Research Article

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Elucidation of the Mechanisms and Molecular Targets of Lianhuaqingwen for Treatment of COVID-19 Based on Network Pharmacology

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ABSTRACT

Lianhuaqingwen (LH) is the widely used in the treatment of Coronavirus disease 2019 (COVID-19). However, its mechanisms of action and molecular targets for treatment of COVID-19 are not clear. The active compounds of LH were collected and their targets were identified through the network pharmacology. The mechanism of compound multi components and multi targets and its relationship with disease are analyzed. COVID-19 targets were obtained by analyzing with TCMSP. In total, 282 active ingredients and 510 targets of LH were identified. Twenty-one target genes associated with LH and COVID-19. Protein-protein interaction (PPI) data were then obtained and PPI networks of LH putative targets and COVID-19-related targets were visualized and merged to identify the candidate targets for LH against COVID-19. Gene ontology and Kyoto Encyclopedia of Genes and Genomes pathway analysis were carried out. The gene-pathway network was constructed to screen the crucial target genes. The functional annotations of target genes were found to be related to immune regulation, host defense, inflammatory reaction and autoimmune diseases and so on. Twenty pathways including immunology, cancer, and cell processing were significantly enriched. Quercetin and luteolin might be the crucial ingredients. IL6 was the core gene and other several genes including IL1B, STAT1, IFNGR1, and NCF1 were the key genes in the gene-pathway network of LH for treatment of COVID-19. The results indicated that LH's effects against COVID-19 might relate to regulation of immunological function through the specific biological processes and the related pathways. This study demonstrates the application of network pharmacology in evaluating mechanisms of action and molecular targets of complex herbal formulations.

Keywords: Lianhuaqingwen; TCM; Network pharmacology

INTRODUCTION

The Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has spread worldwide [1]. As of 14 November, over 252 million confirmed cases and over 5 million deaths have been reported and a huge impact on people's lives and production [2]. At present, there is no specific drug for COVID-19. Investigations showed that LH could significantly relieve cardinal symptoms and reduce the course of the COVID-19. The Scheme for Diagnosis and Treatment of 2019 Novel Coronavirus Pneumonia (The 7th Trial Edition) mentioned during the medical observation period, Lianhuaqingwen (LH) recommended for the fever and fatigue symptoms [3]. LH is a Chinese patent medicine composed of 13 herbs. LH is a Chinese patent medicine composed of 13 kinds of traditional Chinese medicine. The key herbs are LianQiao (latin name: *Forsythiae Fructus*) and Jinyinhua (latin name: *Lonicerae Japonicae Flos*). LH based on the prevention and treatment theory of "plague" of traditional Chinese medicine. It played a positive role in the treatment of SARS-CoV-2 [4]. Study showed that LH could exerts anti-influenza activity through the inhibition of viral propagation and impacts immune function [5]. The study found that LH could signficantly inhibited SARS-CoV-2 replication in cells and markedly reduced pro-inflammatory cytokine [6].

The characteristics of Traditional Chinese medicine (TCM) are multi-component, multi-target, and multipathway. Network pharmacology focuses on the fact that many active ingredients interact with numerous different genes or proteins, emphasizing a holistic thought also shared by TCM [7]. In this study, network pharmacology was used to explore the mechanism and molecular targets of LH in the treatment of COVID-19.

MATERIALS AND METHODS

Screen active ingredients and potential targets

Identified the chemical ingredients of LH from Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform [8] (TCMSP, http://tcmspw.com/tcmsp.php) and selected candidate ingredients which oral bio-availability (OB) ≥ 30% and drug-likeness (DL) ≥ 0.18 [9]. For ingredients not included in TCMSP, checked their ingredients from ETCM [10]. And only targets with a reliability score greater than 0.80 were retained. Then, the candidate ingredients were imported into the TCMSP database to identify and collect the corresponding targets of LH.

Seek COVID-19 potential targets

The disease target genes corresponding to COVID-19 were collected from OMIM database (https://omim.org/) and genecards database (https://www.genecards.org/). The intersection genes of COVID-19 target genes and the herbs of LH target genes were seeked.

Network construction

According to the prediction of herb and disease targets, the network of active ingredients and targets were constructed and visualized by Cytoscape 3.5.1 software. PPI data were obtained from Database of Interacting Proteins (DIP), Biological General Repository for Interaction Datasets (BioGRID), Human Protein Reference Database (HPRD), IntAct Molecular Interaction Database (IntAct), Molecular INTeraction database (MINT), and biomolecular interaction network database (BIND) using the plugin Bisogenet of Cytoscape 3.5.1 software [11]. The PPI networks of LH potential targets and COVID-19 related targets were visualized with Cytoscape software.

Network merge

The PPI networks of LH potential targets and COVID-19 related targets were merged with Cytoscape software. And the nodes with topological importance in the interaction network were screened by calculating Degree Centrality (DC), Betweenness Centrality (BC) with the Cytoscape plugin CytoNCA. These parameters represent the topological importance and computational formulas and used in network pharmacology and systems pharmacology [12].

Bioinformatic analysis

GO analysis with the biological process, cellular component, and molecular function were carried out using the Database for Annotation, Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov, v6.8) [13]. Functional categories were enriched within genes ($P<0.05$) and the top 20 GO functional categories were selected. DAVID that assigned Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used for pathway analysis. Pathways that had significant changes of $P<0.05$ were identified for further analysis. The genes that significantly regulated pathways were selected for gene-pathway network analysis. The gene-pathway network was constructed to screen the key target genes that LH treated COVID-19.

RESULTS

Ingredient-target network analysis

Four hundred and fifty-one eligible ingredients were obtained in Supplementary Table 1. Thirty seven in *Forsythiae Fructus*, 23 in *Lonicerae Japonicae Flos*, 50 in *Ephedra Herba*, 34 in *Amygdalus Communis Vas*, 39 in *Isatidis Radix*, 9 in *Fortunes Bossfern Rhizome*, 7 in *Houttuyniae Herba*, 94 in *Pogostemon Cablin Benth*, 22 in *Radix Rhei Et Rhizome*, 122 in *Licorice*. 14 in *Rhodiola crenulata*. Eventually, 282 candidate ingredients were obtained in total after the duplications were removed.

The targets of 4956 compounds were selected after removing the compounds which did not link to any targets, 489 in *Forsythiae Fructus*, 756 in *Ephedra Herba*, 355 in *Amygdalus Communis Vas*, 276 in *Isatidis Radix*, 93 in *Fortunes Bossfern Rhizome*, 205 in *Houttuyniae Herba*, 811 in *Pogostemon Cablin Benth*, 141 in *Radix Rhei Et Rhizome*, 1644 in *Licorice*. 185 in *Rhodiola crenulata*. A total of 510 targets were finally collected after removing duplication.

Target genes of COVID-19 were collected from OMIM and Gene Cards database. Then, 21 target genes were obtained from the intersection of disease and drug targets, which were as follows: DPP4, GSK3B, SLC6A4, PON1, ADH1C, IL1B, NCF1, STAT1, SOD1, EGFR, IL6, NOS3, HSPB1, MUC1, ATP2C1, IFNGR1, BMPR2, ALB, APC, GPER1, ABCB1.

The ingredient-target network of LH and COVID-19 targets were constructed using the screened ingredients and their targets as shown in Figure 1. The network contained 157 nods (136 compounds in LH and 21 compound targets) and 203 edges which indicated the ingredient-target interactions. Quercetin and luteolin acted on 11 and 4 targets, respectively. There are 5 herbs contain quercetin, and 2 herbs contain luteolin. Therefore, they might be the key active ingredients of LH due to their considerable localization in the network.

Identification of crucial candidate targets for LH against COVID-19

In order to reveal the mechanisms of action underling LH's effects on COVID-19, the PPI network of LH potential targets and the PPI network of COVID-19 related targets

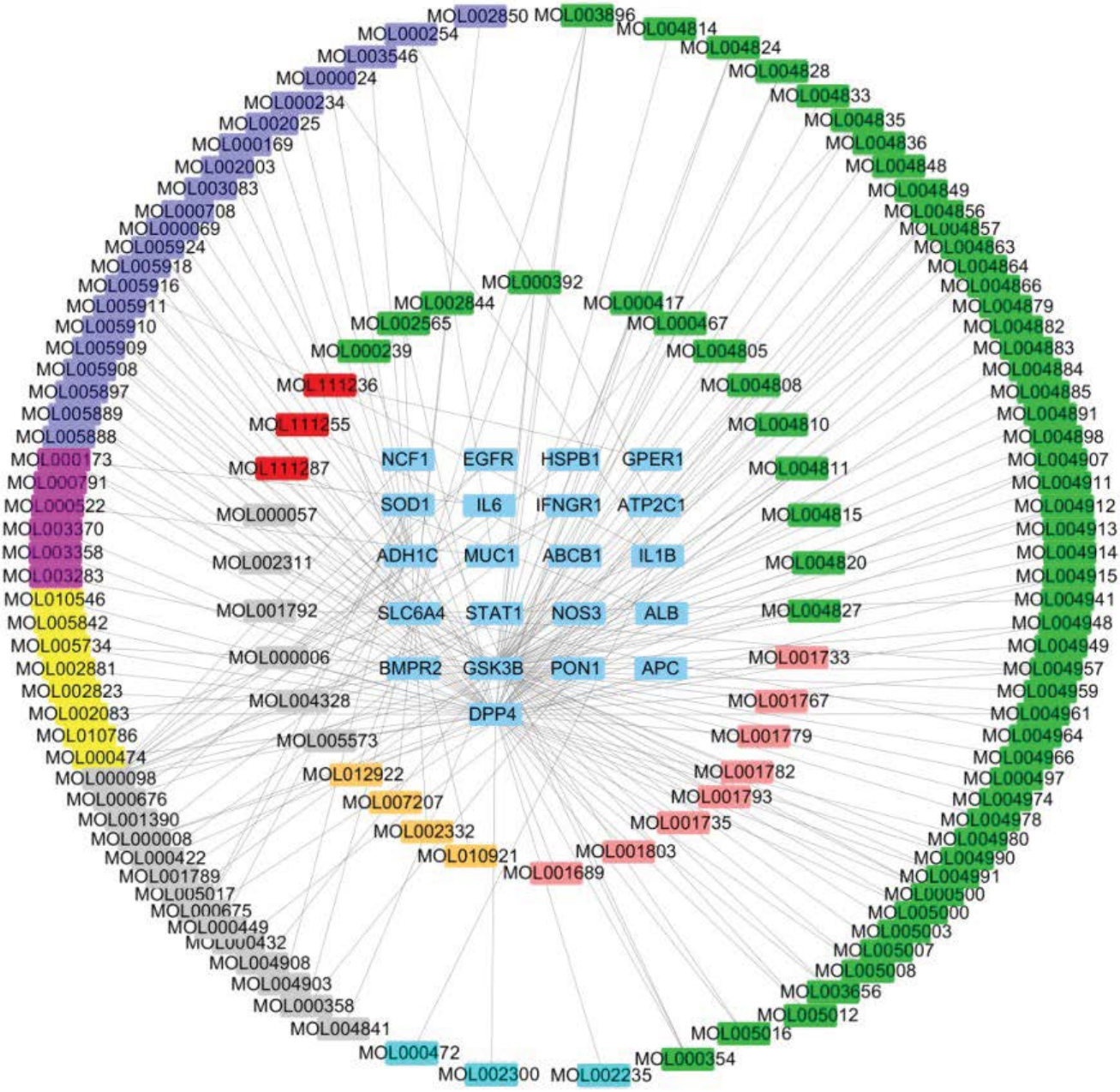


Figure1: Ingredient-target network of LHQWC. The blue rectangle in the central area represent targets. gene; the grey, yellow, rose, red, violet, green, sky blue, orange, and flesh rectangles represent the ingredients from multi Drug, Ephedra Herba, Forsythiae Fructus, Pogostemon Cablin (Blanco) Benth, licorice, Radix Rhei Et Rhizome, Amygdalus Communis Vas, and Isatidis Radix, respectively.

were merged to identify the candidate targets for LH against COVID-19. This network consisting of 3,232 nodes and 39,315 edges was presented in Figure 2A. The median degree of all nodes was 30 and the nodes with more than 60 degrees were identified as significant targets. A network of significant targets for LH against COVID-19 was constructed and it contained 341 nodes and 9,834 edges Figure 2B. The median values of BC was 600. The candidate targets were further screened and 37 targets with BC>600 were identified Figure 2C. Thirty seven target proteins, forming a core network, were eventually identified for LH against COVID-19.

GO and pathway enrichment analysis

DAVID was used to perform GO and KEGG pathway analysis of the 282 candidate targets identified. GO of candidate targets was analyzed based on biological process, cellular component, and molecular function. Six hundred seventy GO terms were significantly enriched (P<0.05), 639 in biological process, 18 in cellular component, and 13 in molecular function. Top 20 terms were shown in Figure 3. The highly enriched GO terms in biological process, cellular component, and molecular function included regulation of positive regulation of blood vessel diameter, regulation of tube diameter, membrane raft, membrane microdomain, ubiquitin-like protein ligase binding, protein phosphatase binding. The pathways that

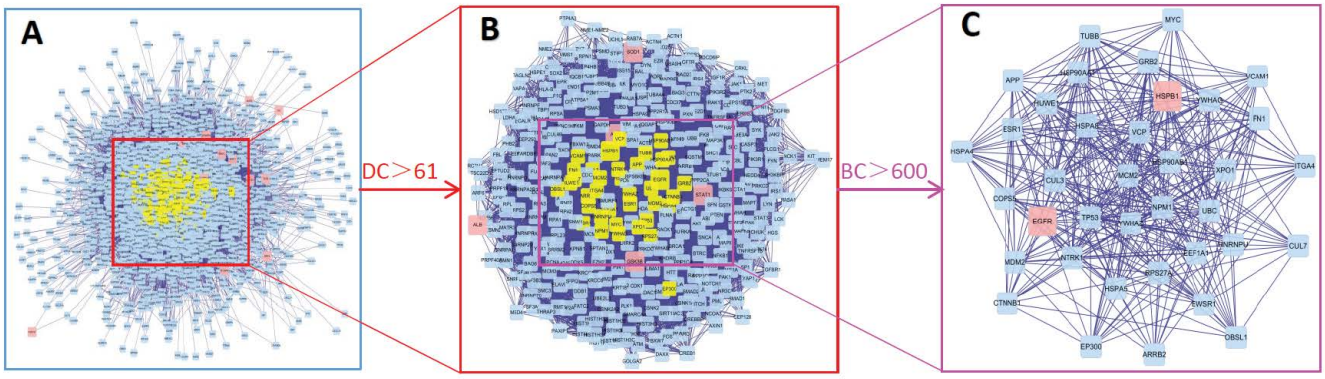
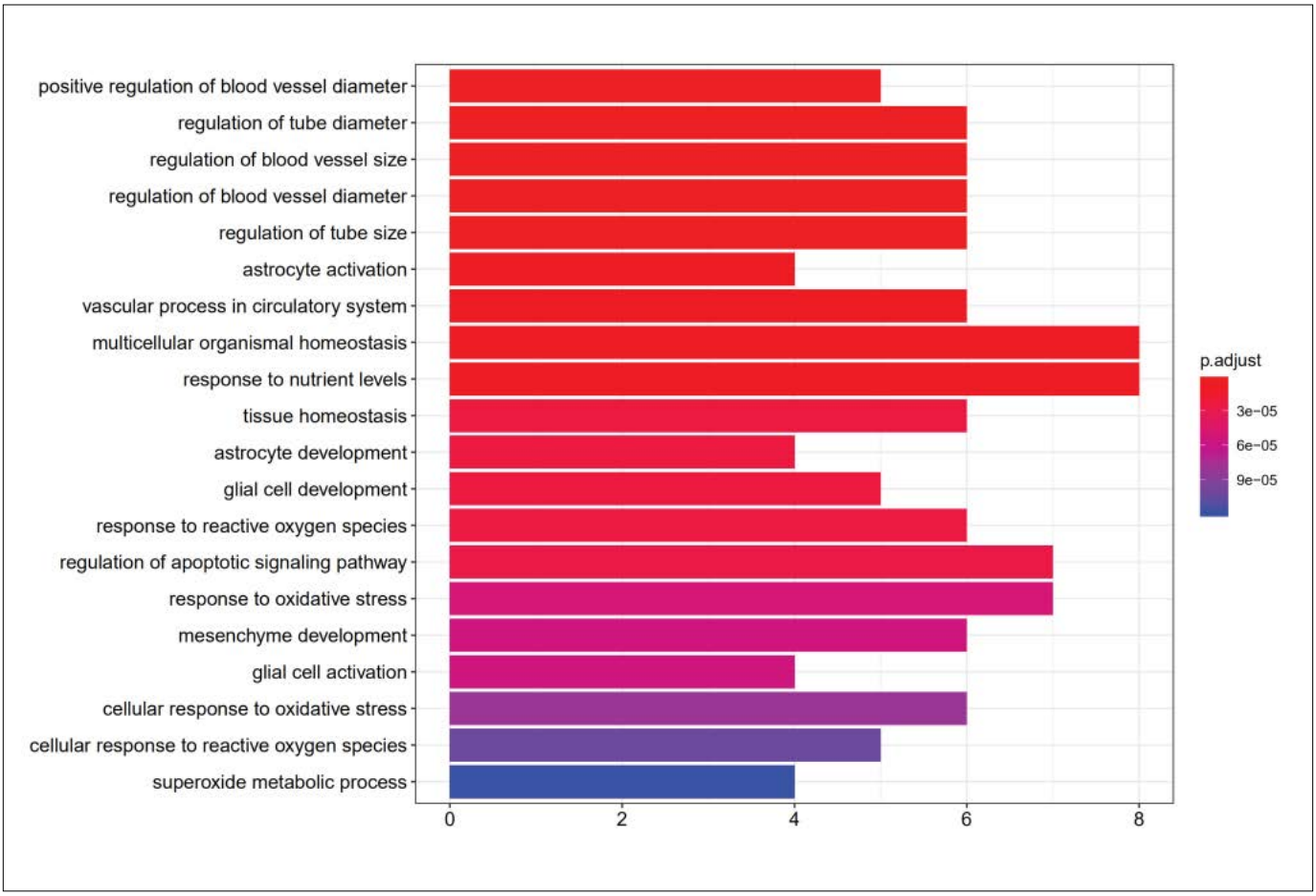


Figure 2: Identification of candidate targets of LHQWC against COVID-19. (A) The interactive PPI network of LHQWC putative targets and COVID-19 related targets. (B) PPI network of significant proteins extracted from A. (C) PPI network of candidate LHQWC targets for COVID-19 treatment extracted from B. DC, degree centrality; BC, between ness centrality.



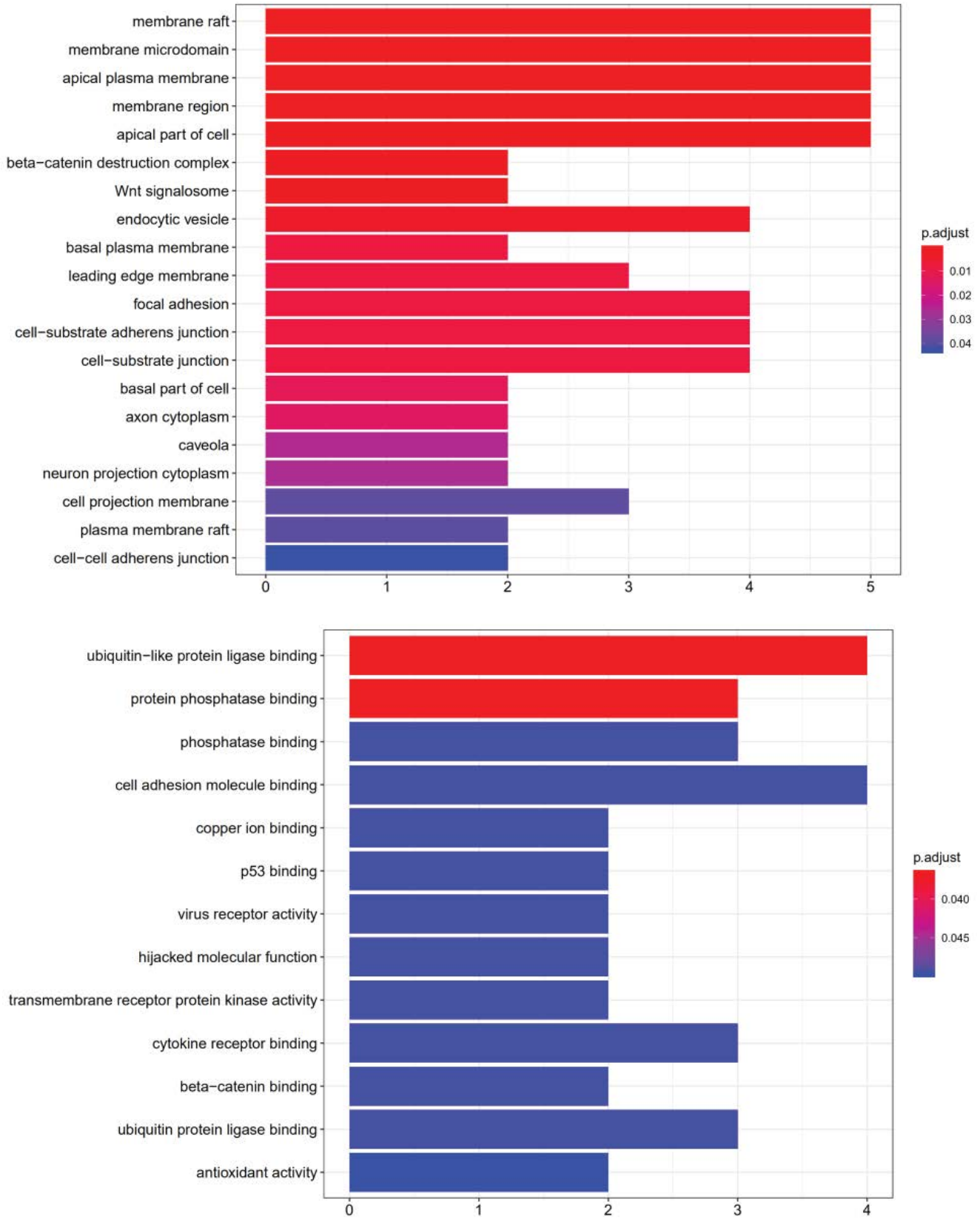


Figure 3: The histogram of Gene ontology terms of candidate targets of LH against COVID-19. Biological Process, Cellular Component, and Molecular Function. The top 20 GO functional categories with P<0.05 were selected.

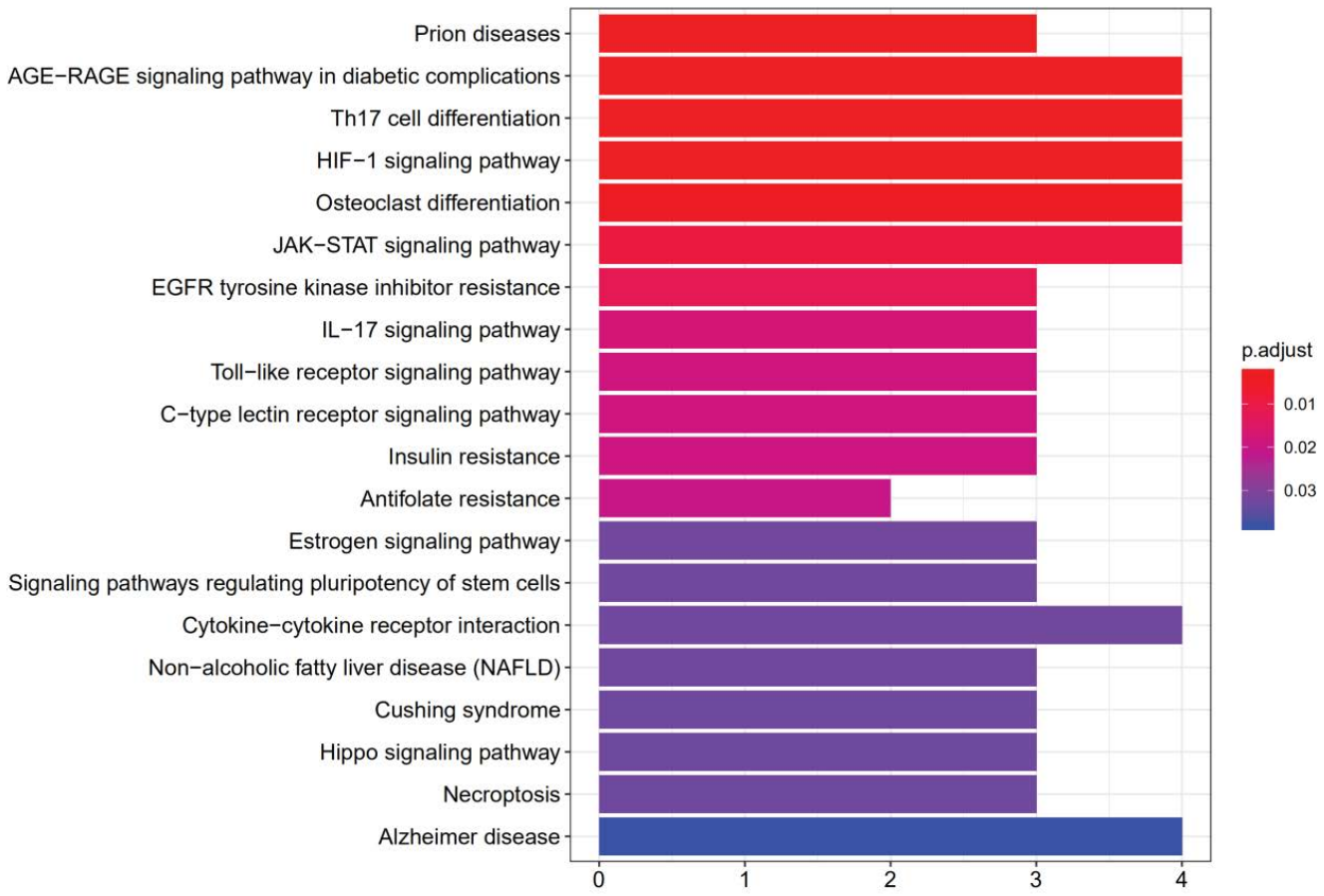


Figure 4: The histogram of KEGG, KEGG pathway enrichment of candidate targets of LH against COVID-19. Pathways that had significant changes of P <0.05 were identified.

were significantly influenced by LH in the process of treating COVID-19 were identified by KEGG pathway analysis. Twenty-four significantly enriched pathways (P<0.05) including Prion diseases, AGE-RAGE signaling pathway in diabetic complications, Th17 cell differentiation, HIF-1 signaling pathway, Osteoclast differentiation, JAK-STAT signaling pathway were identified. The data of KEGG pathway analysis were shown in Supplementary Table 2. As shown in Figure 4, size of the spot represented number of genes and color represented P value.

Gene-pathway network analysis

The gene-pathway network was constructed based on the significantly enriched pathways and genes that regulated these pathways, which was presented in Figure 5. The topological analysis of 20 pathways and 12 genes was carried out with BC. The squares represented target genes and the V-shapes represented pathways in the network. The network diagram suggested that IL6 had the most maximum BC and was the core target gene. Other several genes also had larger BC, such as IL1B, STAT1, IFNGR1 and GSK3B. They might be the key target gens for LH against COVID-19.

DISCUSSION

The unique medical theory of TCM has been formed and developed for thousands of years in China, which is used to treat and prevent diseases. Species compatible herbs are often used as complex herbal prescriptions to improve therapeutic effect through synergism. LH is a prescription based on the SARS period in 2003. At the same time, it can inhibit the activity of MERS-CoV and has been listed in Scheme for Diagnosis and Treatment of Middle East respiratory syndrome case (2015 Edition) [14]. Its main effects is clear away the plague and detoxify, release the lung heat. The indications are fever or high fever, chills, muscle soreness, stuffy or runny nose, cough, headache, dry throat and sore throat, red tongue, yellow or greasy fur, etc. It has a wide spectrum of antiviral, effective antibacterial, antipyretic and anti-inflammatory, cough and phlegm relieving, immune regulating and other systemic intervention effects on viral respiratory infectious

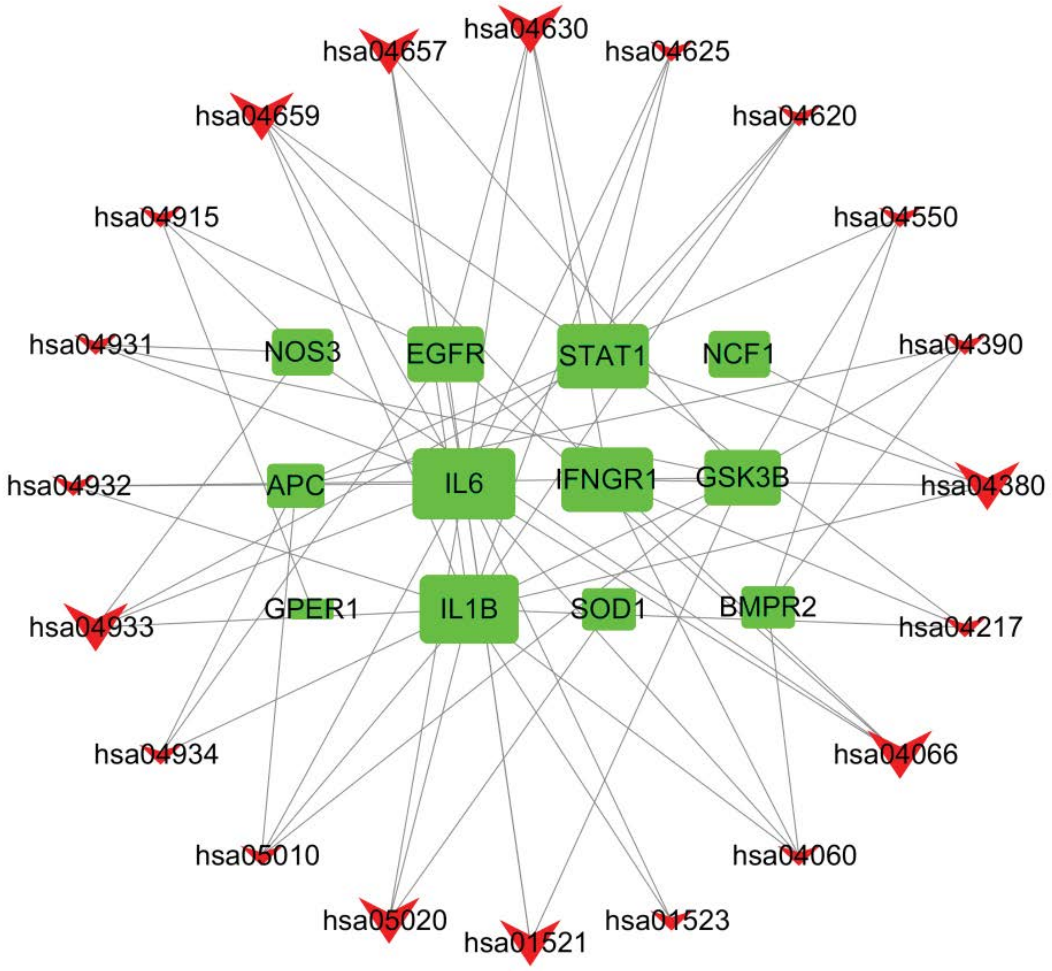


Figure 5: Gene-Pathway Network of LH against COVID-19. The topological analysis of 20 pathways and 12 genes was carried out with between ness centrality. The green squares represent target genes and the red V-shapes represent pathways. Big size represents the larger between ness centrality.

diseases. The outbreak of COVID-19 shows unique advantages of traditional Chinese medicine [15]. Meanwhile, traditional Chinese medicine such as LH had been recommended by the state for epidemic prevention. The retrospective study of anti epidemic in China showed that LH had a significant effect on COVID-19 [16].

It is well known that the effects of TCM on treating diseases are the result of the combination effects of many constituents. However, up to now, there is no method to identify the total effective components of TCM. LH is a traditional chinese prescription, same as the most TCM highly complex system and contains a large number of constituents. We had been tried to verify even more effective chemical components from LH through various approaches including network pharmacology.

The top 9 of the active ingredients of LH are mainly: quercetin, luteolin, apigenin, wogonin, isorhamnetin, formononetin, Vestitol, oleic acid, 7-Methoxy-2-methyl isoflavone, HMO, irisolidone. Reported that quercetin might be an immunosuppressant to decrease the harmful immune responses [17], including chronic inflammation [18], anti-cancer [19], antioxidant [20], and autoimmunity [21]. In the present studies, luteolin has been shown to exert multiple pharmacological activities, such as anti inflammatory reduce leukocytes [22], treatment H1N1 and immunomodulatory properties [23]. Studies on apigenin showed it could anti-inflammatory, regulating immunity [24-26]. Studies had shown that the main components of LH have different effects on pneumonia caused by various bacteria and viruses. Therefore, they might be identified as the representative compounds for LH.

Although the number of putative targets in each single herb were different, the overlapping targets in different herbs were numerous. In another word, multiple compounds of LH may have the same targets providing synergistic effects.

The targets of LH against COVID-19 were enriched in biological processes, cellular components, and molecular function by GO enrichment analysis. Results suggested that LH regulated some biological processes (BP), such as multicellular organismal homeostasis, response to nutrient levels, regulation of apoptotic signaling pathway, response to oxidative stress. Cellular component (CC), such as membrane raft, membrane microdomain, apical plasma membrane, membrane region. Molecular function (MF) such as: ubiquitin-like protein ligase binding, cell adhesion molecule binding. COVID-19 is an infectious disease caused by a novel beta coronavirus. LH could inhibit the binding of virus to various sites in the cell membrane and inhibit the replication of virus in new cells by improving the immune ability of the body, improving cell homeostasis, cell nutritional status, inhibiting apoptosis, anti-oxidation and so on. Results demonstrated that quercetin prevented biofilm formation and also altered the physicochemical cell properties repressed the genes of stress and virulence [27]. Quercetin could inhibit p53 and TNF-α/caspase8 mediated apoptosis and antioxidant stress [28]. Investigation showed that quercetin, and apigenin on stimulation of cholecystokinin release in vitro. Then cholecystokinin released from the endocrine cells in response to the ingestion of nutrients [29]. Research demonstrated acetylation quercetin could interact with F-protein with lower binding energy and better stability to block in vitro human respiratory syncytial viral adhesion [30].

TCM is multi-component, multi-target, and multi-pathway. Therefore, LH treats COVID-19 through multi-pathway. In the present study, a total of 26 KEGG pathways including Th17 cell differentiation, Osteoclast differentiation, IL-17 signaling pathway, Toll-like receptor signaling pathway, and JAK-STAT signaling pathway were significantly enriched. Aforementioned signaling pathways were played the roles of anti-inflammatory, antiviral and immunomodulatory. Study showed in the Th17-differentiation conditions, quercetin suppressed Th17 cell and the production of IL-17, and then decreased osteoclast differentiation [31]. Research showed that LH could inhibit the inflammatory response of acute lung injury [32]. And research showed that LH had a significant therapeutic effect on viral influenza [33].

The target genes of LH therapeutic COVID-19 main contain MDM2, IL6, IL1B, STAT1, IFNGR1 and NCF1. Most of the genes enriched on the signaling pathway, such as AGE-RAGE signaling pathway in diabetic complications, Th17 cell differentiation [34], HIF-1 signaling pathway [35], Osteoclast differentiation, Prion diseases, JAK-STAT signaling pathway, EGFR tyrosine kinase inhibitor resistance and so on, which associated with Human disease. Quercetin could induce the expression of miR-369-3p, inhibit the chronic inflammatory response and reduce the expression of IL6 [36]. Studies suggested that the inhibitory effect of luteolin on intracellular signaling execution and proinflammatory cytokine expression is associated with resolution of oxidative stress and promotion of protein phosphatase activity. And luteolin could attenuate inflammatory response by suppresses NF-κB, STAT1 and IRF-1 signaling [37]. Same with the pathogenic characteristics with COVID-19: related to immune regulation, host defense, inflammatory reaction and autoimmune diseases and so on [38].

In summary, this study used the methods of network pharmacology and molecular docking to explore the chemical composition, target, core active compounds and COVID-19 in LH. The results showed that novel coronavirus could be prevented and treated by multiple targets and multi pathways in LH. In view of the limitations of network pharmacology, the effect of LH on the prevention and treatment of covid-19 needs further clinical study.

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Supplementary Table 1: Part of the selected ingredients in LH for analysis.

Herb	ID	Ingredient	Herb	ID	Ingredient
Guanzhong (Fortunes Bossfern Rhizome)	MOL001040	(2R)-5,7-dihydroxy-2-(4-hydroxyphenyl)chroman-4-one	Banlangen (latin name: Isatidis Radix)	MOL001728	3-[2′ -(5′ - hydroxymethyl) furyl] -1 (2H) -isoquinolinone-7-O-BETA-D-glucoside_qt
	MOL002605	11-Hydroxynumantenine		MOL001792	DFV
	MOL002609	Harmonyl		MOL001756	quindoline
	MOL002610	ZINC00035529		MOL001833	Glucobrassicin-1-Sulfonate_qt
	MOL002614	Flavidin		MOL001689	acacetin
	MOL002619	Albaspidin AA		MOL001820	(E)-3-(3,5-dimethoxy-4-hydroxyb-enzylidene)-2-indolinone
	MOL002628	isopentenyaldenosine		MOL001814	(E)-3-(3,5-dimethoxy-4-hydroxy-benzylidene)-2-indolinone
	MOL002633	Albaspidin AP		MOL001782	(2Z)-2-(2-oxoindolin-3-ylidene) indolin-3-one
	MOL000422	kaempferol		MOL001781	Indigo
Huoxiang (latin name:Pogostemon Cablin Benth)	MOL000119	ZINC02040970		MOL001735	Dinatin
	MOL000126	(-)-nopinene		MOL001798	neohesperidin_qt
	MOL001390	49070_FLUKA		MOL001736	(-)-taxifolin
	MOL003333	acteoside		MOL001767	hydroxyindirubin
	MOL001606	BB_NC-0668		MOL001774	Ineketone
	MOL000169	alpha-Guaiene		MOL001722	2-O-beta-D-glucopyranosyl-2H-1,4-benzoxazin-3(4H)-one
	MOL000170	guaiene		MOL001793	(E)-2-[(3-indole)cyanomethylene-]-3-indolinone
	MOL000193	(Z)-caryophyllene		MOL001749	ZINC03860434
	MOL002025	Tereton		MOL001733	EUPATORIN
	MOL000204	-cis-.beta.-Elemene diastereomer		MOL001721	Isaindigodione
	MOL002066	neophytadiene		MOL001803	Sinensetin
	MOL002338	136458-42-9		MOL001779	Sinoacutine
	MOL000234	L-Limonen		MOL001734	3-[[(2R,3R,5R,6S)-3,5-dihydroxy-6-(1H-indol-3-yloxy)-4-oxooxan-2-yl] methoxy]-3-oxopropanoic acid
	MOL000024	alpha-humulene		MOL001750	glucobrassicin
	MOL000250	cis-Cinnamaldehyde		MOL001726	pinoresinol-4-O-beta-D-apiosyl-beta-D-glucopyranoside

Supplementary Table 2: The KEGG pathway analysis.

ID	Description	pvalue	p.adjust	qvalue	geneID	Count
hsa05020	Prion diseases	8.03E-05	0.002967949	0.001927672	IL1B/SOD1/IL6	3
hsa04933	AGE-RAGE signaling pathway in diabetic complications	9.03E-05	0.002967949	0.001927672	IL1B/STAT1/IL6/NOS3	4
hsa04659	Th17 cell differentiation	0.00011752	0.002967949	0.001927672	IL1B/STAT1/IL6/IFNGR1	4
hsa04066	HIF-1 signaling pathway	0.000126296	0.002967949	0.001927672	EGFR/IL6/NOS3/IFNGR1	4
hsa04380	Osteoclast differentiation	0.000235122	0.004420299	0.002870967	IL1B/NCF1/STAT1/IFNGR1	4
hsa04630	JAK-STAT signaling pathway	0.000577878	0.009053426	0.005880165	STAT1/EGFR/IL6/IFNGR1	4
hsa01521	EGFR tyrosine kinase inhibitor resistance	0.000906072	0.012167254	0.007902584	GSK3B/EGFR/IL6	3
hsa04657	IL-17 signaling pathway	0.001500424	0.017629979	0.011450602	GSK3B/IL1B/IL6	3
hsa04620	Toll-like receptor signaling pathway	0.002006916	0.018865015	0.012252753	IL1B/STAT1/IL6	3
hsa04625	C-type lectin receptor signaling pathway	0.002006916	0.018865015	0.012252753	IL1B/STAT1/IL6	3
hsa04931	Insulin resistance	0.002235987	0.019107525	0.012410262	GSK3B/IL6/NOS3	3
hsa01523	Antifolate resistance	0.002599977	0.020366485	0.013227952	IL1B/IL6	2
hsa04915	Estrogen signaling pathway	0.00447945	0.032389868	0.021037092	EGFR/NOS3/GPER1	3
hsa04550	Signaling pathways regulating pluripotency of stem cells	0.004853098	0.032494345	0.02110495	GSK3B/BMPR2/APC	3
hsa04060	Cytokine-cytokine receptor interaction	0.005185268	0.032494345	0.02110495	IL1B/IL6/IFNGR1/BMPR2	4
hsa04932	Non-alcoholic fatty liver disease (NAFLD)	0.005551618	0.032615754	0.021183804	GSK3B/IL1B/IL6	3
hsa04934	Cushing syndrome	0.006196472	0.032902308	0.02136992	GSK3B/EGFR/APC	3
hsa04390	Hippo signaling pathway	0.00642104	0.032902308	0.02136992	GSK3B/BMPR2/APC	3
hsa04217	Necroptosis	0.006650467	0.032902308	0.02136992	IL1B/STAT1/IFNGR1	3
hsa05010	Alzheimer disease	0.008131332	0.038217259	0.02482196	GSK3B/IL1B/IL6/APC	4
hsa04370	VEGF signaling pathway	0.009181241	0.040205126	0.026113071	NOS3/HSPB1	2
hsa04621	NOD-like receptor signaling pathway	0.009502554	0.040205126	0.026113071	IL1B/STAT1/IL6	3
hsa04151	PI3K-Akt signaling pathway	0.009953587	0.040205126	0.026113071	GSK3B/EGFR/IL6/NOS3	4
hsa04623	Cytosolic DNA-sensing pathway	0.010418496	0.040205126	0.026113071	IL1B/IL6	2
hsa04062	Chemokine signaling pathway	0.010692853	0.040205126	0.026113071	GSK3B/NCF1/STAT1	3
hsa04917	Prolactin signaling pathway	0.012751523	0.04610166	0.029942848	GSK3B/STAT1	2