widespread attention[19,21]. As demonstrated by ancient records and modern application, MXSGD is beneficial to cure respiratory tract diseases with symptoms of short breath, fever, cough, and body ache, which are highly consistent with the symptoms of COVID-19[1,9,22]. Integrating network pharmacology underlines the concept of a "multicomponent, multitarget therapeutic network" and the overall thinking of TCM. Network pharmacology provides a novel concept for comprehending the multitargeted mechanism of the treatment of sophisticated diseases with TCM[23]. In this study, we attempted to provide references for the potential therapeutic mechanism of MXSGD in COVID-19 treatment utilizing integrating network pharmacological measures.

Materials and Methods

Constructing Database of Candidate Compounds

The constituents of the compounds in MXSGD were retrieved from the online public database Traditional Chinese Medicine Systems Pharmacology (TCMSP) (http://lsp.nwu.edu.cn/tcmsp.php) and TCM database@Taiwan (http://tcm.cmu.edu.tw) [24-27]. The druggability of candidate components was analysed by oral bioavailability (OB) and drug-likeness (DL). OB is the degree and speed by which drugs are absorbed into the circulatory system and serves as an important indicator to evaluate the intrinsic quality of drugs objectively. DL reflects the sum of the pharmacokinetic properties and safety derived from the

interactions of physicochemical properties and structural factors, such as solubility, permeability, and stability. The chemical compounds of *Herba Ephedra*, *Semen Armeniacae Amarum*, and *Radix Glycyrrhizae* with OB ≥30% and DL ≥0.18 were considered to exhibit good pharmacokinetic properties and were screened out for analysis[28,29]. All the chemical compounds were standardized utilizing the PubChem database (https://pubchem.ncbi.nlm.nih.gov/).

Predicting Targets of Active Compounds

The validated targets, which were confirmed by experiments, of the active ingredients of MXSGD were collected from the TCMSP database[16]. Then, the prediction of the potential targets of these active ingredients was performed utilizing PharmMapper (http://lilab.ecust.edu.cn/pharmmapper/index.php) and SwissTargetPrediction (http://www.swisstargetprediction.ch/) based on the chemical similarities and pharmacophore models[30, 31]. It is worth noting that the *Gypsum Fibrosum* belongs to the mineral category of Chinese medicine. Among the ingredients of *Gypsum Fibrosum*, Fe, Mn, Zn, and $CaSO4{\cdot}nH_2O$ (n=0 or 2) were accepted for further study in manual retrieval determined on account of their known properties[32] and the targets of *Gypsum Fibrosum* were obtained from STITCH (http://stitch.embl.de/)[33]. The targets of the active ingredients of MXSGD were screened out after eliminating the duplicates of the validated and predicted targets. All the targets were standardized as gene names and UniProt IDs utilizing the UniProtKB (https://www.uniprot.org/) database with the "*Homo sapiens*" species[34].

Visualization Networks Construction and Analysis

To visualize the complex interactions between components and potential targets, we established the compound-target networks using Cytoscape 3.7.2 (https://cytoscape.org/), a well-known tool for network pharmacology research, to comprehend the molecular mechanisms. Cytoscape provides basic features for data integration, analysis, and visualization for complicated network analysis[35]. In the network, nodes represent molecules (compounds or targets), and edges indicate internode interactions.

Conducting Protein-protein Interaction (PPI) Network

Proteins seldom achieve assigned functions solely, which indicates that the proteins tend to form macromolecular complexes through interactions to complete biological functions within cells. Hence, it is important to explore protein interactions and their interaction networks to understand cellular organization, bioprocesses, and functions. All the targets obtained above were input to the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING, Version: 11.0) (https://string-db.org/) to obtain relevant information on protein interactions, among which the genes were determined as nodes and interactions as lines in a network[36]. STRING is an online database for functional protein association networks, providing associations between proteins according to experimentally determined data, literature mining, databases, and gene associations such as neighbourhood, fusions, cooccurrence, coexpression, or protein homology[37]. In this study, we set the minimum score to the highest confidence of 0.9 to capture the broader scope of protein, which ensured high interaction confidence information. We excluded the disconnected proteins in the network. Protein interactions were analysed according to exported statistics. Furthermore, the main modules of the PPI network were screened out by the Molecular Complex Detection (MCODE) plugin of Cytoscape with MCODE scores set as ≥ 3.

Gene Ontology (GO) Enrichment Analysis

Gene Ontology (GO) is an international standardized gene functional classification system that offers a dynamic-updated controlled vocabulary and a strictly defined concept to comprehensively describe properties of genes and their products in any organism[38]. GO generally has three ontologies: molecular function (MF), cellular component (CC), and biological process (BP). The GO term is the basic unit of GO, which belongs to a type of ontology. GO enrichment analysis provides all GO terms that are significantly enriched in targets compared to the genome background and filters the targets that correspond to biological functions. In the present study, all the targets were mapped into GO terms in the Gene Ontology database (http://www.geneontology.org/), gene numbers were calculated for every term, and significantly enriched GO terms in targets compared to the genome background were defined by a hypergeometric test. The calculated *p*-value was then subjected to False Discovery Rate (FDR) Correction. In this study, FDR ≤ 0.05 was set as a threshold, and the statistics were collected by ClueGO and CluePedia (Cytoscape plug-in)[39-41].

Pathway Enrichment Analysis

Pathway-based analysis is employed to characterize the biological functions of targets. Pathway enrichment analysis identified significantly enriched metabolic pathways or signal transduction pathways in targets compared with the whole genome background in the KEGG pathway database (http://www.genome.jp/kegg/), the major public pathway-related database[42]. The statistics were collected by the ClueGO and CluePedia plugins with the FDR set as ≤ 0.05[43,44]. In addition, R software version 3.6.1 (http://www.r-project.org) with several R packages, such as clusterProfiler, org.Hs.eg.db, enrichplot, and ggplot2, was applied to draw the barplot, bubble diagram, and signalling pathway map during GO and KEGG enrichment analysis[45]. The R packages are available on Bioconductor (https://www.bioconductor.org/)[46].

Results

Compound-Target Network of MXSGD

A total of 103 chemical ingredients were collected with thresholds of OB≥ 30% and DL≥ 0.18, including 9, 7, 82, and 5 from the four natural products: *Herba Ephedra*, *Semen Armeniacae Amarum*, *Radix Glycyrrhizae*, and *Gypsum Fibrosum*, respectively (Table I). Removing the duplicated part, 97 active chemical ingredients were identified and selected for the following investigation. It is shown that 169 targets of the active ingredients of MXSGD were screened out with the elimination of repetitive component-targets. The correspondence between compound names and letters is shown in ***supplementary Table I*** of the appendix.

The compound-target network comprised 266 nodes and 2071 edges. As shown in Figure 1, circular nodes represent targets and rectangular nodes represent compounds. Take Radix Glycyrrhizae (Gan Cao) as an example. Radix Glycyrrhizae has 82 compounds and 126 corresponding targets, demonstrating that Radix Glycyrrhizae modulated a wide range of targets by complicated components. This finding suggests that MXSGD may exert pharmacological effects on COVID-19 by a complex network of compounds and targets, which requires further investigation (Figure 1).

Table I. Main compounds and targets of MXSGD.

Medicine names	Number of components	Main components	Number of targets	Main targets
*Herba Ephe*dra *(Ma Huang)*	9	Quercetin Kaempferol Herbacetin Delphinidin Resivit	107	TNF, IL-1β, IL-2, DPP4, PIM1, MAPK14, F2, HSP90AB1, MAPK1, TP53
Semen Armeniacae Amarum (Xing Ren)	7	Estrone Stigmasterol CLR Sitosterol	57	NOS2, AR, PGR, PTGS2, NCOA2, ESR1, CDK2, DPP4, CA2
Radix Glycyrrhizae (Gan Cao)	82	Isotrifoliol Inflacoumarin A Kanzonol F Quercetin Formononetin	126	JUN, VEGFA, PLAT, VCAM1, IL-10, IL6, CDK1/2/4
Gypsum Fibrosum (Shi Gao)	5	CaSO4 CaSO4. 2H2O Fe Mn Zn	33	TUBGCP6, AKT1, TF, HSP90AA1, TUBGCP3, NOS3

Abbreviations. AKT1: RAC-alpha Serine/threonine-protein Kinase; AR: Androgen Receptor; CA2: Carbonic Anhydrase II; CDK: Cell Division Protein Kinase; DPP4: Dipeptidyl peptidase 4; ESR1: Estrogen Receptor 1; F2: Prothrombin; HSP90AA1: Heat Shock Protein 90 Alpha Family Class A Member 1; HSP90AB1: Heat Shock Protein 90 Alpha Family Class B Member 1; IL: Interleukin; JUN: Transcription Factor AP-1; MAPK: Mitogen-activated Protein Kinase; NCOA2: Nuclear Receptor Coactivator 2; NOS: Nitric Oxide Synthase; PIM1: Serine/threonine-protein Kinase Pim1; PGR: Progesterone Receptor; PLAT: Tissue-type Plasminogen Activator; PTGS2: Prostaglandin G/H Synthase 2; TF: Serotransferrin; TNF: Tumor Necrosis Factor; TP53: Cellular Tumor Antigen p53; TUBGCP: Gamma-tubulin Complex Component; VCAM1: Vascular Cell Adhesion Protein 1; VEGFA: Vascular Endothelial Growth Factor A.

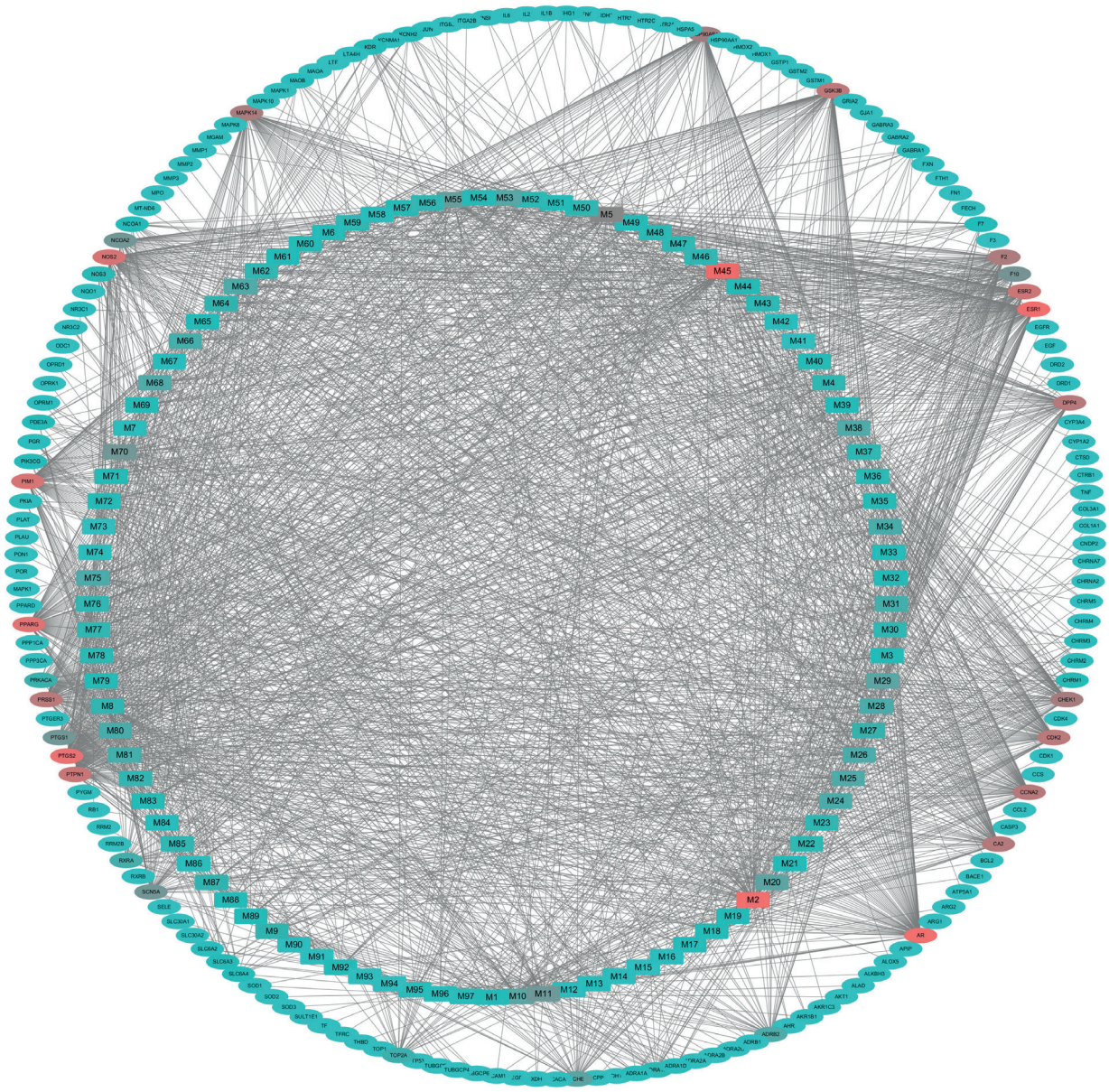

Figure 1. Compound-target network. Circular nodes represent targets and rectangular nodes represent compounds. Edges represent interactions between ingredients and targets. Node colour was regulated by degree centrality, and the nodes with red tended to have a high degree.

Analysis of Targets in PPI Network

To identify functional connections between the predicted targets of MXSGD, protein-protein interactions were predicted using STRING. We obtained the PPI network with the highest degree (degree $\geq$ 0.9) of confidence level and rejected the target protein independent of the network. The PPI network finally contains 146 nodes (representing active proteins) and 486 edges (representing the interaction between the active proteins and proteins, Figure 2A). All the nodes were query proteins and the first shell of interactors, and the protein-protein associations represented as edges were implied specific and meaningful. The degree of nodes was contained in the most elemental quantitative properties and the nodes with high degrees are considered hubs. The following showed the network topology analysis results: 6.658 was the average de-

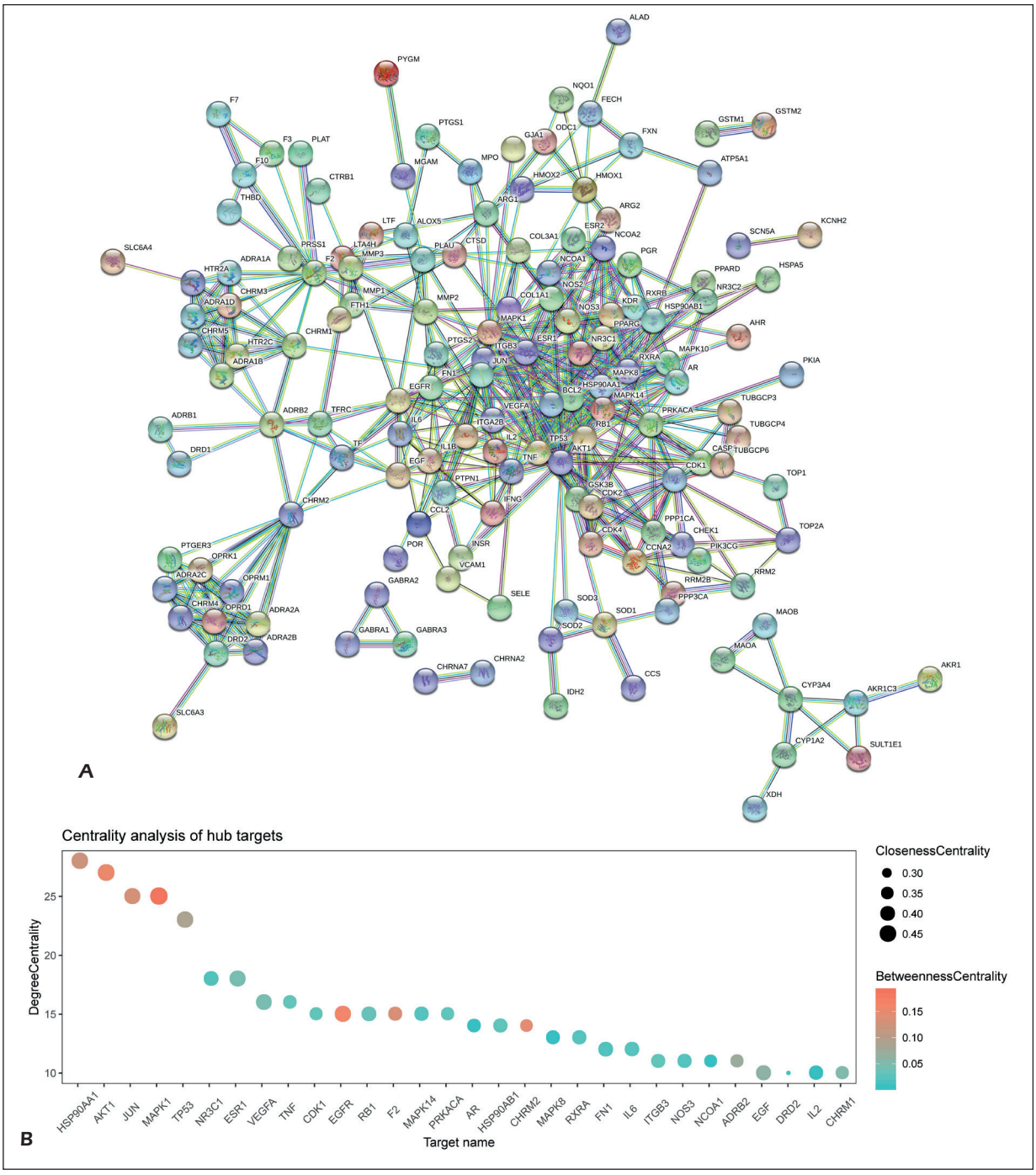

Figure 2. PPI network of MXSGD and hub target analysis. **A**, The nodes indicate proteins, and edges represent protein-protein associations. The cyan edge indicated that the interaction was from a curated database, and the purple edge was experimentally determined. The green, red, and blue edges represent the interactions that were judged from the gene neighbourhood, fusions, and cooccurrence, respectively. **B**, The centrality of targets was evaluated by degree centrality, betweenness centrality, and closeness centrality, which exhibited the variation of the y-axis value, the colour of nodes, and the size of nodes.

gree of nodes, and there were 63 nodes higher than the average degree; 0.023 was the average betweenness centrality of nodes, and there were 27 nodes higher than the average betweenness centrality. Based on the critical value of degree ≥10, we chose 30 critical nodes and shown them in bubble charts (Figure 2B) as the hub targets of MXSGD. The value of hub proteins was as-

sessed comprehensively by the degree centrality, betweenness centrality, and closeness centrality. For example, HSP90, AKT1, JUN, MAPK1, TP53, ESR1, VEGFA, and TNF tended to execute more critical roles in the target network of MXSGD, which suggested that MXSGD probably exerted its therapeutic effects on COVID-19 by targeting these hub targets.

GO Functional Enrichment Analysis

To elucidate the diverse mechanisms of MSXGD from a systematic level, we performed GO enrichment analysis for 169 targets. GO terms in the categories of biological process (blue bar), molecular function (orange bar), and cellular component (green bar) are shown in Figure 3A. We also displayed the top 20 BP, MF, CC terms in bubble charts as shown in Figure 3B, Figure 3C, and Figure 3D, respectively. Among these terms, in the BP category, MXSGD was significantly enriched in the response to oxygen-containing compound, response to organic substance, response to drug, cellular response to chemical stimulus, response to organic cyclic compound, response to chemical, cellular response to oxygen-containing compound, response to endogenous stimulus, cellular response to organic/inorganic substance, response to hormone, response to toxic substance, and response to reactive oxygen species. These results help to elucidate the biological function changes in the body after treatment with MXSGD.

KEGG Pathway Enrichment Analysis

To further reveal the potential therapeutic mechanism of MXSGD on COVID-19, we conducted KEGG pathway enrichment analysis on 169 targets and screened 174 pathways based on the kappa score level setting as $\geq$0.4 (Figure 4). The overview map of KEGG results was displayed in Figure 4B. The most significant KEGG terms of the target genes included the IL-17 signalling pathway, the advanced glycation end-products and their receptor (AGE-RAGE) signalling pathway in diabetic complications, fluid shear stress and atherosclerosis, the Phosphatidylinositol 3-kinase (PI3K)-AKT signalling pathway, human cytomegalovirus infection, and dopaminergic synapses (Figure 4C). As shown in Figure 4A, several immune response-related and inflammation-related pathways were significantly enriched, such as the IL-17 signalling pathway, the p53 signalling pathway, the MAPK signalling pathway, the P13K-AKT signalling pathway, apoptosis, the Toll-like receptor signalling pathway, the Nucleotide-binding oligomerization domain (NOD)-like receptor signalling pathway, the TNF signalling pathway, T helper (Th) type 17 cell differentiation, the Nuclear factor-kappa B (NF-κB) signalling pathway, the Retinoic acid-inducible gene I (RIG-I)-like receptor signalling pathway, and the Hypoxia-inducible factor (HIF)-1 signalling pathway. The pathways regulating viral infection were also highly enriched, involving prion diseases, hepatitis B, hepatitis C, influenza A, human papillomavirus infection, Epstein-Barr virus infection, human T-cell leukaemia virus 1 infection, and Kaposi sarcoma-associated herpesvirus infection. In addition, a certain degree of enrichment was also exhibited in other contagious pulmonary diseases such as pertussis and tuberculosis. We speculated that taking advantage of regulating inflammation-related pathways, immunomodulation-related pathways, and viral infection-related pathways, MXSGD may be beneficial for the treatment of COVID-19.

Recognition and Analysis of Main Modules of PPI Network

A Cytoscape plugin, MCODE, was available to carry out module analysis, resulting in 8 main modules (Figure 5B) identified from the PPI network (Figure 5A) with the score set as $\geq$3.0. Module I included 10 nodes and 45 interactional pairs. Module II had 9 nodes and 36 interactional pairs. Module III included 14 nodes and 30 interactional pairs. Module IV included 17 nodes and 53 interactional pairs. Module V included 17 nodes and 30 interactional pairs. Module VI included 8 nodes and 12 interactional pairs. Module VII included 9 nodes and 20 interactional pairs. Module VIII included 6 nodes and 7 interactional pairs.

To comprehend MXSGD by disclosing its inner functional modules, GO functional enrichment and KEGG pathway enrichment analysis of the genes in each module were performed by ClueGO and CluePedia plugins (Figure 6 and 7). As presented in Figure 6, the GO terms of Module I were mainly related to G protein-coupled neurotransmitter receptor activity, the adenylate cyclase-inhibiting G protein-coupled acetylcholine receptor signalling pathway, and the opioid receptor signalling pathway. Kappa-type opioid receptor (OPRK1), Mu-type opioid receptor (OPRM1), D2 dopamine receptor (DRD2), Alpha-2B ad-

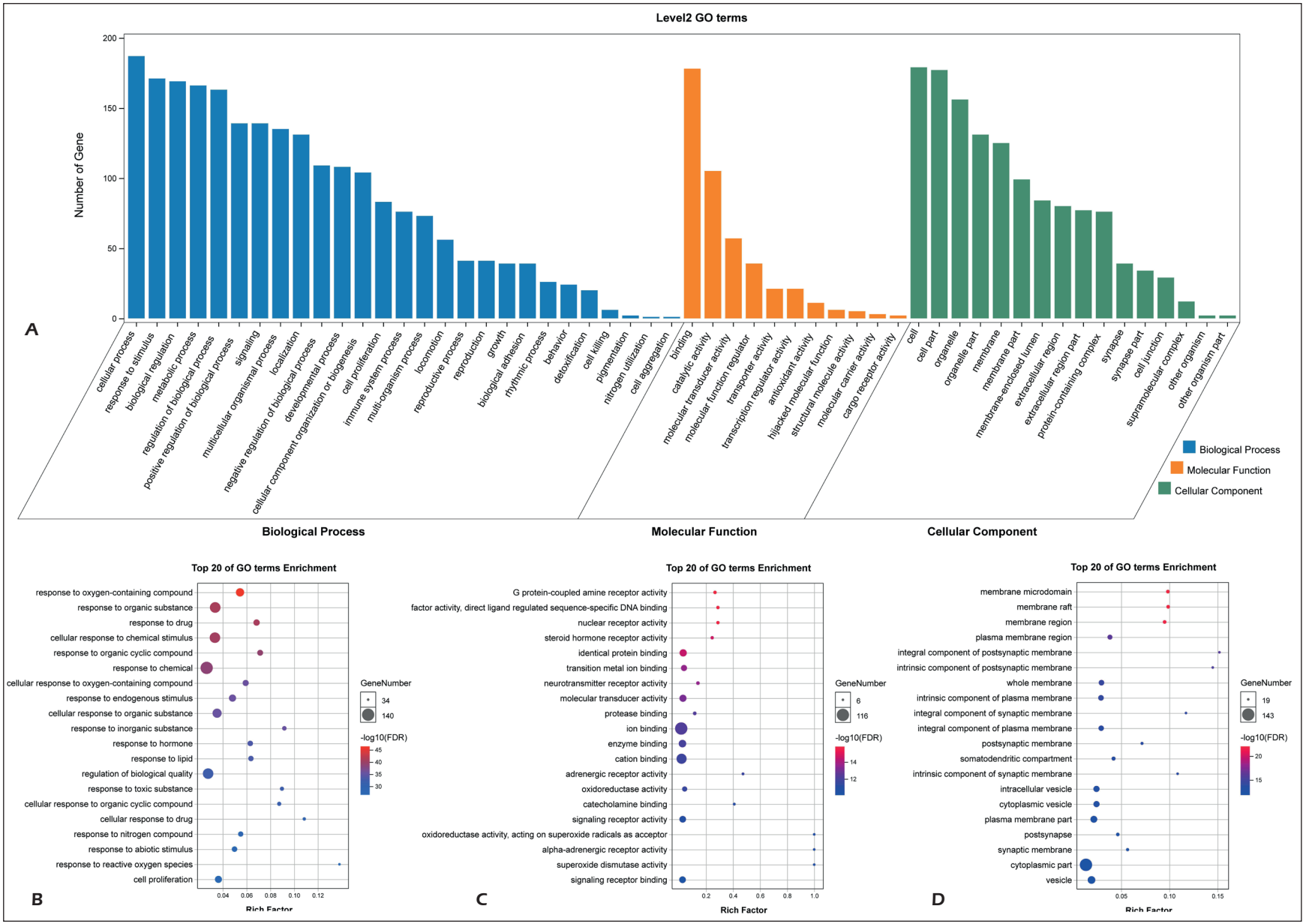

Figure 3. GO functional enrichment analysis. **A**, The second level GO enrichment statistics of 169 targets. **B**, The top 20 GO enrichments in BP. **C**, The top 20 GO enrichments in MF. **D**, The top 20 GO enrichments in CC. Each node signalled a GO term, and its size represented the gene number. The colour indicates the *p*-value.

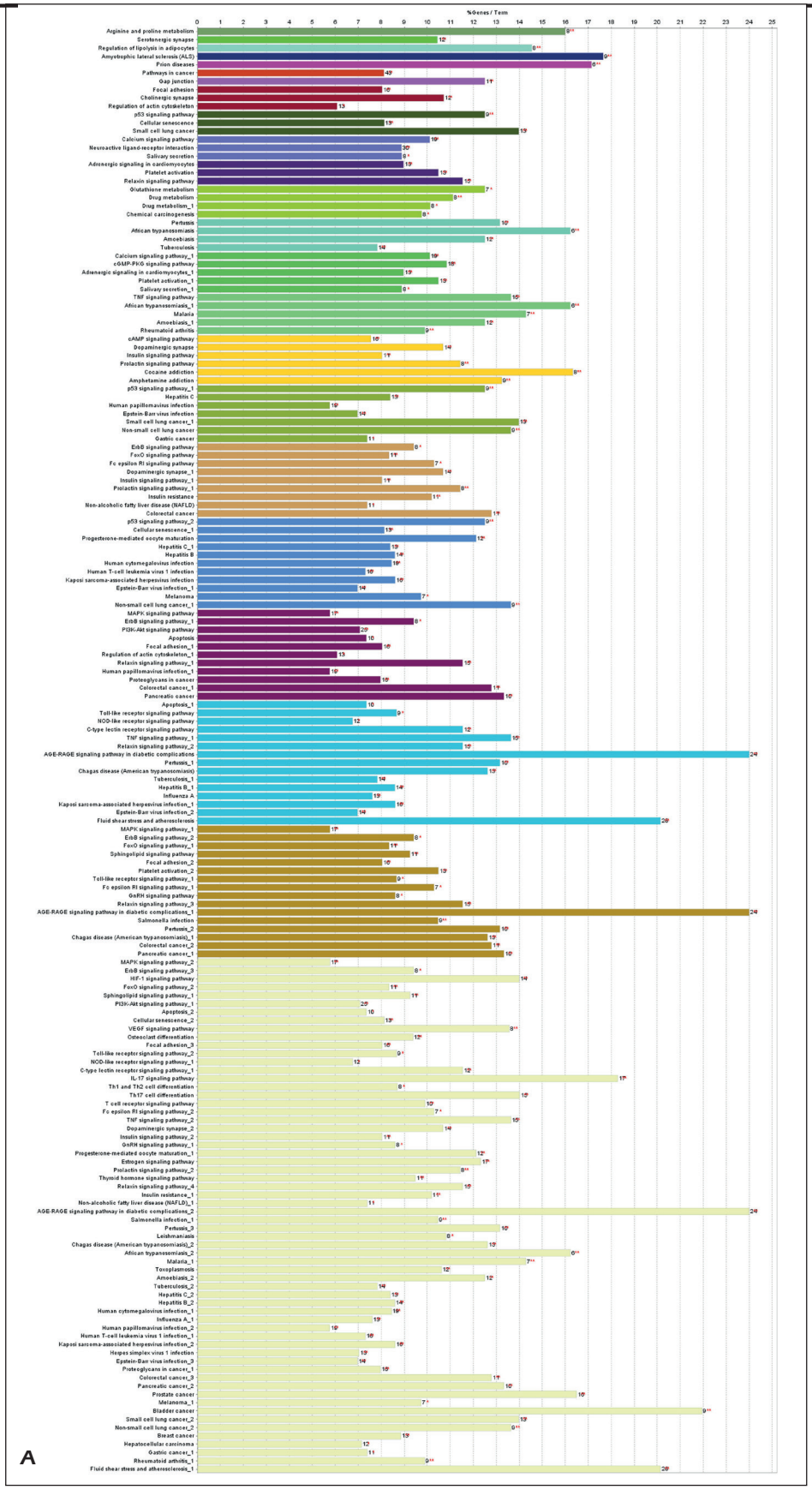

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Figure 4. KEGG enrichment analysis and pathway mapping. **A**, The bars show the percentage of genes in pathway terms. *$p<0.05$, **$p<0.01$.

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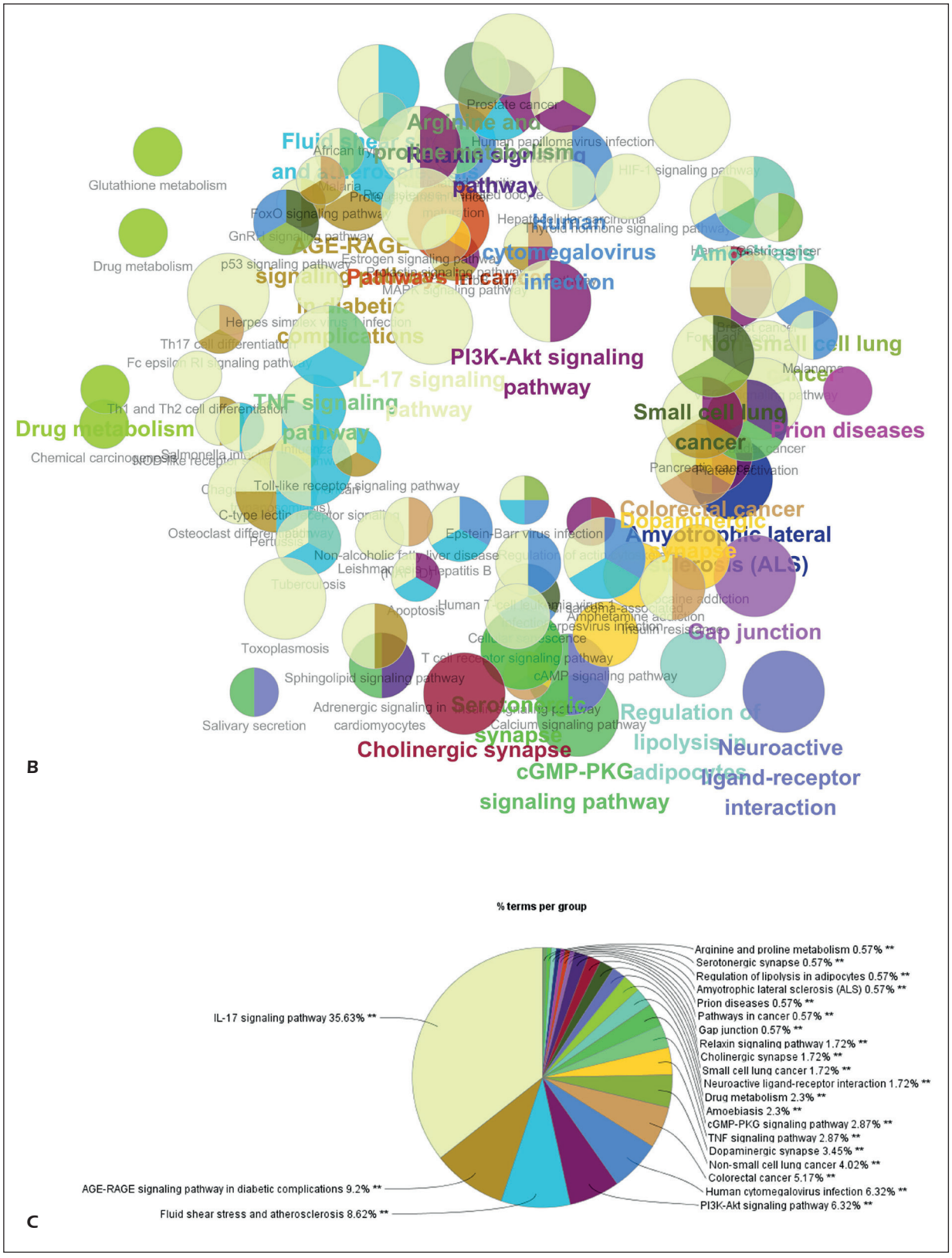

Figure 4 (continued). **B**, The functionally grouped network of enriched categories was generated for the target genes using ClueGO and CluePedia. Pathway terms were represented as nodes, and the node colour represented the grouping information. **C**, The pie chart shows the proportion of each group associated with 169 targets.

renergic receptor (ADRA2B), Alpha-2A adrenergic receptor (ADRA2A), Alpha-2C adrenergic receptor (ADRA2C), Delta-type opioid receptor (OPRD1), and Muscarinic acetylcholine receptor M4 (CHRM4) were the most associated genes contained in Module I. The mechanism of Module II was mainly related to positive regulation of blood circulation, positive regulation of blood pressure, positive regulation of smooth muscle contraction, adrenergic receptor activity and signalling, regulation of vasoconstriction, and negative regulation of blood vessel diameter. The potential targets of Module II were Alpha-1B adrenergic receptor (ADRA1B), Muscarinic acetylcholine receptor M3 (CHRM3), Alpha-1D adrenergic receptor (ADRA1D), Alpha-1A adrenergic receptor (ADRA1A), Muscarinic acetylcholine receptor M5 (CHRM5), 5-hydroxytryptamine 2A receptor (HTR2A), and Muscarinic acetylcholine receptor M1 (CHRM1). Activation and differentiation of CD4-positive cells and alpha-beta T cell, regulation of B cell mediated immunity, regulation of hormone metabolic process, positive regulation of oxidoreductase activity, monocyte chemotactic protein-1 production, positive regulation of adaptive immune response, negative regulation of chemokine production like IL-6 and IL-17, positive regulation of IL-8 production, positive regulation of immunoglobulin production, chemokine biosynthetic process, regulation of acute inflammatory response, and positive regulation of receptor signaling pathway via Signal transducers and activators of transcription (STAT) were the significantly enriched GO terms of Module III. Pro-epidermal growth factor (EGF), TNF, mRNA of PKA Catalytic Subunit C-alpha (PRKACA), Beta-2 adrenergic receptor (ADRB2), HSP90AA1, Transferrin receptor protein 1 (TFRC), IL-2, IL-6, Interferon gamma (IFNG), IL1β, TUBGCP3, TUBGCP4, and TUBGCP6 were classified into the potential targets of Module III. The Module IV modulated Fibronectin (FN1), TNF, AKT1, Epidermal growth factor receptor (EGFR), PTGS2, IL-6, JUN, HSP90AA1, VEGFA, NOS3, Integrin beta-3 (ITGB3), PRKACA, VEGF receptor 2 (KDR), C-C motif chemokine 2 (CCL2), EGF, 72 kDa type IV collagenase (MMP2), and TP53 to exert biological effects through the release of cytochrome c from mitochondria, glial cell apoptotic processes, cell migration involved in sprouting angiogenesis, regulation of endothelial cell proliferation, negative regulation of lipid localization, and positive regulation of smooth muscle cell proliferation. Module V played a role in mediating oxidative stress via regulation of the oxidative stress-induced intrinsic apoptotic signaling pathway, cellular response to superoxide and oxygen radical, removal of superoxide radicals, and oxidoreductase activity. The key targets of Module V were Superoxide dismutase [Cu-Zn] (SOD1), Mitochondrial superoxide dismutase [Mn] (SOD2), Extracellular superoxide dismutase [Cu-Zn] (SOD3), ESR1, MAPK14, Ferrochelatase (FECH), Heme oxygenase (HMOX) 1/2, CDK1, Apoptosis regulator B-cell lymphoma 2 (BCL2), Frataxin (FXN), and AKT1. Module VI exhibited positive regulation of fibroblast proliferation and the cellular response to cadmium ions via EGFR, MAPK1, JUN, and FN1. Module VII affected the positive regulation of DNA replication, replicative senescence, and histone phosphorylation by TP53, Ribonucleoside-diphosphate reductase subunit M (RRM) 2/2B, Cyclin-A2 (CCNA2), CDK1, CDK2, and Serine/threonine-protein kinase Chk1 (CHEK1). Module VIII was involved in extracellular matrix (ECM) disassembly and collagen catabolic processes by regulating Interstitial collagenase (MMP1), MMP2, Stromelysin 1 (MMP3), Chymotrypsinogen B (CTRB1), and Serine protease 1 (PRSS1).

Then, we performed KEGG enrichment analysis for each module. The most significant KEGG terms of the Module I network included the adenylate cyclase-inhibiting opioid receptor signalling pathway, alpha2-adrenergic receptor activity, glial cell-derived neurotrophic factor secretion, enkephalin receptor activity, negative regulation of Wnt protein secretion, negative regulation of uterine smooth muscle contraction, and phospholipase C-activating adrenergic receptor signalling pathway (Figure 6). Module II had a high degree of enrichment in the phospholipase C-activating serotonin receptor signalling pathway, alpha1-adrenergic receptor activity, positive regulation of vascular smooth muscle contraction, cytolysis in other organisms involved in symbiotic interactions, and the phospholipase C-activating serotonin receptor signalling pathway. Module III is mainly involved in regulating the HIF-1 signalling pathway, ferroptosis, intestinal immune network for IgA production, hematopoietic cell lineage, IL-17 signalling pathway, and Th17 cell differentiation. Module IV's high-level enrichment focused on the VEGF signalling pathway, fluid shear stress and atherosclerosis, the AGE-RAGE signalling pathway in diabetic complications, and focal adhesion. Terms of module V enriched in high-yield specific pathways, such as photody-

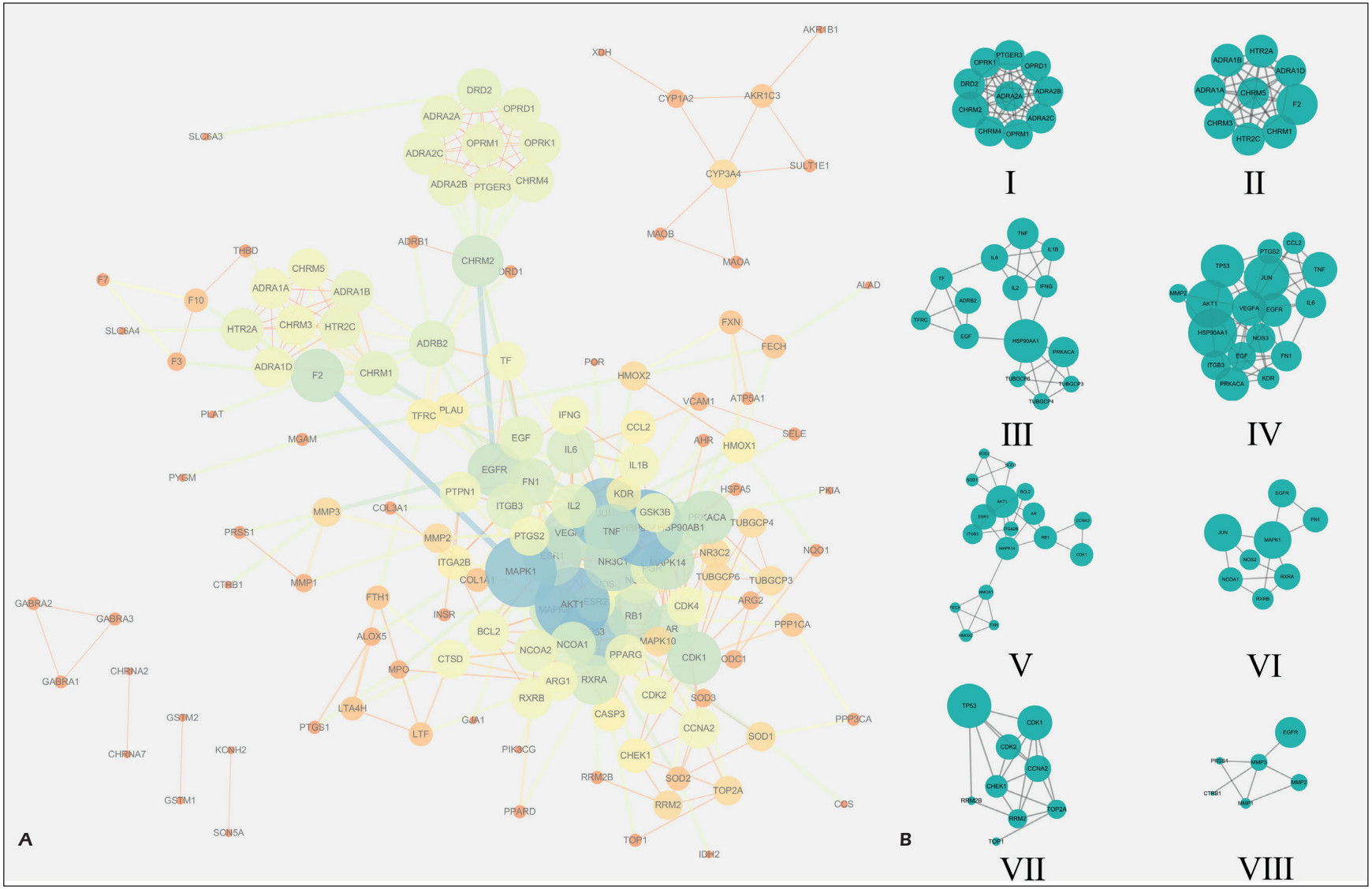

Figure 5. Recognition of the main modules of the PPI network. **A**, The PPI network of 169 targets of MXSGD. The nodes indicate proteins, and edges represent protein-protein associations. **B**, Clusters of the 8 main modules resolved from the PPI network by MCODE.

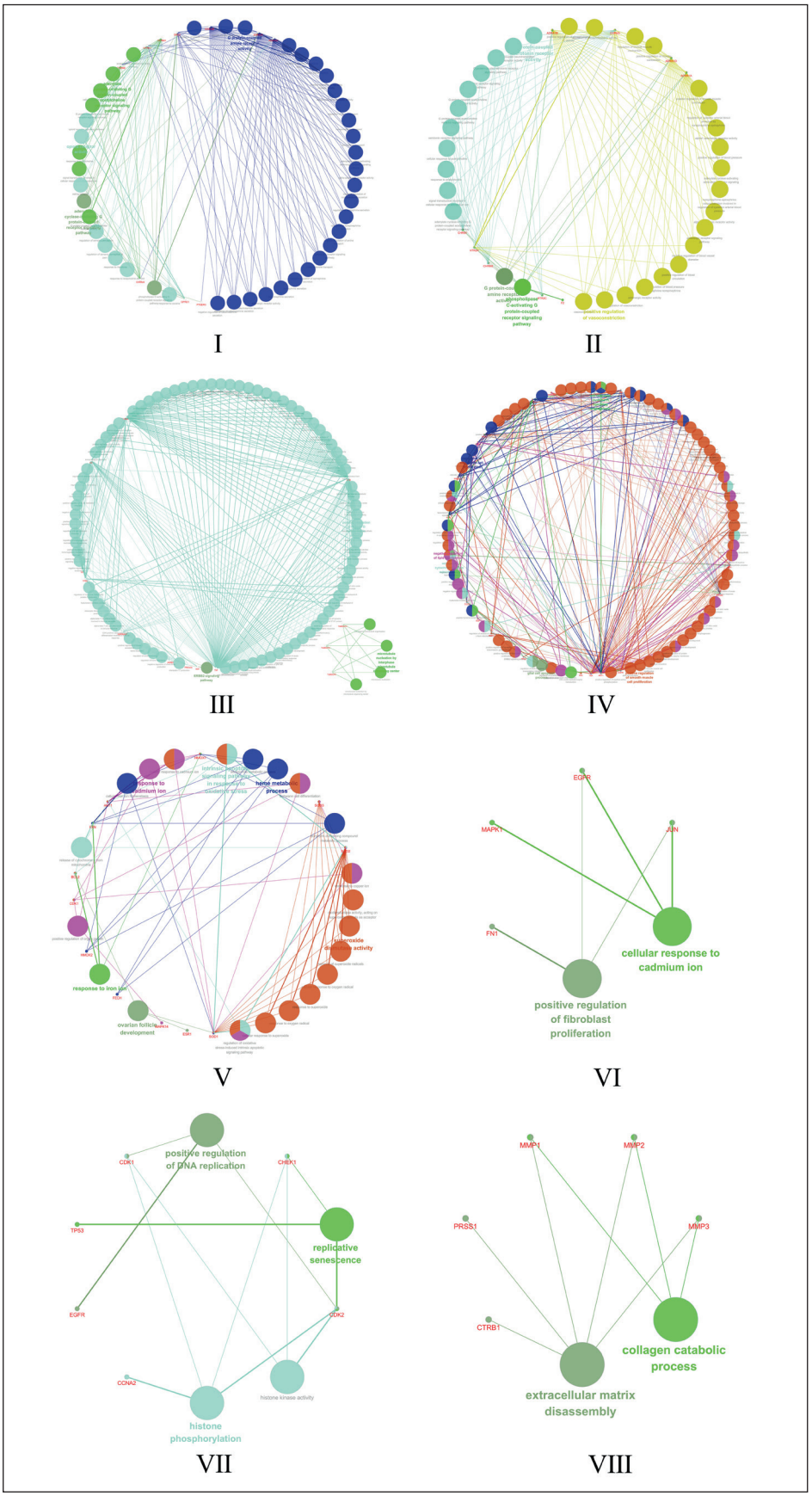

Figure 6. GO functional enrichment analysis of 8 modules. Each node signaled a GO-term. Edge indicates the existence of common genes: the thicker line indicates a larger overlap. Diverse functional groups of GO-terms were reflected by different colours.

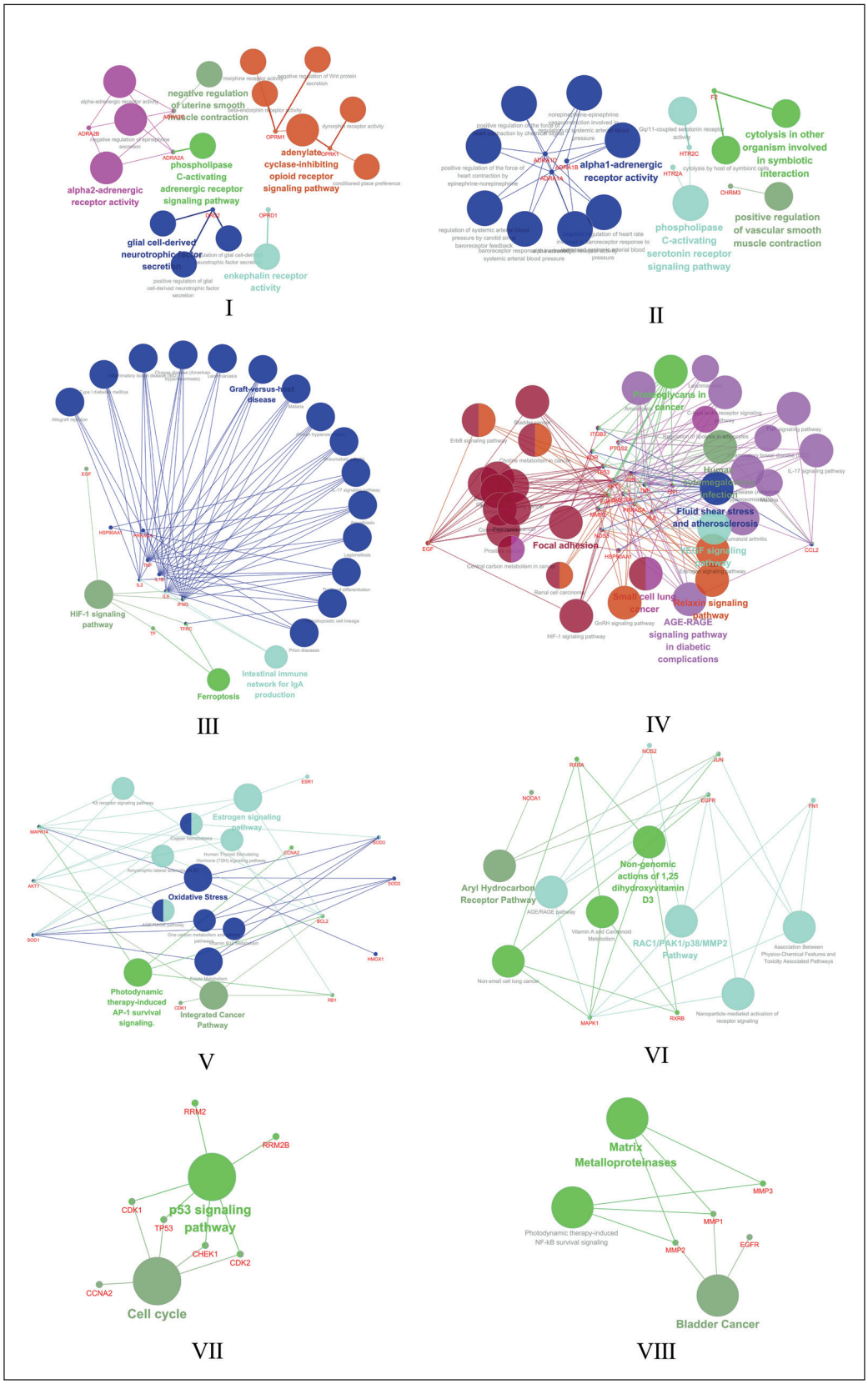

Figure 7. KEGG enrichment analysis of the main modules of the PPI network and pathway mapping. The nodes were pathway terms, whose sizes represented the significance of term enrichment.

namic therapy-induced AP-1 survival signalling, estrogen signalling pathway, and oxidative stress. The aryl hydrocarbon receptor pathway, non-genomic actions of 1,25 dihydroxyvitamin D3, and the RAC1/PAK1/p38/MMP2 pathway were classified in high-yield enrichment in Module VI.

In summary, the eight main modules of the PPI network seemed to play synergistic roles in such processes as regulating the acute inflammatory response, chemokine production, vascular permeability, response to oxygen radicals, oxidative stress-induced apoptosis, T cell differentiation involved in the immune response, immunoglobulin secretion, and ECM disassembly. Comprehending the functional modules of MXSGD was conducive to disclosing the underlying curative mechanism from different perspectives.

Discussion

In a brief period since December 2019, a severe acute respiratory illness has broken out in Wuhan city, China and then discovered in over one hundred countries and regions worldwide. The most ordinary and outstanding clinical symptoms of COVID-19 were succedent respiratory insufficiency and viral pneumonia, and some patients promptly developed serious and even lethal respiratory diseases, such as acute respiratory failure or ARDS[1, 8-10]. To date, no specific and efficient therapies have been found for COVID-19 treatment, while the main strategies include respiratory support, kidney replacement therapy, corticosteroid therapy, antiviral therapy, and symptomatic treatment[10]. Fortunately, the combination of TCM treatment and effective life support has been widely adopted and is useful to decrease the severity and promote the recovery of COVID-19. TCM can be employed for treating viral pneumonia via anti-inflammatory effects, modulating cytokine production, controlling viral replication, and regulating metabolism and immunological function[47-51]. Although the Chinese government is advising doctors to consider combining Western antiviral drugs with TCM remedies in combating COVID-19, there have been few studies to help select suitable herbal drugs before undertaking costly biological experiments and clinical trials[52]. Among the multiple TCM prescriptions formulated for treating COVID-19 in China, a traditional formula named the Ma Xing Shi Gan Decoction is considered as one of the fundamental prescriptions[15, 53]. Meanwhile, MXSGD was the basic part of "*Qing Fei Pai Du Decoction*" issued by China's National Health Commission and has been applied widely in China for treating COVID-19. Four components contained in MXSGD, which are decocted into a decoction, have been used for treating respiratory tract infections for nearly two thousand years since Han Dynasty. Additionally, MXSGD has been shown to have anti-infection or antiviral effects *in vitro* or *in vivo*[19,54-58]. In this study, we used integrating network pharmacological approaches to determine the latent mechanism of MXSGD in treating COVID-19.

The virus surmounts the host defence and results in disease in some cases. The 2019 n-CoV causes lower respiratory tract infection and mostly brings about clusters of pneumonia with difficulty breathing, among which the median duration of ARDS and dyspnea is 8 days and 5 days, respectively[1,8,9,59,60]. The clinical manifestation, laboratory abnormalities, radiological evidence, and postmortem results collectively indicated that the pulmonary pathological characteristics had a close connection with the inability of the lung to exchange gas, which could be caused by various conditions, such as alveolar structure loss, airway obstruction, and pulmonary edema[9,10,59,61-63]. The continual inflammation, which was demonstrated as the major fatal mechanism of COVID-19[10], was raised from direct viral infection and immune system response recruitment, but it consequently brought about downstream lung injury and serious respiratory distress. It may be beneficial to maintain vitality and gain a precious time window to remove the virus by focusing on the immune pathways that could amplify the inflammatory signals and lead to diffuse alveolar injury[61]. Since clinical evidence manifested that corticosteroid treatment is controversial for treating COVID-19 at present, TCM therapies, such as MXSGD, could function as a valid adjuvant therapy[64].

As described in the Results section, the MXSGD is widely involved in mediating such processes as inflammation, the immune response, cytokine production, cellular structural integrity, vascular permeability, hypoxia, apoptosis, and oxygen-free radical accumulation. As many pathways were significantly enriched (Figure 4), we used the PI3K-AKT signalling pathway as an example (Figure 8). We found that the three herbs and gypsum of MXSGD all contained ingredients that targeted this pathway. As shown in Figures 1 and 2, key proteins of the PI3K-AKT signalling pathway manifested a high centrality during both compound-target network analysis

and PPI network analysis. Typical critical proteins included HSP90, Akt, PI3K, endothelial NOS (eNOS), BCL-2, and p53. By interacting with and stabilizing the receptor interacting kinase, Hsp90 plays an important role in the TNF-mediated activation of NF-κB, a key component of the inflammatory response[65]. After virus infection, the activated PI3K/AKT/eNOS signalling pathway leads to the ascension of histamine and nitric oxide (NO), which increases microvascular permeability and then damages human endothelial cells in the lung[66]. BCL-2, an anti-apoptotic member that is increased in alveolar epithelial cells of patients with diffuse alveolar damage, could also be regulated by the activation of AKT[67,68]. Additionally, p53 is a key participant in the type I interferon antiviral defence mechanism[69]. Based on the above evidence, the PI3K-AKT signalling pathway might have a close connection with the development and treatment of COVID-19, indicating that MXSGD could treat COVID-19 via intervening in the complex functional network.

Cytokine storm is a mortal systemic complication that is chiefly caused by virus-induced abnormal immune activation[70]. Correlating with pro-inflammatory cytokines, such as IL-6 and IL-1β, ARDS is a common consequence of cytokine storm[71]. Laboratory examinations and pathological findings of COVID-19 showed evidence of ARDS and immune injury, confirming that cytokine storms could be a crucial factor linked to the severity and mortality of COVID-19[1,10,63]. In the body of COVID-19 patients, concentrations of diverse associated cytokines were aberrant, such as TNF-α, IL-1β, IL-6, and IL-2, which also indicated the association between cytokine storm and disease severity[1,72,73]. As illustrated in Figure 9, MXSGD seemed to widely intervene in the production of inflammatory factors, including TNF-α, IL-1β, IL-6, and IL-2. TNF-α, widely known as a typical pro-inflammatory cytokine, is the key effector of a lethal cytokine storm[74]. IL-1β is also a pro-inflammatory cytokine that has garnered extensive attention[75]. Triggered by an initial infective or physical insult, TNF-α and IL-1β are released from leucocytes or monocytes, and circulating cytokines enhance the adherence of polymorphonuclear neutrophils to endothelial cells by upregulating the expression of VCAM-1. Activation of neutrophils generates reactive oxygen species (ROS), and secondary inflammatory mediators trigger a sharp inflammatory response, as well as inducing endothelial cell dysfunction. Exosmosis of cytokines and inflammatory cells then zooms up inflammation and initiates pulmonary parenchymal cell injury. Cytokines leaking into the bloodstream may induce damage to remote organs and eventually cause multiple organ dysfunction syndromes or even death[76]. IL-6 is a typical cytokine that is beneficial to host defence against various infections. Nevertheless, excessive IL-6 may cause acute cytokine storms during the anti-infection process, which can progress to multiple organ dysfunction[70]. Moreover, although low-dose IL-2 could be used to treat autoimmune and inflammatory diseases, high-dose IL-2 may lead to nausea, malaise, and a flu-like syndrome, which are ordinary symptoms of a cytokine storm[77]. Quercetin, a typical flavonoid, is one of the main compounds of *Herba Ephedra* and *Radix Glycyrrhizae* in MXSGD and has been shown to inhibit TNF-α, ROS, IL-6, IL-1β, and IL-2, thereby contributing to anti-inflammatory activity[74,78-80]. Similar to quercetin, kaempferol could suppress TNF-α, IL-1β, and IL-6[74,78,81]. Based on these results, we speculated that through downregulating TNF-α, IL-1β, and ROS, kaempferol and quercetin could suppress endothelial cell dysfunction, extravasation of inflammatory cells and cytokines, parenchymal cell injury, and reflux of cytokines into circulation. Quercetin and kaempferol might also inhibit the coagulation pathway by diminishing tissue factor (TF) triggered by exaggerated IL-6, suppressing disseminated intravascular coagulation and ultimately reducing lethal cytokine storm[70]. Additionally, quercetin could diminish the positive regulatory loop of IL-2 autoactivation by decreasing IL-2 receptor alpha expression, thereby inhibiting cytokine storms[79]. The results of the GO enrichment analysis for the main modules of the PPI network (Figure 6 III and Figure 6 IV) also demonstrated that MXSGD was associated with IL-6 secretion, IL-8 production, acute inflammatory response, and cytokine production, thereby possibly reducing extravasation of inflammatory cells and disseminated intravascular coagulation via inhibiting TNF-α, ROS, IL-6, IL-1β, IL-2, and TF. In summary, MXSGD may treat COVID-19 through cytokine storm suppression.

Pathological findings of diffuse alveolar damage, pulmonary edema, and hyaline membrane formation indicated severe pulmonary damage in COVID-19 patients[63]. The diffuse alveolar damage triggered by virus infection and inflammatory dysregulation was the main cause of diminished respiratory function, such as dyspnea and ARDS. A major function of the alveolar epithelium is to maintain alveolar fluid balance resulting in minimal epithelial lining fluid. The

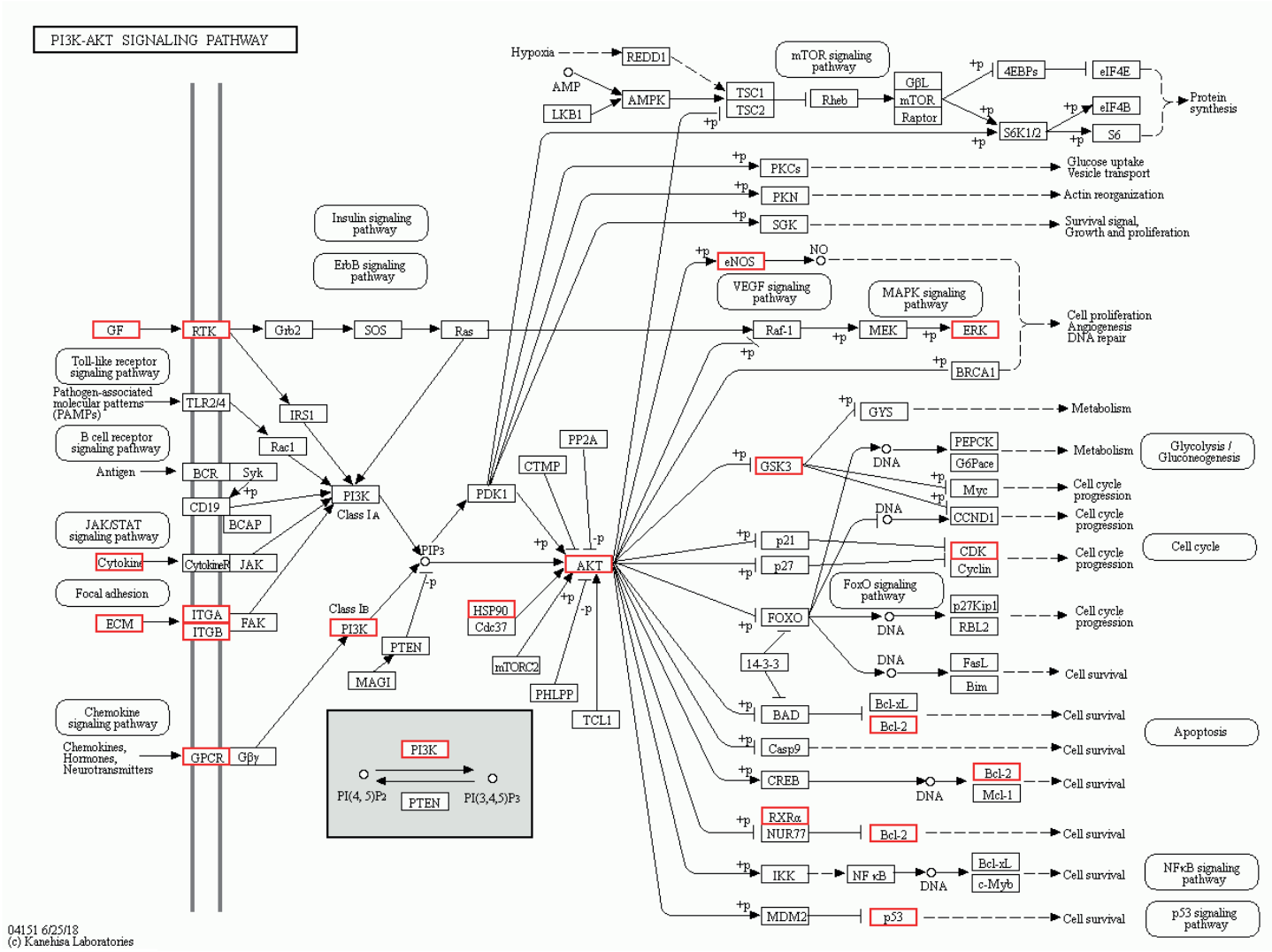

Figure 8. KEGG pathway: map04151. Red boxes mark the proteins or pathways targeted by MXSGD.

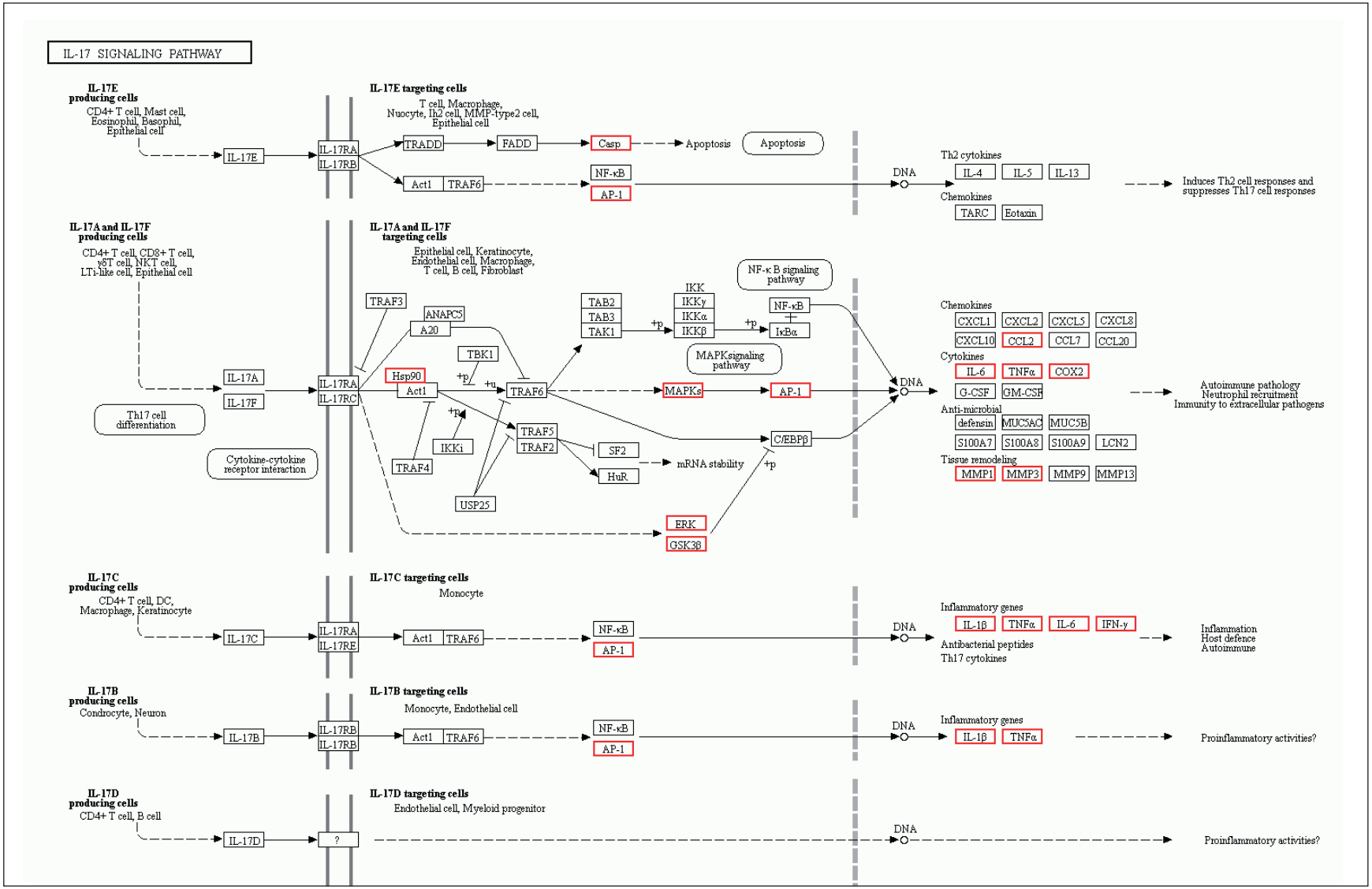

Figure 9. KEGG pathway: map04657. Red boxes mark the proteins or pathways targeted by MXSGD.

disruption of alveolar endothelial and epithelial barriers leads to increased permeability accompanying focal hemorrhages and extravascular edema fluid accumulation, ultimately resulting in pulmonary alveolar-capillary barrier dysfunction[82-84]. Integrated intercellular junctions, including tight junctions (TJs) and adherens junctions (AJs), and basement membrane/ECM coverage collectively form the barrier[85]. IL-1β, which is secreted primarily by monocytes and macrophages, could alter the TJs through claudins and disassemble the AJs complex by phosphorylating β-catenin in endothelial cells. TNF could disturb zonula occludens-1 (ZO-1) localization and downregulate the level of ZO-1, as well as other claudin family members in the lung, functionally opening the TJs barrier[86]. In MXSGD, we found that quercetin and kaempferol could reduce IL-1β and TNF by blocking the MAPK pathway[81,87]. As shown in Figure 6 II, MXSGD was related to vasoconstriction and vascular space reduction. Through the molecular mechanism mentioned above, MXSGD might reduce inflammatory fluid exudation and promote the absorption of edema fluid, thereby alleviating and improving pathological changes in the lungs of COVID-19 patients. Consequently, MXSGD might protect alveolar endothelial and epithelial barriers by maintaining the integrity of intercellular junctions, eventually alleviating COVID-19 by improving pulmonary ventilation function.

Aside from the AJs and TJs, the ECM is also critical for maintaining the barrier[88]. As shown in Figure 2, VEGFA was a hub target in the network, as was the VEGF signalling pathway. The binding of VEGFA to VEGF receptor (VEGFR) increases vascular permeability and results in vascular leakage by cleaving ECM and disassembling endothelial junctions[89,90]. Virus-mediated upregulation of VEGF seems to participate in the pathogenesis of many viral diseases[91], which is consistent with the laboratory findings among the COVID-19 patients[1]. Quercetin has been shown to inhibit the activity of VEGFA/VEGFR[92,93]. Furthermore, quercetin exhibited anti-inflammatory effects and promoted cell survival by regulating AKT signalling and decreasing the levels of the inflammatory enzymes Cyclo-oxygenase-2 (COX-2), inducible NOS (iNOS), and NO[94]. Quercetin seemed to protect the pulmonary capillary barrier by inhibiting VEGFA/VEGFR-induced ECM disruption and relieving inflammatory injury. Matrix metalloproteinases (MMPs), which are generated primarily from epithelial and inflammatory cells in response to inflammatory signals, are a family of enzymes that cleave ECM proteins, such as collagen and proteoglycan[95 96]. With the degradation of ECM induced by MMPs, immunocytes are recruited rapidly to invade and destroy the vascular matrices, thereby increasing alveolar and pulmonary capillary permeability[97,98]. The upregulated expression of VEGFR in endotheliocytes could also be mediated by MMP through the Protease-activated receptor-1 (PAR-1)/NF-κB signalling pathway[99,100]. Anti-MMP treatment profoundly inhibits VEGF production and maintains vascular integrity[101]. Several experiments indicated the importance of MMPs in destructive pulmonary pathology and respiratory failure, suggesting that the inhibition of MMPs plays a protective role in viral pneumonia[102-104]. The quercetin in MXSGD could downregulate the expression of multiple kinds of MMPs via MAPK, Peroxisome proliferator-activated receptors (PPAR), and NF-κB signalling pathways and profoundly suppress ECM degradation and vascular injury[105,106]. Moreover, there were some other signal transduction pathways involved in ECM remodelling. The 2019-nCoV infection revealed coagulation activation, followed by an abnormally elevated level of intra-alveolar fibrin that contributed to severe lung injury[107]. Mediated by the inhibition of PLAT driven by Serpine1, excess fibrin elevated vascular permeability, stimulated inflammatory cell migration and proliferation, and recruited neutrophils to the lung[107]. Quercetin in MXSGD targeted Serpine1 and PLAT and has been shown to increase the expression of PLAT and suppress Serpine1[108,109]. Figure 6 VIII illustrates that MXSGD exerted significant impacts on ECM disassembly and collagen catabolic processes, potentially decreasing MMPs and suppressing ECM degradation via targeting MAPK, PPAR, and NF-κB signalling pathways, Serpine1, and PLAT. In summary, MXSGD may protect the alveolar endothelial and epithelial barriers from disruption, alleviate pulmonary capillary leakage, enhance the removal of edema fluid, suppress the pulmonary inflammatory environment, and ultimately improve respiratory function.

In addition to the targets of MXSGD mentioned above, JUN, one of the hub targets (Figure 2B) enriched in the KEGG enrichment analysis (Figure 6), plays a role in positive regulation of myeloid cell differentiation, regulation of myeloid leukocyte differentiation, positive regulation of myeloid leukocyte differentiation and macrophage activation, which involves several aspects of positive regulation of myeloid cell differentiation and regulation of adaptive immune response. Among the functions, c-Jun NH_2-terminal kinas-

es (JNK) participate in T helper cell proliferation, differentiation, and maintenance of Th1/Th2 polarization. JNK1 participates in the survival of activated T cells in immune responses, and JNK2 is involved in the control of $CD8^+$ T cell expansion *in vivo*[110]. When induced by virus, phosphorylation of c-Jun through activation of the JNK pathway can lead to an increase in AP-1 activity. AP-1 consists of members of the Jun and Fos families that dimerize and bind to promoter regions, among which the transcription factor AP-1 is also one of the 21 hub targets (Figure 2B). This protein regulates the transcription of many cytokine genes affected in infection, which is related to an aggravated pro-inflammatory immune response, especially the pro-inflammatory cytokines responsible for the cytokine storm[111]. The results of Figure 6 III and Figure 6 IV also proved that MXSGD regulated immune processes, such as leukocyte activation, T cell differentiation, and acute inflammatory response, that are inclined to limit pro-inflammatory cytokines and control myeloid cell differentiation through the aforementioned molecules. As AP-1 was targeted by quercetin, kaempferol, and formononetin in MXSGD, the immune response and cytokine storm were suggested to be modulated for treating COVID-19.

In addition, one of the most common and typical symptoms of COVID-19 is fever, which is often initiated by the immediate activation of the innate immune system via exogenous pyrogens, specifically of the complement cascade and Toll-like receptors. Activated Toll-like receptors induces the generation of pro-inflammatory cytokines and synthesizing enzymes of prostaglandin E2 (PGE2), such as COX-2, thereby facilitating further production of PGE2, the proximal mediator of fever. COX-2 is an indispensable enzyme in the synthesis of PGE 2 from arachidonic acid. Pro-inflammatory cytokines like IL-1β, IL-6, and TNF-α tend to induce the expression of COX-2 and hence PGE2, thereby playing a significant role in the maintenance of fever[112-114]. As mentioned above, both quercetin and kaempferol in *Herba Ephedra* and *Radix Glycyrrhizae* could inhibit the expression of IL-1β, IL-6, and TNF-α[79-81,115]. *Herba Ephedra* was also reported to attenuate hypothalamic IL-1β overexpression through NF-κB inhibition and restrain an increase in COX-2 protein expression, thereby helping to suppress the increased body temperature[116-118]. Moreover, research showed that *gypsum* possessed a powerful anti-pyretic effect by decreasing the PGE2 level in the hypothalamus. Cotreatment with *Herba Ephedra* and *gypsum* can have synergistic effects to manage fever compared with single use[119]. Since *Herba Ephedra*, *Radix Glycyrrhizae*, and *gypsum* are all ingredients of MXSGD, we concluded that MXSGD could treat COVID-19 by alleviating fever, possibly through reducing PGE2 by diminishing IL-1β, IL-6, TNF-α, and COX-2.

Conclusions

In this study, we investigated the underlying mechanism of MXSGD in treating COVID-19 utilizing the approaches of integrating network pharmacology. The therapeutic effects potentially focus on the following aspects: reducing inflammation, suppressing cytokine storm, protecting pulmonary alveolar-capillary barrier, alleviating pulmonary edema, regulating immune response, and decreasing fever. As an important adjuvant therapy, traditional Chinese medicine, such as MXSGD, combined with modern medicine approaches would benefit patients with COVID-19 and help to overcome the current 2019 n-CoV epidemic. Further experiments are needed to validate the specific molecular mechanisms governing the effect of MXSGD on COVID-19.

Authors' contributions

Conceptualization, L.H., W.M.H., and L.Z.G.; methodology, W.Y.X. and M.J.R.; software, W.S.Q.; formal analysis, M.J.R. and W.S.Q.; investigation, Z.Y.Q. and Z.C.Y.; resources, R.Y.H., and Z.L.; data curation, L.Z.G.; writing—original draft preparation, W.Y.X., M.J.R., W.S.Q., and Z.Y.Q.; writing—review and editing, W.M.H.; supervision, L.H.; project administration, W.Y.X.; funding acquisition, L.H., W.M.H., and L.Z.G. All authors have read and agreed to the published version of the manuscript.

Funding

This research was funded by grants from the National Natural Science Foundation of China (81673412), the National Administration of Traditional Chinese Medicine (K2019BZ07), the Jiangsu Province Administration of Traditional Chinese Medicine (ZX2016A2), the 333 high level talents training project in Jiangsu (BRA2016507), and the Social Development Program Fund of Jiangsu Province by Jiangsu Science and Technology Department (BE2016808).

Acknowledgements

We appreciate all the people who are fighting the COVID-19 epidemic around the world. We thank American Journal Experts (AJE) for English language editing.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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