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Identification of biomarkers of acne based on transcriptome analysis and combined with network pharmacology to explore the therapeutic mechanism of Jinhuang ointment

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Abstract

The incidence of acne is on the rise due to unhealthy diet and living habits. Jinhuang ointment (JHO) is a classic prescription composed of 10 kinds of commonly used Chinese herbal medicine, which has been widely used in clinical prevention and treatment of skin inflammatory diseases since ancient times. However, the pharmacological mechanism and target of JHO are not clear. The acne microarray dataset was downloaded from gene expression omnibus database to identify differentially expressed genes (DEG). Immune infiltration was analyzed by CiberSort algorithm. HUB gene was identified by protein-protein interaction network. The gene expression omnibus dataset validates the biomarkers of acne with high diagnostic value. The potential active components and targets of JHO were obtained through Traditional Chinese Medicine Systems Pharmacology database, and the therapeutic targets were obtained by crossing with disease targets. R-packet is used for enrichment analysis. Molecular docking using Auto Dock Tools. A total of 202 DEGs were identified from 12 skin samples in the GSE6475. Immune infiltration analysis showed that there were a large number of macrophages and mast cells in acne skin. Gene set enrichment analysis analysis showed that DEGS was mainly involved in bacterial reaction, inflammatory reaction and so on. Six central genes and gene cluster modules were identified by Cytoscape software. A total of 185 JHO active components and 220 targets were obtained, of which 10 targets were potential targets for JHO in the treatment of acne. Kyoto encyclopedia of genes and genomes enrichment analysis showed that JHO treatment of acne was mainly related to Toll-like receptors, IL-17 and other signal pathways. The results of molecular docking showed that 5 active compounds in JHO had strong binding activity to the core protein receptor. IL-1 β, CXCL8, toll-like receptor 2, CXCL2, LCN2, and secretory phosphoprotein 1 may be potential biomarkers for early diagnosis of acne. JHO active components may regulate skin cell metabolism and inflammatory response and improve cellular immune microenvironment by acting on core targets (CXCL8, ESR1, IL-1 β, MMP1, MMP3, secretory phosphoprotein 1), thus achieving the purpose of treating acne. This is the result of the joint action of multiple targets and multiple pathways. It provides an idea for the development of a new combination of drugs for the treatment of acne.

Abbreviations: AUC = area under curve, DEG = differentially expressed genes, GEO = gene expression omnibus, GO = gene ontology, GSEA = gene set enrichment analysis, IL-1β = interleukin-1β, IL-8 = interleukin-8, JHO = Jinhuang ointment, KEGG = Kyoto encyclopedia of genes and genomes, PPI = protein-protein interaction, SPP1 = secretory phosphoprotein 1, TCMSP = traditional Chinese medicine systems pharmacology, TLR2 = toll-like receptor 2.

Keywords: acne, biomarkers, Jinhuang ointment, molecular docking, network pharmacology, transcriptome analysis

1. Introduction

Acne is a common chronic inflammatory disease of the skin involving hair follicle sebaceous glands.[1,2] According to incomplete statistics, the disease is found in about 80% of young people and adolescents.[3] Strauss et al[4] defined acne as a chronic inflammatory skin disease, including open acne (blackhead), closed acne (white head) and inflammatory lesions such as nodules, pustules and papules. As the skin lesions caused by acne will affect the beauty of the face, it brings a greater psychological burden to some patients. In addition, it is related to serious negative effects on mental health, including emotional disorders, mental disorders, absenteeism, unemployment, and increased suicide rates.[5] It is estimated that the annual economic burden of acne in the United States is $3 million.[6] There are many factors that

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The datasets generated during and/or analyzed during the current study are publicly available.

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cause or aggravate acne, including heredity, stress, smoking, unhealthy diet, abnormal, and Rogen secretion and so on.[7,8] The pathological factors include increased sebum production, hyperkeratosis of hair follicles, proliferation of Propionibacterium acne, and local inflammatory reaction.[9] There is a lack of effective biomarkers in the diagnosis and treatment of acne, which brings some limitations to the targeted treatment of acne. At present, the treatment of acne is mainly treated with retinoic acid, local antibiotics, fungicides and laser, but the long-term use of these treatments is easy to cause facial skin allergic reaction, and skin intolerance.[10,11] Therefore, it is necessary to explore biomarkers for early diagnosis and treatment of acne and to find safer and more effective alternative drugs to treat acne.

Traditional Chinese medicine has a history of nearly 5000 years and has played an important role in the treatment of many diseases. The description of acne in traditional Chinese medicine can be traced back to Huangdi Nei Jing. Traditional Chinese medicine believes that acne belongs to the category of "heat" and "depression". Jinhuang ointment (JHO) is a classic prescription of traditional Chinese medicine surgery, also known as Jinhuang Powder. The prescription originated from the authentic surgery of the Ming Dynasty (AD 1617). JHO is composed of Arisaematis Rhizoma (Shengnanxing), Atractylodis (Cangzhu), Licorice (Gancao), Citrus Reticulata (Chenpi), Trichosanthis Radix (Tianhuafen), Angelica dahurica (Baizhi), Curcumaelongae Rhizoma (Jianghuang), Magnolia officinalis (Houpu), Rheum officinale (Dahuang), and Phellodendron chinense (Huangbai). JHO has the effect of clearing heat and detoxification, reducing swelling and relieving pain. JHO has been widely used in the clinical treatment of acne in China, and achieved satisfactory results.[12,13] Previous studies have shown that JHO can reduce the level of inflammatory factors, promote wound healing and improve local skin state.[14] JHO can effectively regulate the expression of TNF- α, IL-1, interleukin-8 (IL-8), and other inflammatory factors to achieve anti-infection effect.[15] Modern medicine has proved that external use of this drug can reduce the proportion of leukocytes and neutrophils in peripheral blood, reduce vascular permeability, and has anti-swelling and anti-inflammatory effects. It can also improve local blood circulation, effectively control infection, significantly improve efficacy and high safety.[16] However, the specific mechanism and related targets of JHO in the treatment of acne have not been fully studied.

At present, transcriptome and microarray analysis have been widely used in a variety of diseases, including various skin inflammatory diseases, to identify new biomarkers to improve diagnosis and treatment.[17–19] Compared with western medicine, the treatment of traditional Chinese medicine has the characteristics of multi-components, multi-targets and multi-approaches.[20] In recent years, as a systematic research method of multi-discipline, network pharmacology coincides with the holistic view of traditional Chinese medicine, which provides new ideas and methods for the research of complex prescriptions.[21] Network pharmacology is based on systems biology, multi-directional pharmacology and other multi-disciplinary theories, using biological network data, biological network construction analysis, and visualization and other information and technology to reveal the interaction between organism and drugs.[22,23] Therefore, in this study, transcriptome, bioinformatics, network pharmacology and molecular docking techniques were used to explore the biomarkers of diagnosis and treatment of acne and to explore the effective components of JHO in the treatment of acne at the molecular level, and to predict its potential therapeutic targets and pathways. The purpose of this study is to provide reference for further research, better application in clinic and new drug development of this prescription. The specific flow chart is shown in Figure 1.

2. Materials and methods

2.1. Collection of acne-related microarray data

The microarray data of acne skin tissue were obtained by using gene expression omnibus (GEO) database. The screening criteria include: the subjects are "Homo sapiens"; the samples are facial skin tissues of acne patients and healthy patients; the data set information is complete and the sample size is not <5. Finally, 2 data sets were obtained, including GSE6475 (GPL571 platform, [HG-U133A_2] Affymetrix Human Genome U133A 2.0 Array) and GSE122592 (GPL20301, I llumina HiSeq 4000 [Homo sapiens]). GSE6475 included 6 acne skin samples and 6 healthy skin samples as test sets. GSE122592 included 3 acne skin samples and 4 healthy skin samples as a verification set. The study has been approved by the Ethics Committee of the Shandong Women's University.

2.2. Data preprocessing and differentially expressed genes (DEGs) screening

The original chip data downloaded from (GEO, https://www.ncbi.nlm.nih.gov/geo/) database is normalized by R software (v4.0.5). The genetic analysis of the differences between samples was carried out by using Limma package, and the threshold of corrected P was adjusted to $P < .05$. The screening criteria of differential genes were log2 (Fold change) > 1 or < −1, and adjusted P value (Q value) < .05. In order to better show these changes, R software is used to analyze PCA and draw heat map and volcano map. The volcano map is drawn with the ggplot2 [3.3.3] package, and the heat map is made with the ComplexHeatmap [2.2.0] package.

2.3. Acquisition of the final target of disease

Search for acne-related targets in Human Gene Database (GeneCards, https://www.genecards.org/) and Comparative Toxicogenomics Database (CTD, http://ctdbase.org/) database, with "acne" and "acne vulgaris" as keywords, and species as "Homo Sapiens." After the target is sorted out, the repetitive value is deleted and intersected with DEGs to get the final disease target.

2.4. Enrichment analysis

gene set enrichment analysis (GSEA) is a method for analyzing the microarray data of full gene expression profile.[24] The functional enrichment analysis of GSE6475 data set was carried out by using GSEA 4.1.0, and the background base factor set was set to c5 (c5.bp.v7.4 symbols. gmt). R package org.Hs.e.g..db (v3.10.0) was used for gene ID conversion of the final acne target, and then Cluster Profiler (v3.14.3) package was used for gene ontology (GO) and Kyoto encyclopedia of gene and genome (KEGG) analysis. Taking the adjusted $P < .05$ as the screening condition, the first 15 biological processes (BP) and KEGG pathways were selected to construct bar chart and bubble chart respectively. The z-score value is calculated by R package GO plot (v1.0.2), and the chord diagram and circle diagram are drawn respectively.

2.5. Construction of protein-protein interaction (PPI) Network and screening of core genes

The acne target was inputted into STRING database (https://string-db.org), and minimum required interaction score was set as high confidence (score > 0.7). Protein-protein interaction network was constructed. Download the "tsv" format file, import it into Cytoscape (v3.9.1), and use Cytoscape software to optimize the network. The protein-protein interaction network was analyzed by molecular complex detection (setting conditions:

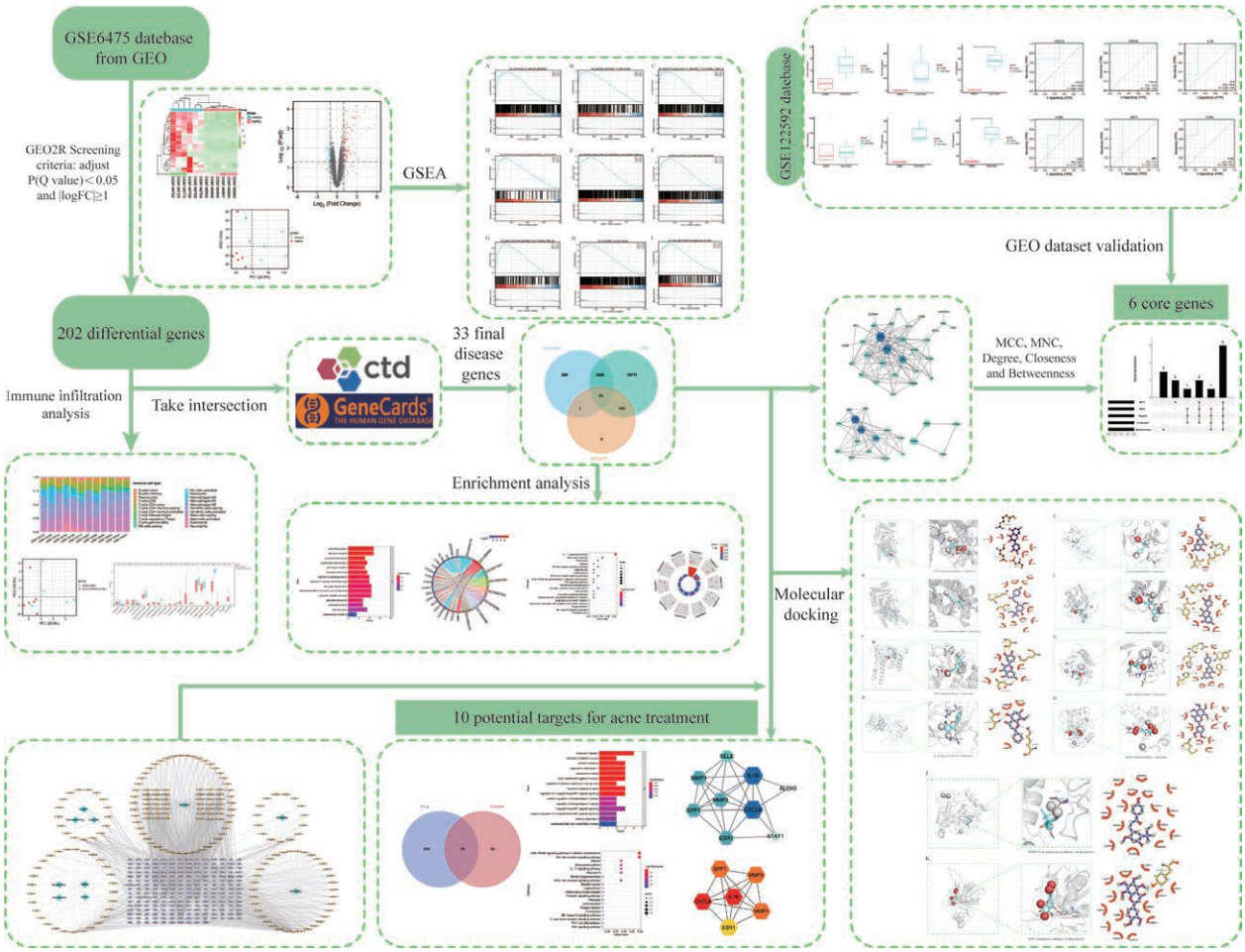

Figure 1. The flowchart of the analysis procedures of the study.

Degree Cutoff = 2, Node score Cutoff = 0.2, k-score = 2, Max. Depth = 100),[25] and meaningful protein modules and clustering scores were obtained. The core gene was identified by Cytoscape plug-in CytoHubba.[26] The core genes were screened by maximal clique centrality, maximum neighborhood component, Degree, Closeness, and Betweenness.

2.6. Statistics analysis and immune infiltration analysis

R package ggpubr was used for statistical analysis, *t* test was used to compare the differences between the 2 groups, and ggplot2 package was used to draw box diagram. Use IBM SPSS (v26.0) to analyze the data and draw ROC curves. The normalized microarray data set was uploaded to CIBERSORT (https://cibersortx.stanford.edu/) for immune infiltration analysis, and the abundance ratio of 22 kinds of immune cells was obtained. These immune cells include immature CD4 + T cells, resting memory CD4 + T cells, M0 macrophages, M1 macrophages, activated mast cells, eosinophils, neutrophils and so on. According to $P < .05$, the percentage of immune cells in the sample and the immune infiltration level of all kinds of immune cells between the 2 groups were calculated by R software. PCA was used to analyze the difference of immune cell infiltration between acne patients and healthy controls.

2.7. Screening of active components of herb in JHO and construction of "herb-active ingredient-target" network

The herb contained in JHO were inputted into the pharmacological database and analysis platform of traditional Chinese medicine systems pharmacology[21] (TCMSP, http://tcmspw.com/tcmsp.php), and all the chemical components of the drugs were obtained. According to the principle of pharmacokinetics (ADME), the screening conditions were set as oral bioavailability ≥ 30% and drug-likeness ≥ 0.18, and the active components in JHO were screened. The selected active components were searched by TCMSP platform, and the corresponding targets were obtained and standardized with the help of Uniprot database. The active components of JHO and their corresponding targets are used to construct the "Herb-Active ingredient-Target" network. The network is constructed by using Cytoscape (v3.7.2), and the topology of the network is analyzed.

2.8. Potential target prediction and enrichment analysis of JHO in the treatment of acne

We intersect the target genes regulated by the active components in JHO with the final target of acne and draw the venn diagram. On this basis, the potential targets of JHO in the treatment of acne were obtained. The potential targets of Danshen granule in the treatment of gout were obtained. Then we used R software to analyze the potential targets of JHO for acne treatment by GO and KEGG enrichment analysis. Set the screening condition to $P < .05$. The first 15 BP were selected to draw the bar chart and the first 15 KEGG paths were selected to draw the bubble chart.

2.9. PPI network analysis and molecular docking verification

Input the potential targets of JHO in the treatment of acne into the STRING database for PPI network construction.

Cytoscape software is used for network optimization. The top 6 core therapeutic targets were screened out with the help of cytoHubba plug-in. The active components with the top 5 degree values in the "Herb-Active ingredient-Target" network were selected as docking small molecules. The 3D structure of small molecules was downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/) database, and the mechanics of small molecules was optimized by Chem3D (v19.0). Then import the small molecules into Auto Dock Tools (v1.5.6) to determine the rotatable key and save it as a "PDBQT" format file. We downloaded the 3D structure of the core therapeutic target from the RCSBPDB database (http://www.rcsb.org).as receptor proteins. The water molecules and excess small molecular ligands in the receptor protein were removed by PyMOL (v2.3.0). Then the treated receptor protein was introduced into Auto Dock Tools (v1.5.6) for hydrogenation, charge calculation, atomic type addition and other operations, and saved as a "PDBQT" file. Finally, Auto Dock Vina is used for molecular docking, and PyMOL is used to visualize the best docking results. LigPlot + (v2.2.5) is used to visualize 2D structures. The binding energy is less than −5kcal/mol, indicating that the compound has a strong binding force.

3. Results

3.1. Identification of DEGS in acne and acquisition of final disease target

The data set GSE6475 consists of 6 acne skin samples and 6 healthy control samples for analyzing and identifying DEGs and mapping PCA (Fig. 2A). Compared with the genes in healthy control samples, we identified a total of 202 DEGs in acne skin samples, including 67 down-regulated genes and 135 up-regulated genes. Subsequently, heat maps and volcano map analysis are used to visualize these DEGs, as shown in Figure 2B, C.A total of 21,654 acne-related targets were collected from 2 disease databases, including 20,115 in the CTD database and

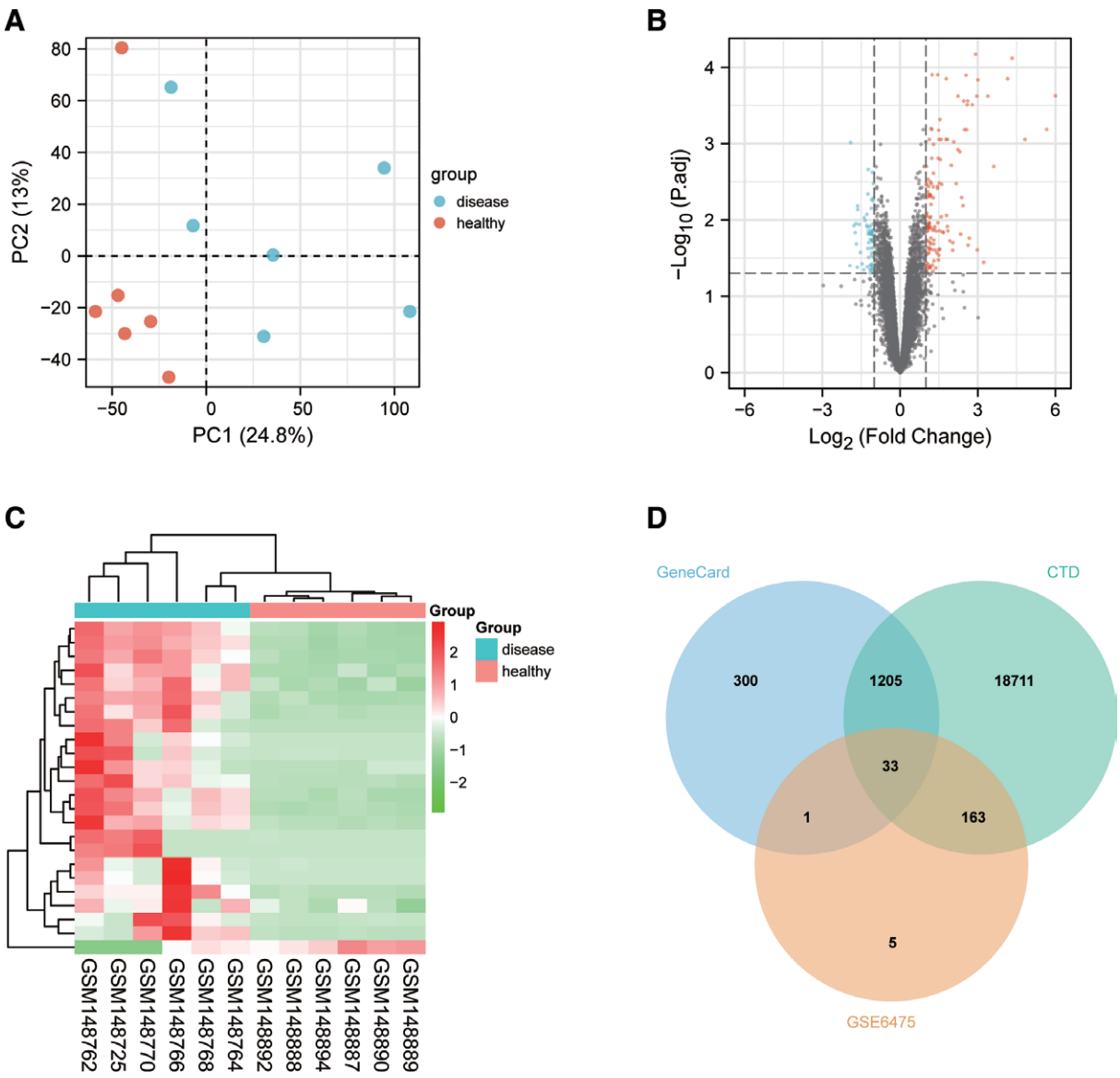

Figure 2. Analysis of differential genes related to acne. (A) Principal component analysis. (B) Differential gene heat map between acne skin samples and healthy samples: each cell represents each gene, and the color depth indicates the expression of the gene. The higher the expression level, the darker the color (red up-regulated, green down-regulated). (C) Differential gene volcano map between acne skin samples and healthy samples, blue dots represent down-regulated genes, red dots represent up-regulated genes, and gray dots represent genes with little difference. (D) Wayne map to obtain the final disease target of acne.

1539 in the Gene Cards database. Delete the duplicate value and intersect with DEGs to get the final disease target (Fig. 2D).

3.2. Enrichment analysis results

GSEA software, R package org.Hs.e.g..db and Cluster Profiler were used to analyze the function and pathway of the final acne disease target. First of all, we upload the expression profiles of all genes in GSE6475 to GSEA and select the background gene set as [c5.bp.v7.4 symbols. Gmt] carried out BP enrichment analysis on the expression profile at the overall level. The screening criterion of significant genome is $P < .05$. We found that most of the rich gene sets are related to immune inflammatory response and cytokine stimulation mediated by bacterial invasion (Fig. 3). Next, GO and KEGG enrichment analysis was performed on the final acne disease targets using R software. GO enrichment analysis showed that the immune inflammatory response of acne skin samples was stronger than that of healthy skin samples, which mainly involved biological processes such as Toll-like receptor binding, RAGE receptor binding, interleukin-1 receptor binding and so on. The details of the top 10 BP items in GO enrichment analysis and the top 10 items in KEGG enrichment analysis are shown in Table 1. The first 15 BP were selected according to the $P < .05$.Bar graphs and chord graphs were drawn (Fig. 4A and B). KEGG pathway enrichment analysis showed that acne disease targets

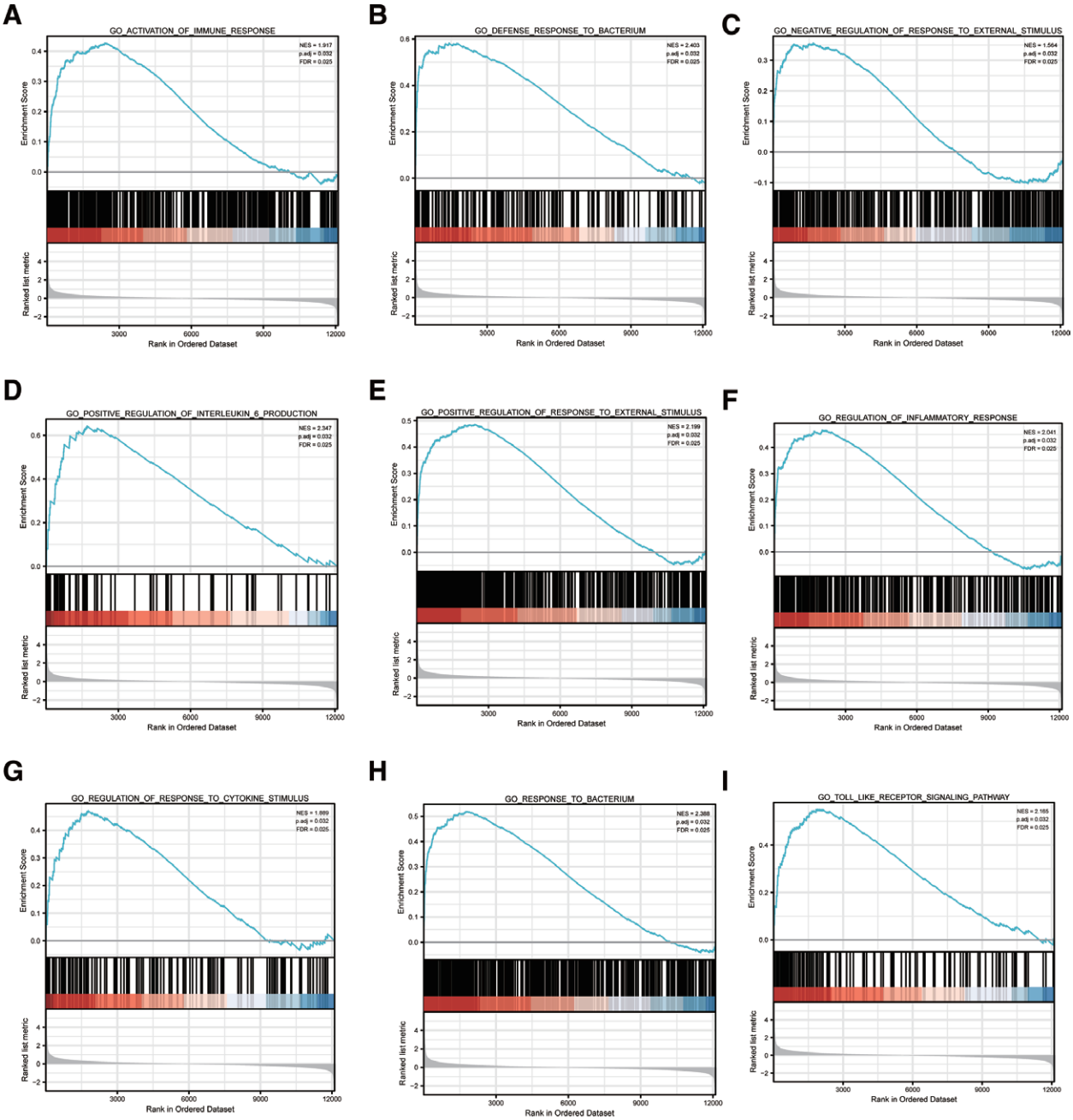

Figure 3. GSEA analysis. (A) Activation of immune response (NES = 1.917, $P < .05$). (B) Defense response to bacterium (NES = 2.403, $P < .05$). (C) Negative regulation of response to external stimulus (NES = 1.564, $P < .05$). (D) Positive regulation of interleukin-6 production (NES = 2.347, $P < .05$). (E) Positive regulation of response to external stimulus (NES = 2.199, $P < .05$). (F) Regulation of inlammatory response (NES = 2.041, $P < .05$). (G) Regulation of response to cytokine stimulus (NES = 1.889, $P < .05$). (H) Response to bacterium (NES = 2.388, $P < .05$). (I) Toll-like receptor signaling pathway (NES = 2.165, $P < .05$). GSEA = gene set enrichment analysis.

were mainly concentrated in IL-17 signaling pathway, Toll-like receptor signaling pathway, Nod-like receptor signaling pathway, Staphylococcus aureus infection and NF-κB signaling pathway. According to the $P < .05$, we selected the first 15 KEGG pathways and displayed them in the bubble diagram and loop diagram (Fig. 4C and D).

3.3. PPI network analysis and core genes identification

The PPI network consists of 30 nodes and 113 edges (3 targets are not involved in the construction of the network). The average node value is 7.53. the network is optimized by Cytoscape, as shown in Figure 5A. According to the screening criteria, 2 protein modules were identified by Molecular Complex Detection, as shown in Figure 5B and C. Module 1 has the highest clustering score of 8.308, including 14 nodes and 54 edges, while module 2 has a score of 2.667, including 4 nodes, and 4 edges. Then, CytoHubba was used to screen the core genes by maximal clique centrality, maximum neighborhood component, Degree, Closeness and Betweenness. A total of 6 core genes were obtained, namely IL-1 β, CXCL8, toll-like receptor 2 (TLR2), CXCL2, LCN2, and secretory phosphoprotein 1 (SPP1). These genes are the most important genes in the PPI network and may play an important role in the pathogenesis of acne. See Figure 5D.

3.4. Immune infiltration analysis

Using the CIBERSORT algorithm, we first studied the difference of immunoosmosis between acne skin and healthy skin in 22 immune cell subsets. Figure 5E summarizes the results of 6 acne patients and 6 normal subjects. Through PCA analysis, the proportion of immune cells from acne patients skin tissue and healthy control skin tissue showed significant population bias aggregation and individual differences (Fig. 5F). Compared with normal skin tissue, the proportion of macrophage M0, macrophage M1, Mast cells activated, T cells CD4 memory activated, Plasma cells, NK cells resting and Neutrophils in acne patient skin was generally higher, while the proportion of macrophage M2, T cells CD4 memory resting, Dendritic cells resting and Mast cells resting was relatively lower (Fig. 5G).

3.5. Verification of 6 core expressed genes using GSE122592 dataset

The GSE122592 dataset (including 3 acne skin samples and 4 healthy skin samples) was selected to verify the expression of 6 core genes. T-test statistical analysis was carried out with R-packet ggplot2 and ggpubr and box diagram was drawn. Consistent with our prediction, the expression level of 6 core genes in acne skin samples was significantly higher than that in healthy skin samples ($P < .05$) (Fig. 6). Using IBMSPSS (v26.0), 6 core gene expression profiles of acne skin samples and healthy skin samples in GSE122592 were analyzed by ROC, and the ROC curve was drawn. The area under the ROC curve surrounded by the axis is defined as area under curve (AUC). The closer the AUC is, the higher the authenticity of the detection method is, and when it is equal to 0.5, the authenticity is the lowest and has no application value.[25] Through the analysis, it is found that, compared with healthy skin samples, these 6 core genes have higher diagnostic value in acne samples. Among them, the diagnostic value of CXCL8 in acne skin samples is relatively high (AUC:1.000). The diagnostic values of other core genes in acne are: IL1β (AUC:0.833), TLR2 (AUC:0.917), CXCL2 (AUC:0.833), SPP1 (AUC:0.750), LCN2 (AUC:0.667), as shown in Figure 7. Because they show good diagnostic performance in acne skin samples, according to the current samples, we speculate that these 6 core genes may be biomarkers for the diagnosis of acne diseases.

3.6. Identification of active components of JHO and analysis of "herb-active component-target" network

A total of 1137 JHO compounds were retrieved from TCMSP database. Taking oral bioavailability ≥ 30% and drug-likeness ≥ 0.18 as indexes, a total of 198 active compounds were screened. Including 7 from Arisaematis Rhizoma (Shengnanxing), 9 from Atractylodis (Cangzhu), 92 from Licorice (Gancao), 5 from Citrus Reticulata (Chenpi), 2 from Trichosanthis Radix (Tianhuafen), 23 from Angelica dahurica (Baizhi), 3 from Curcumaelongae Rhizoma (Jianghuang), 3 from Magnolia officinalis (Houpu), 16 from Rheum officinale (Dahuang) and 38 from Phellodendron chinense (Huangbai). After eliminating the repeated values, a total of 185 active components were obtained. Some active ingredients in JHO are shown in Table 2. The number

Table 1

Details of the top 10 entries for BP and KEGG pathways.

Ontology	ID	Description	GeneRatio	BgRatio	*P* value	P. adjust	Q value
BP	GO: 0042119	Neutrophil activation	12/33	498/18,670	2.43e–11	1.59e–08	9.87e–09
BP	GO: 0050900	Leukocyte migration	12/33	499/18,670	2.48e–11	1.59e–08	9.87e–09
BP	GO: 0071621	Granulocyte chemotaxis	8/33	123/18,670	3.41e–11	1.59e–08	9.87e–09
BP	GO: 0097529	Myeloid leukocyte migration	9/33	210/18,670	7.41e–11	2.59e–08	1.61e–08
BP	GO: 0097530	Granulocyte migration	8/33	141/18,670	1.03e–10	2.87e–08	1.78e–08
BP	GO: 0030595	Leukocyte chemotaxis	9/33	224/18,670	1.32e–10	3.07e–08	1.91e–08
BP	GO: 0032496	Response to lipopolysaccharide	10/33	330/18,670	1.68e–10	3.35e–08	2.08e–08
BP	GO: 0002237	Response to molecule of bacterial origin	10/33	343/18,670	2.44e–10	4.28e–08	2.65e–08
BP	GO: 0043312	Neutrophil degranulation	11/33	485/18,670	3.75e–10	5.61e–08	3.48e–08
BP	GO: 0002283	Neutrophil activation involved in immune response	11/33	488/18,670	4.00e–10	5.61e–08	3.48e–08
KEGG	hsa04657	IL–17 signaling pathway	9/29	94/8076	2.20e–11	2.11e–09	1.48e–09
KEGG	hsa05323	Rheumatoid arthritis	6/29	93/8076	7.61e–07	3.65e–05	2.56e–05
KEGG	hsa05146	Amoebiasis	5/29	102/8076	2.72e–05	5.73e–04	4.02e–04
KEGG	hsa05144	Malaria	4/29	50/8076	2.76e–05	5.73e–04	4.02e–04
KEGG	hsa04620	Toll-like receptor signaling pathway	5/29	104/8076	2.99e–05	5.73e–04	4.02e–04
KEGG	hsa05134	Legionellosis	4/29	57/8076	4.64e–05	7.43e–04	5.21e–04
KEGG	hsa05140	Leishmaniasis	4/29	77/8076	1.51e–04	0.002	0.001
KEGG	hsa04621	NOD–like receptor signaling pathway	5/29	181/8076	4.10e–04	0.004	0.003
KEGG	hsa04933	AGE-RAGE signaling pathway in diabetic complications	4/29	100/8076	4.14e–04	0.004	0.003
KEGG	hsa04668	TNF signaling pathway	4/29	112/8076	6.37e–04	0.006	0.004

GO = gene ontology, KEGG = Kyoto encyclopedia of genes and genomes.

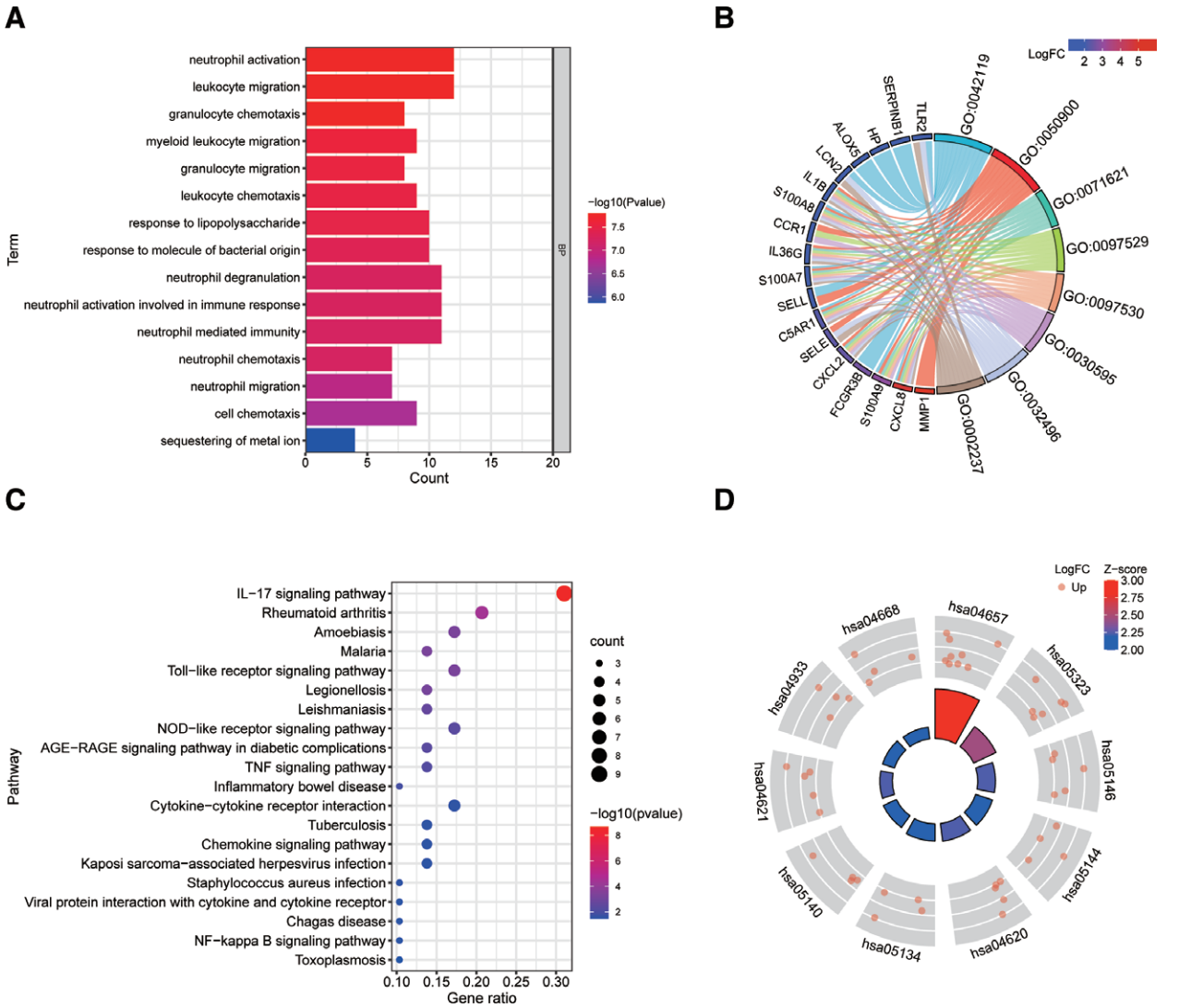

Figure 4. Enrichment analysis of final disease targets in Acne. (A) BP bar chart. (B) BP chord diagram. (C) KEGG bubble diagram, Y axis represents the name of the pathway, X axis represents the percentage, bubble area represents the number of enrichment genes in the pathway, the larger the bubble, the more the number of enrichment genes, the color of the bubble represents the size of *P* value, and the redder the color is, the more significant the enrichment degree is. (D) KEGG circle diagram. The screening criteria was $P < .05$. KEGG = Kyoto encyclopedia of genes and genomes.

of active compounds as well as the number of targets for each herb in JHO are shown in Table 3. A total of 220 active component targets were obtained by using TCMSP database. The corresponding standard gene name of each target was obtained by UniProt database.The "Herb-Active ingredient-Target" network was constructed using Cytoscape (Fig. 8A). The network consists of 415 nodes (including 10 Herb nodes, 185active component nodes and 220 target nodes) and 2044 edges. Carry on the topology analysis to the network through the network analysis function. Select the node with larger value for analysis. These nodes play an important role in the network and play a more critical role. The average value of active components in the network was 11.05. The first 5 active components were MOL000098- quercetin, MOL000173-wogonin, MOL004328- naringin, MOL005828-nobiletin and MOL000497-licochalcone a, and their degrees of substitution were 135, 38, 34, 29, and 28 respectively.

3.7. Potential target acquisition and enrichment analysis of JHO in the treatment of acne

The 33 final disease targets were intersected with 220 drug-related targets, and 10 potential targets for acne treatment with JHO were obtained (Fig. 8B). The 33 final disease targets were intersected with 220 drug-related targets, and 10 potential targets for acne treatment with JHO were obtained. Then R software was used to analyze the enrichment of potential therapeutic targets. GO enrichment analysis showed that the potential therapeutic targets were mainly related to biological processes such as response to interleukin-1, androgen metabolic process, hormone metabolic process, leukocyte migration, and so on. In addition, KEGG pathway enrichment analysis shows that the process of JHO in the treatment of acne is mainly achieved by affecting Toll-like receptor signaling pathway, IL-17 signaling pathway, Nod-like receptor signaling pathway, NF-κB signaling pathway, and so on. BP and KEGG pathways ranked 15 were selected for visualization (Figure 8C,D).

3.8. PPI network analysis of potential therapeutic targets and molecular docking

The PPI network consists of 9 nodes and 25 edges (of which NR3C2 is not involved in the network construction). Six core therapeutic targets were screened by cytoHubba, which were

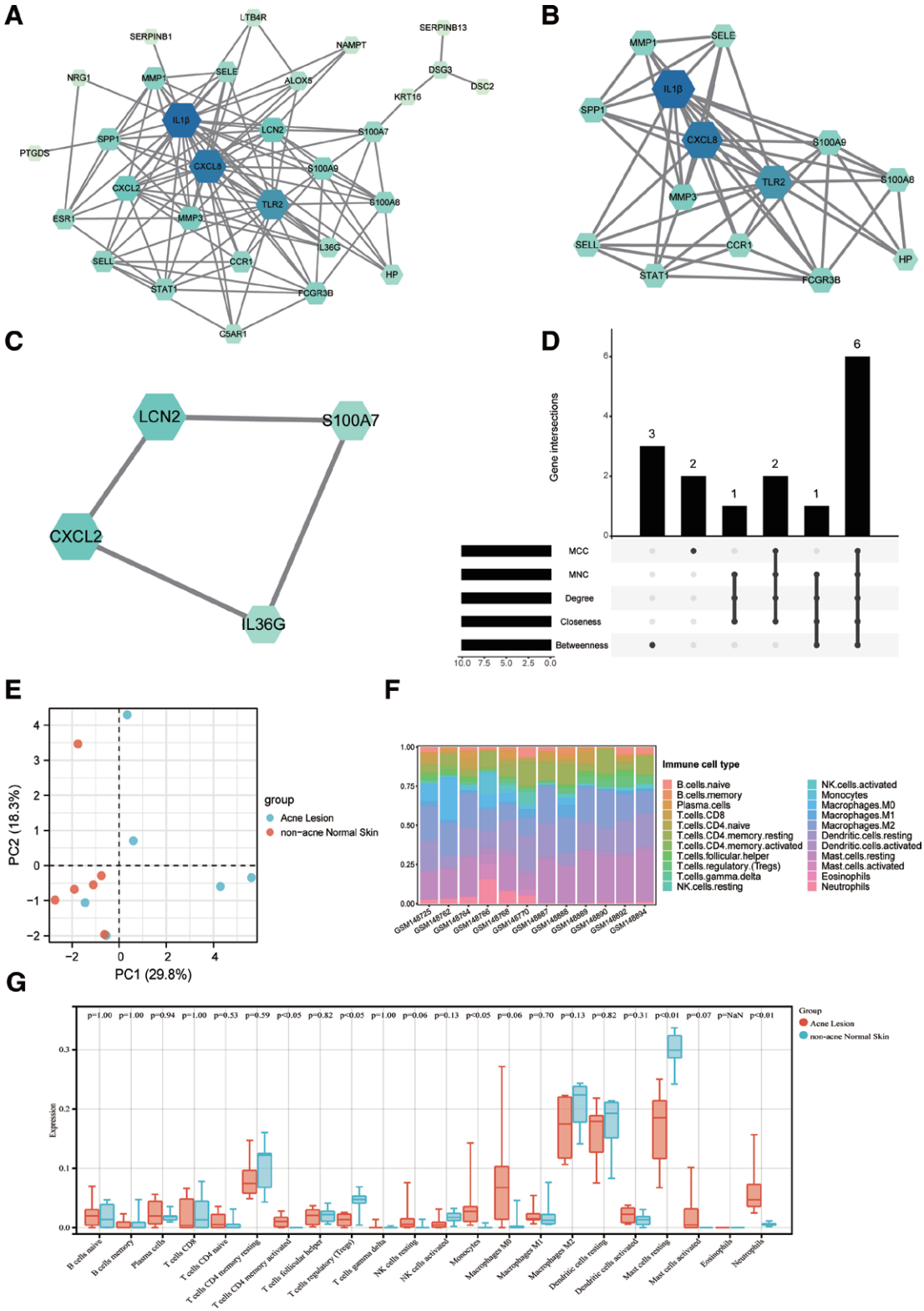

Figure 5. Core genes Identification and Immune infiltration analysis. (A) PPI network, the larger the graph, the greater the degree value. (B) MCODE1 (score = 8.308). (C) MCODE2 (score = 2.667). (D) Five commonly used algorithms (MCC, MNC, Degree, Closeness and Betweenness) were used to screen core genes. (E) Principal component analysis of all samples. The relative percentage of 22 immune cell subsets in 12 samples in (F) GSE6475 data set. (E) The difference of immune infiltration between acne group and healthy group (marked green in healthy group and red in acne group). MCC = maximal clique centrality, MNC = maximum neighborhood component, MCODE = molecular complex detection, PPI = protein-protein interaction.

CXCL8, ESR1, IL1β, MMP1, MMP3, and SPP1 (Fig. 8E). Then, the selected active components (including quercetin, wogonin, naringin, nobiletin and licochalcone a) were docked with the core therapeutic targets (CXCL8, ESR1, IL1β, MMP1, MMP3, and SPP1) by AutodockVina software. The higher the binding activity of small molecular compounds to the core target protein receptor is, the lower the binding energy is. The docking results showed that the binding energies of 5 small molecular

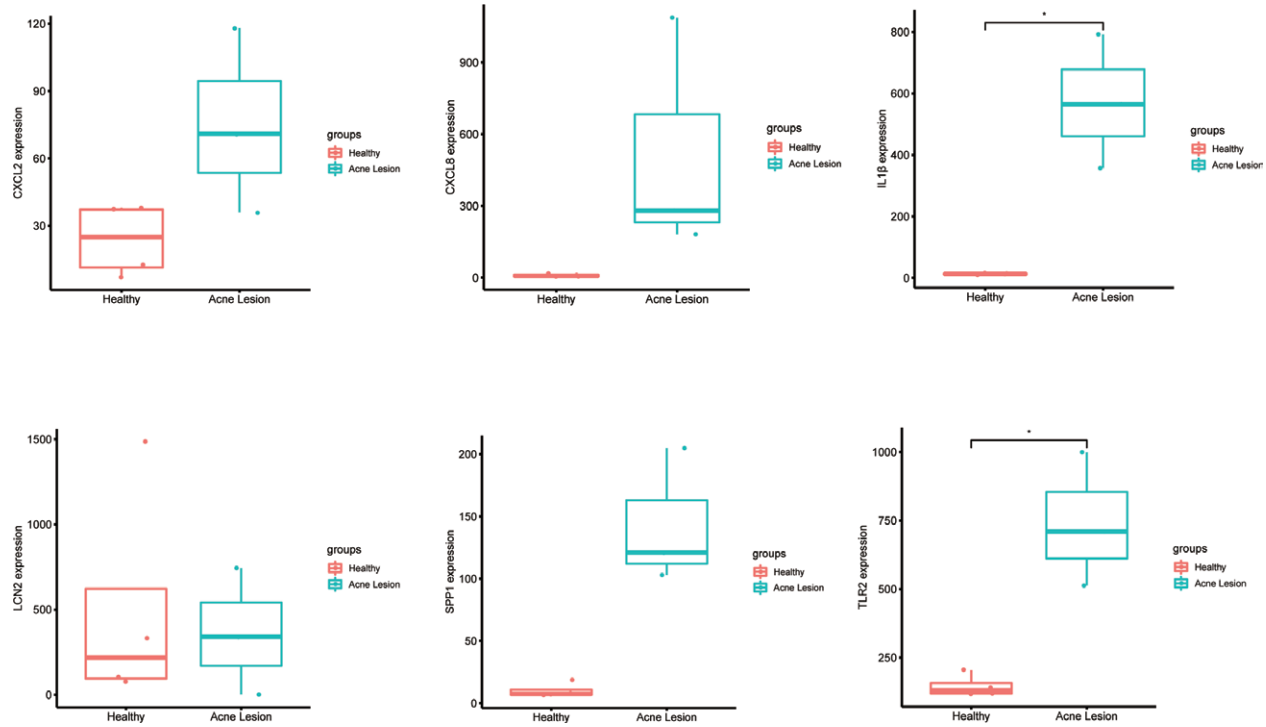

Figure 6. Verification of 6 core expressed genes using GEO dataset. The expression of 6 core genes was verified by using a data set in GEO database. Dataset: GSE122592. The expression of all core genes in acne skin was significantly higher than that in healthy skin ($P < .05$). GEO = gene expression omnibus.

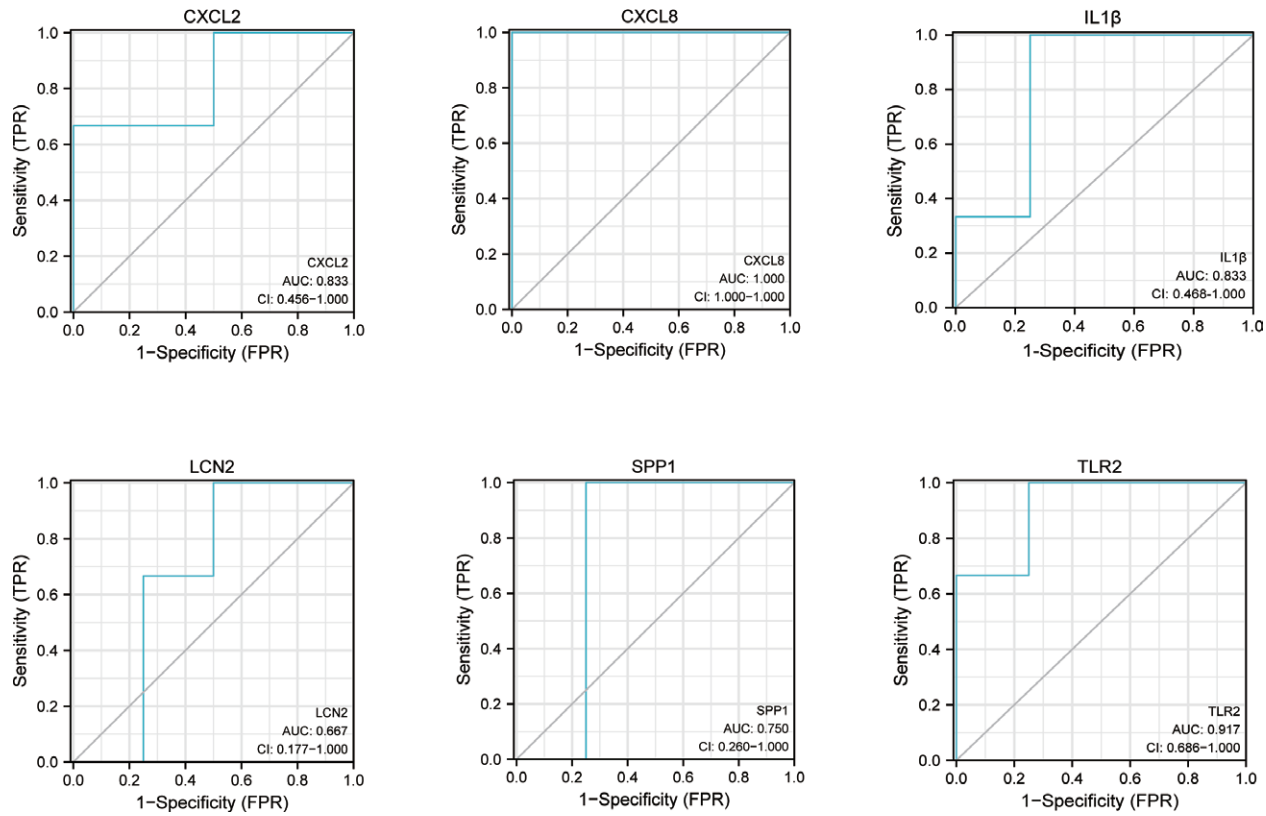

Figure 7. Subject working characteristic curves of six core expressed genes. The receiver working characteristic curve of six core genes, and AUC represents the area under the subject working characteristic curve. AUC = area under curve.

compounds to 6 core target protein receptors were all < −5kcal/mol (Table 4). The results of molecular docking are shown in 2D images and 3D structures (Fig. 9).

4. Discussion

Acne is a chronic inflammatory disease of the sebaceous glands of hair follicles, which is the most common skin disease. Studies

Table 2

Some active compounds of JHO.

Herb	Coding	Active compounds	OB (%)	DL
Licorice	MOL000098	Quercetin	46.43	0.28
Licorice	MOL000497	Licochalcone a	40.79	0.29
Licorice	MOL004328	Naringenin	59.29	0.21
Licorice	MOL000354	isorhamnetin	49.60	0.31
Licorice	MOL000359	sitosterol	36.91	0.75
Atractylodis	MOL000173	wogonin	30.68	0.23
Atractylodis	MOL000179	2-Hydroxyisoxypropyl-3-hydroxy-7-isopentene-2,3-dihydrobenzofuran-5-carboxylic	45.2	0.2
Atractylodis	MOL000184	NSC63551	39.25	0.76
Atractylodis	MOL000186	Stigmasterol 3-O-beta-D-glucopyranoside_qt	43.83	0.76
Atractylodis	MOL000188	3β-acetoxyatractylone	40.57	0.22
Rheum officinale	MOL002235	EUPATIN	50.8	0.41
Rheum officinale	MOL002251	Mutatochrome	48.64	0.61
Rheum officinale	MOL002259	Physciondiglucoside	41.65	0.63
Rheum officinale	MOL002260	Procyanidin B-5,3'-O-gallate	31.99	0.32
Rheum officinale	MOL002268	Rhein	47.07	0.28
Phellodendron Chinense	MOL001454	Berberine	36.86	0.78
Phellodendron Chinense	MOL001458	Coptisine	30.67	0.86
Phellodendron Chinense	MOL002636	Kihadalactone A	34.21	0.82
Phellodendron Chinense	MOL013352	Obacunone	43.29	0.77
Phellodendron Chinense	MOL002641	Phellavin_qt	35.86	0.44
Curcumaelongae rhizoma	MOL000449	Stigmasterol	43.83	0.76
Curcumaelongae rhizoma	MOL000493	Campesterol	37.58	0.71
Curcumaelongae RHIZOMA	MOL000953	CLR	37.87	0.68
Angelica dahurica	MOL001494	Mandenol	42.00	0.19
Angelica dahurica	MOL001939	Alloisoimperatorin	34.8	0.22
Angelica dahurica	MOL001941	Ammidin	34.55	0.22
Angelica dahurica	MOL001942	Isoimperatorin	45.46	0.23
Angelica dahurica	MOL001956	Cnidilin	32.69	0.28
Arisaematis rhizoma	MOL013146	8,11,14-Docosatrienoic acid, methyl ester	43.23	0.3
Arisaematis rhizoma	MOL013156	[(2R)-2-[[[(2R)-2-(benzoylamino)-3-phenylpropanoyl] amino] methyl]-3-phenylpropyl] acetate	38.88	0.56
Arisaematis rhizoma	MOL001510	24-epicampesterol	37.58	0.71
Arisaematis rhizoma	MOL000358	Beta-sitosterol	36.91	0.75
Magnolia officinalis	MOL005970	Eucalyptol	60.62	0.32
Magnolia officinalis	MOL005972	OBOVATOL	69.45	0.18
Magnolia officinalis	MOL005980	Neohesperidin	57.44	0.27
Citrus reticulata	MOL005100	5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl) chroman-4-one	47.74	0.27
Citrus RETICULATA	MOL005815	Citromitin	86.9	0.51
Citrus reticulata	MOL005828	Nobiletin	61.67	0.52
Trichosanthis radix	MOL004355	Spinasterol	42.98	0.76
Trichosanthis radix	MOL006756	Schottenol	37.42	0.75

JHO = Jinhuang ointment, OB = oral bioavailability.

Table 3

Number of active compounds and number of targets per herb in JHO.

Herb name	Number of active compounds	Number of targets
Arisaematis rhizoma	7	42
Atractylodis	9	49
Licorice	92	187
Citrus reticulata	5	56
Trichosanthis radix	2	3
Angelica dahurica	23	49
Curcumaelongae rhizoma	3	26
Magnolia officinalis	3	33
Rheum officinale	16	55
Phellodendron Chinense	38	178

JHO = Jinhuang ointment.

have found that acne lesions initially develop after abnormal desquamation of keratinocytes in the sebaceous gland hair follicles, resulting in hyperkeratosis and microacne formation. With the arrival of puberty, the secretion of androgen in the body increases, which stimulates the production of sebum in hair follicles, thus promoting the pathological process of acne. Excessive keratinization of the skin and increased secretion of androgens lead to the overreproduction of Propionibacterium acne, which in turn leads to a variety of immune inflammatory responses and chemokines.Early diagnosis and treatment of acne can effectively prevent further skin damage and reduce the formation of acne marks and scars. However, due to the lack of effective biomarkers, the diagnosis and treatment of early acne is difficult to be targeted. Although JHO is a classic clinical prescription, the molecular mechanism of it in the treatment of acne is not clear. Therefore, looking for new and effective biomarkers and exploring the mechanism of JHO in the treatment of acne are very important for the early diagnosis and treatment of acne.

In our study, we identified 202 DEGs by comparing the expressed genes in acne skin samples and healthy skin samples. GO enrichment analysis of all genes and acne final targets showed that the immune inflammatory response of acne samples was stronger than that of healthy samples. Immune infiltration analysis showed that the proportion of MacrophagesM0, Mastcells activated and Neutrophils in acne skin samples was higher than that in healthy skin samples. In addition, the final target of acne and the enrichment analysis of KEGG pathway, the potential target of JHO in the treatment of acne, include cytokine-cytokine receptor interaction, Toll-likereceptorsignalingpathway, IL-17signalingpathway and other inflammatory pathways. The network pharmacological analysis also shows that the treatment of acne with JHO is mainly achieved by reducing the

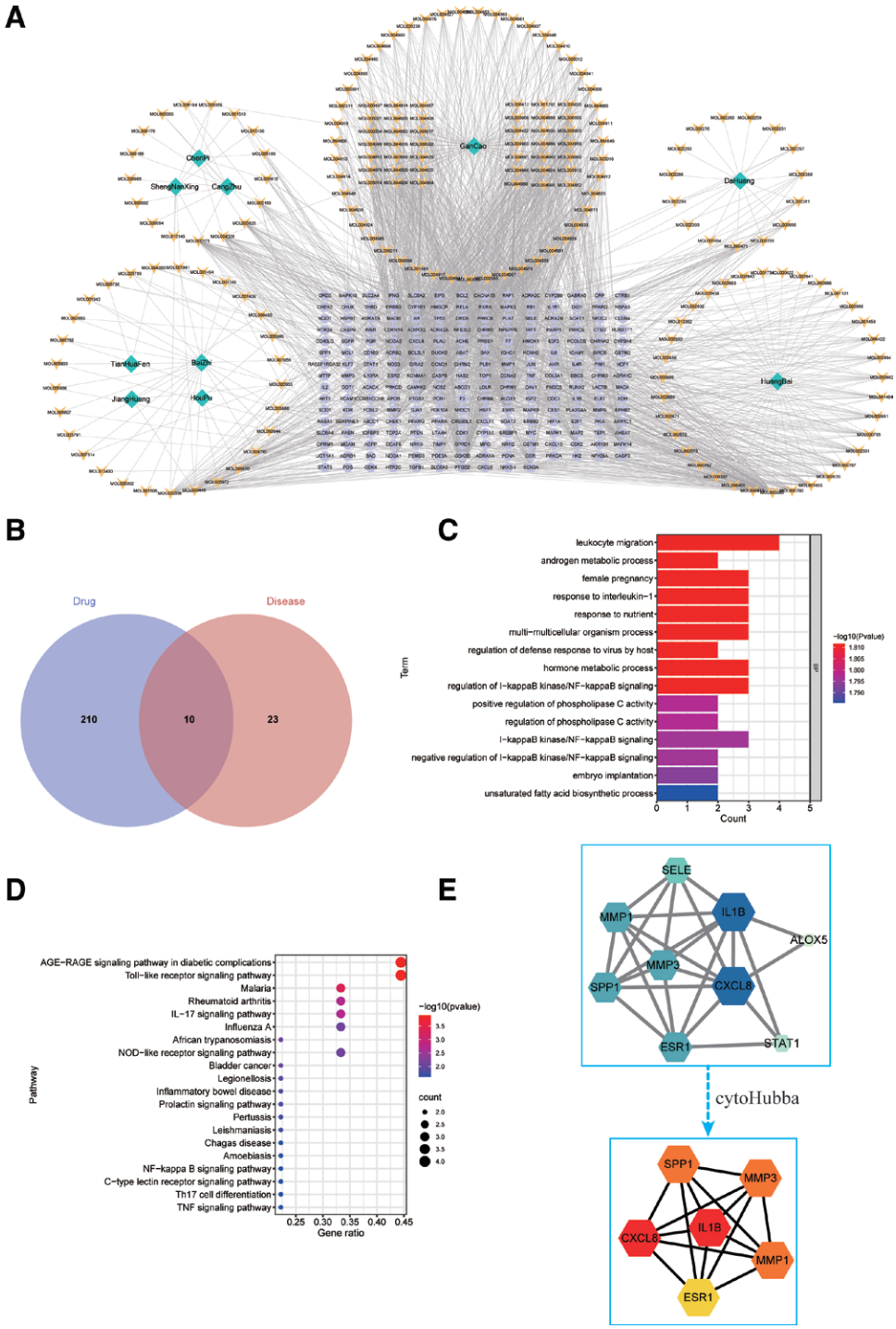

Figure 8. Potential target prediction and enrichment analysis of JHO in the treatment of acne. (A) "Herb-active ingredient-target" network, with green diamond representing drugs, yellow arrows representing active components, and blue circles representing targets. Prediction of potential targets for (B) JHO treatment of acne. (C) KEGG enrichment analysis of potential therapeutic targets. (D) GO enrichment analysis of potential therapeutic targets. (E) PPI network of potential therapeutic targets and screening of core therapeutic targets. GO = gene ontology, JHO = Jinhuang ointment, KEGG = Kyoto encyclopedia of genes and genomes, PPI = protein-protein interaction.

expression of inflammatory factors, regulating skin cell metabolism and improving cellular immune microenvironment. Using GSE122592 dataset to verify PPI network, 6 core genes were screened. ROC curve analysis shows that these genes are of high value in the diagnosis of early acne. Combined with network pharmacology, it is found that 3 core therapeutic targets of JHO in the treatment of acne coincide with the identified 6 biomarkers, they are CXCL8, IL1β and SPP1. The expressions of CXCL8, interleukin-1β (IL-1β), and SPP1 were all up-regulated in acne diseases. Therefore, according to our current samples, we speculate that IL1β and CXCL8, TLR2, CXCL2, LCN2, and SPP1 may be biomarkers for early diagnosis of acne, while CXCL8, IL-1β, and SPP1 may be the key targets for JUO in the treatment of acne.

Chemokine CXCL8, also known as IL-8, is a member of the chemokine family, which is mainly secreted by macrophages and plays an important role in activating and regulating immune cells, inflammatory response, neutrophil chemotaxis

Table 4

Docking binding energy of core target proteins with active component.

Gene ID	PDB ID	Affinity-Quercetin (kcal/mol)	Affinity-Wogonin (kcal/mol)	Affinity-Naringin (kcal/mol)	Affinity-Nobiletin (kcal/mol)	Affinity-Licochalcone a (kcal/mol)
CXCL8	6lfl	−7.0	−6.0	−6.7	−5.8	−7.6
ESR1	7baa	−7.8	−7.3	−7.3	−6.4	−7.2
IL1β	4gai	−8.3	−7.6	−7.6	−5.5	−6.3
MMP1	1hfc	−9.2	−8.2	−8.6	−6.6	−8.1
MMP3	4wk7	−8.6	−8.7	−8.6	−7.4	−8.6
SPP1	2wbn	−8.1	−7.6	−7.2	−6.6	−7.4

SPP1 = secretory phosphoprotein 1.

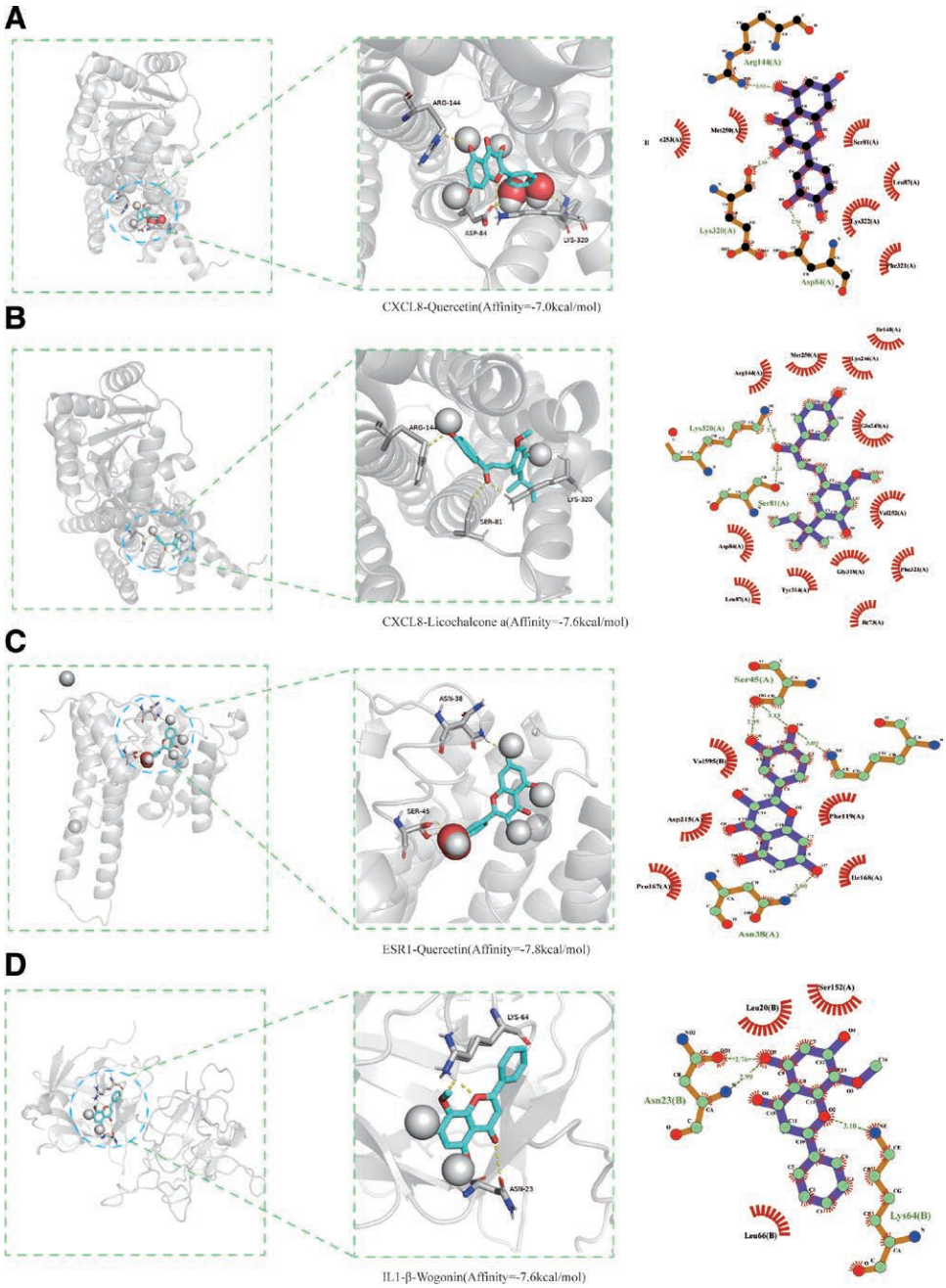

Figure 9. The six core targets of JHO in the treatment of acne dock with the molecules of five active ingredients. JHO = Jinhuang ointment.

and angiogenesis.[26–28]A number of studies have reported that compared with the expression level of CXCL8 in healthy people, the expression level of CXCL8 in skin tissue of patients with acne is increased.[29,30] Grange et al[31] also proved that the expression of CXCL8 in sebaceous cells of acne patients was stronger than that of healthy controls. It is suggested

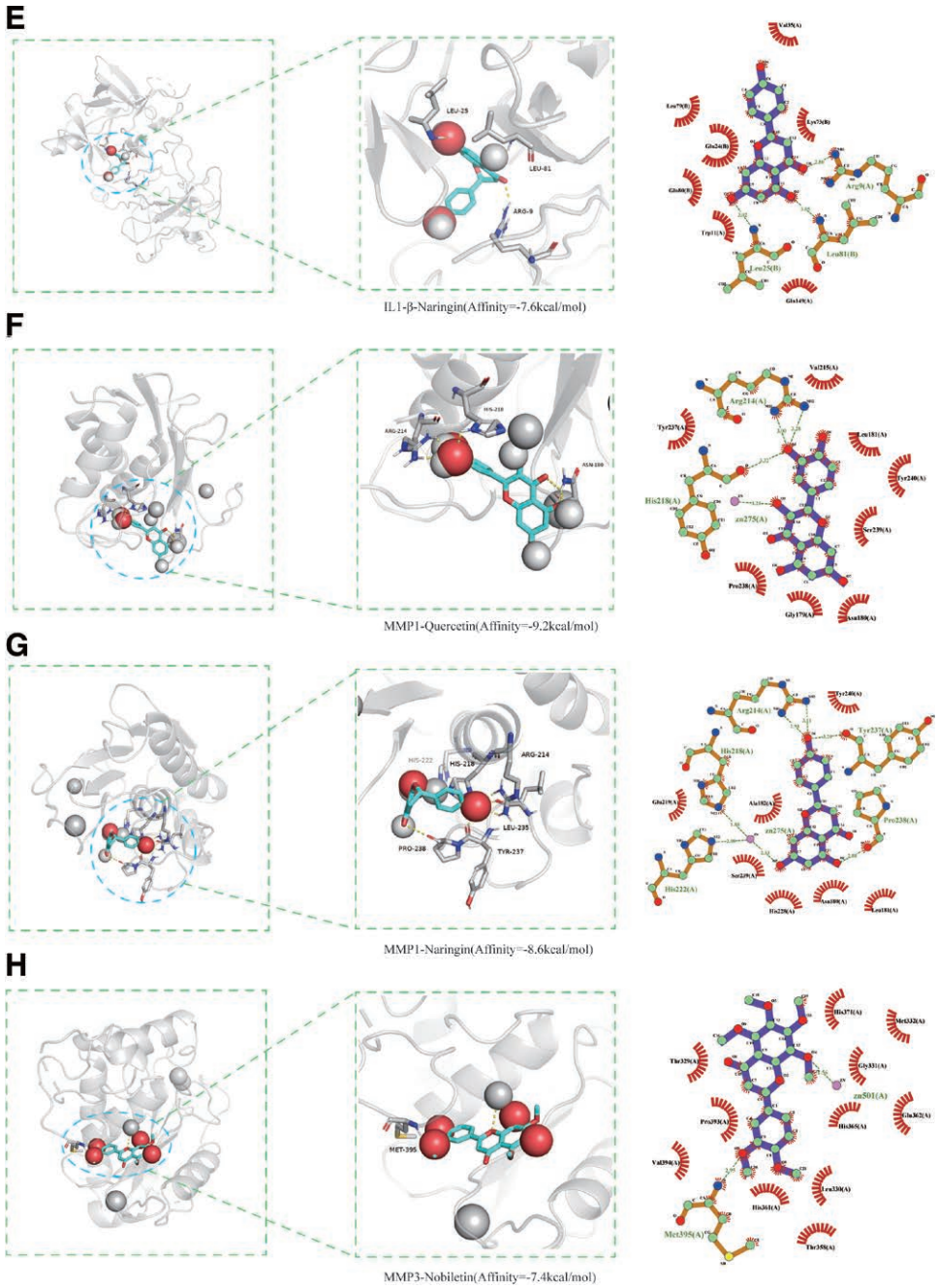

Figure 9. Continued

that CXCL8 plays an important role in the pathogenesis of acne. Consistent with this study, our study found that CXCL8 expression was up-regulated in acne skin samples. CXCL8's ROC curve shows that it has high diagnostic value for acne diseases (AUC = 1.000). We believe that CXCL8 is a very effective biomarker for the diagnosis of acne. Through skin biopsy, it was found that the increased expression of CXCL8 was closely related to epidermal hyperplasia and hyperkeratosis of hair follicles in inflammatory acne vulgaris.[32] Recent studies have shown that CXCL8 gene polymorphism plays an important role in cell metabolism in the pathogenesis of acne.[33] In addition, through the network pharmacological analysis of JHO, it is found that CXCL8 is also the core target of JHO in the treatment of acne, so we speculate that CXCL8 plays a very important role in the diagnosis and treatment of acne.

IL-1β is an important pro-inflammatory cytokine, mainly released by activated T lymphocytes and macrophages.[34] IL-1β plays a very important role in the inflammatory cascade of acne.[35] The overreproduction of Propionibacterium acne is one of the main factors leading to inflammation. When Pseudomonas acne leaves the hair follicle sebaceous glands, contact with the myeloid cells around the hair follicles causes cell infiltration and the release of IL-1β, which induces inflammation around the hair follicles where neutrophils gather.[36] In the current study, the immunohistochemical expression of IL-1β was positive in all patients with acne.[37] In our study, the expression of IL-1β was up-regulated in acne skin samples. At the same time, the study of Kelhletal et al[38] also found a significant increase of Th17 type cytokines including IL-1β in the skin of patients with acne. In addition, network pharmacological analysis showed that IL-1β was the core target of JHO in the treatment of acne.

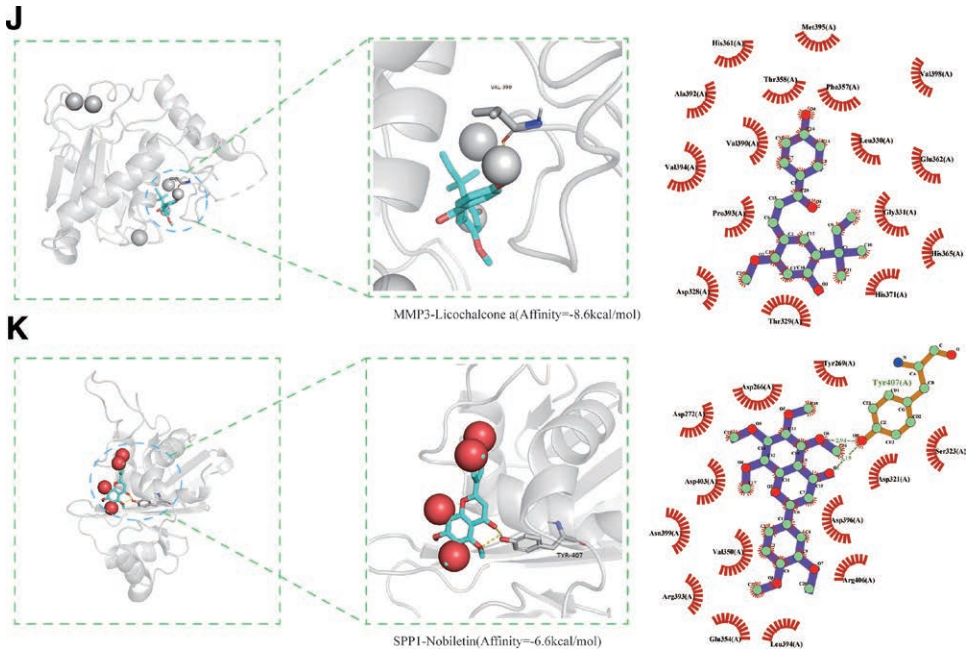

Figure 9. Continued

Molecular docking showed that IL-1β had good docking activity with the main active components of JHO. Therefore, IL-1β may play a key role in the pathogenesis and treatment of acne.

SPP1, also known as osteopontin, is a member of the small integrin binding ligand N-junction glycoprotein family, which is expressed in a variety of cells and tissues.[39] Recent studies have found that SPP1 exists in basal keratinocytes, hair follicles and sebaceous glands.[40] SPP1 can enhance the inflammatory response mediated by Th17 and Th1 by stimulating dendritic cells and macrophages, which plays an important role in the regulation of immune response.[41] In addition, studies have shown that SPP1 can induce angiogenesis and participate in wound repair and healing.[42] In our study, we found that SPP1 is not only highly expressed in acne skin samples, but also is the core target of JHO in the treatment of acne like CXCL8 and IL-1-β. Therefore, we speculate that SPP1 not only has high diagnostic value (AUC = 0.750) for acne, can be used as an early biomarker for the diagnosis of acne, but also plays a very important role in the pathological process and treatment of acne.In addition, the mechanism of cytokine release activated by Pseudomonas acne is through a pathway dependent on TLR2.[43] TLR2 is a transmembrane protein that plays a key role in various innate immune responses.[44] A variety of immune cells, including macrophages, neutrophils and dendritic cells, can express TLR2. The activation of TLR2 promotes the production of pro-inflammatory cytokines and chemokines. It is reported that activated TLR2 is expressed in skin keratinocytes, monocytes and macrophages, and its expression is activated by Pseudomonas acne.[45,46] Studies have found that by stimulating TLR2, the immune system can identify invasive microorganisms and induce the secretion of cytokines/ chemokines in the pathological process of acne. This leads to excessive inflammation in patients with acne, making the lesions visible and, in severe cases, scarring. Therefore, regulating the expression of TLR2 may provide a feasible target for the treatment of acne.[47,48]

In the network pharmacological analysis of JHO, we found that quercetin, wogonin, naringenin, nobiletin and licochalcone a an are the main components of JHO in the treatment of acne through the topological analysis of drug-active ingredient-target network. Quercetin is a kind of flavonoids, which exists widely in many plants and has a variety of biological activities, including anti-inflammatory, antioxidant, anticancer and antivirus.[49] Lim et al[50] found that quercetin could significantly inhibit the production of pro-inflammatory cytokines and chemokines IL-1β, IL-8, IL-6, and TNF-α in HaCaT and THP-1 cells stimulated by Pseudomonas acne. In this study, through molecular docking, we found that the docking binding energy of quercetin to IL-1-β is –8.3kcal/mol and to CXCL8 is –7.0kcal/mol. Quercetin has good binding activity with both of them, which is consistent with other studies. In addition, the antioxidant function of quercetin contributes to skin care and appearance.[51]Wogonin, also a flavonoid, has been shown to inhibit bacterial growth and inflammation.[52] Previous studies have shown that wogonin has been proved to be the best anti-acne compound in Coptis chinensis[53] through in vivo and in vitro experiments. Recently, wogonin has been found to significantly inhibit the expression of IL-1β and IL-8 in THP-1 cells induced by Pseudomonas acne, alleviate the inflammatory response induced by Pseudomonas acne, and has strong anti-acne activity.[54] Wogonin can also inhibit the phosphorylation of MAPKs and the translocation of nuclear factor-kB.[54]Naringenin is a polyphenol phytochemical, which is widely distributed in citrus fruits. Naringenin has anticancer, antioxidant and anti-inflammatory pharmacological properties.[55] Although there is no direct evidence that naringin can treat acne, Martinez et al[56] found that naringenin can reduce skin inflammation by inhibiting skin edema, neutrophil recruitment, MMP-9 activity and the release of pro-inflammatory factors TNF-α, IFN-γ, IL-1 β, IL-6, IL-12, and IL-23. At the same time, naringin can also inhibit oxidative stress by reducing the production of superoxide anions.Nobiletin is the main component of cruciferous citrus, which has anti-adipogenesis, anti-proliferation, anti-acne and anti-inflammatory effects, and anti-proliferation and anti-inflammatory effects on sebaceous glands and acne infiltrating inflammatory cells.[57] Some studies have shown that nobiletin is a new type of anti-acne drug with unique therapeutic effect, which can not only inhibit fat production and inflammatory cell proliferation in sebaceous glands, but also promote sebum consumption by increasing sebum excretion.[58] Licochalcone a is a chalcone compound isolated from licorice. It is reported that it can inhibit IL-1β-mediated inflammation and has anti-inflammatory activity.[59] Yang et al[60] found that licochalcone a has many inhibitory effects on acne inflammation. On the one hand, licochalcone a can resist Pseudomonas acne and slow down bacterial proliferation, on

the other hand, it can inhibit the expression of NLRP3 inflammatory bodies. In short, the main active components of JHO can regulate a variety of biological mechanisms, so as to inhibit the biological activity of Pseudomonas acne, regulate sebum metabolism, reduce inflammatory response, so as to prevent or alleviate the development of acne.

The immune cells that make up the skin epidermal innate immune system mainly involve neutrophils, macrophages and mast cells. Through immune infiltration analysis, we found that in acne skin samples, the proportion of macrophage M0 and M1 increased, while the proportion of macrophage M2 decreased. Macrophages have both protective and pathogenic functions in the skin, in which macrophage M1 can promote inflammation and antimicrobials, macrophage M2 can promote wound healing, and M0 is immature macrophages.[61] KEGG enrichment analysis of acne final disease target and KEGG enrichment analysis of acne potential target of JHO treatment both involve Toll-like receptor, IL-17, Nod-like receptor signaling pathway and NF-κB signaling pathway. It shows that these 3 pathways play a very important role in the pathogenesis and treatment of acne. The innate immune system recognizes pathogens through pathogen-associated molecular patterns (PAMP), which are targeted. The receptors responsible for recognizing PAMP are called pattern recognition receptors (PRRs), which mainly include Toll-like receptors and Nod-like receptors.[62,63] After PAMP recognition, PRRs activates specific signal pathways, such as Toll-like receptor signal pathway and Nod-like receptor signal pathway, and participates in immune inflammatory response.[64]It has been found that acellular extract of Pseudomonas acne can activate NF-κB pathway in human sebum cells and up-regulate the secretion of pro-inflammatory cytokines through TLR2-dependent Toll-like receptor signal pathway.[65]Interleukin 17 is secreted by Th17 cells and can activate related signaling pathways to aggravate the severity of acne.[38,66] He et al[67] proved that Th17 cells based on IL-17 signal pathway play a key role in sebaceous lipoprotein metabolism and acne development. Inhibition of IL-17 signal pathway may be a new method for the treatment of acne.This is consistent with the results of this study. These results may be of great significance to further study and determine the molecular target of JHO in the treatment of acne, the pathogenesis of acne and the application in the development of new drugs for acne treatment. However, there are still some shortcomings in this study. In the later stage, in vivo and in vitro experiments, metabonomics and pathway verification are needed to further identify the biomarkers of acne and the mechanism of JHO in the treatment of acne.

5. Conclusion

In this study, we systematically described the possible biomarkers of acne and the active components and molecular mechanism of JHO in the treatment of acne. Through transcriptome analysis, the possible biomarkers of acne: IL-1 β and CXCL8, TLR2, CXCL2, LCN2, and SPP1 were screened and verified by GEO data set. The main active component of JHO, quercetin, wogonin, naringin, nobiletin, and licochalcone a, was screened by network topology analysis, and then docked with the core therapeutic target (CXCL8, ESR1, IL-1 β, MMP1, MMP3, and SPP1) selected by PPI. These core therapeutic targets have stable binding activity with the main active components. In addition, we believe that CXCL8, IL-1β and SPP1 are not only possible biomarkers of acne, but also core targets of JHO in the treatment of acne. Acne DEGs enrichment analysis and core treatment target enrichment analysis showed that both of them were involved in IL-17, Nod-like receptor, Toll-like receptor and NF-κB signal pathway. Therefore, we infer that CXCL8, IL-1β, and SPP1 may play a key role in the pathogenesis of acne. JHO may regulate IL-17, Nod-like receptor, Toll-like receptor and NF-κB signaling pathways by targeting CXCL8, IL-1β and SPP1, which in turn regulates skin cell metabolism, reduces local inflammatory response, improves cellular immune microenvironment as well as local skin vascular microcirculation, thus achieving the purpose of acne treatment. It is the result of the synergistic effect of multi-components, multi-targets and multi-signaling pathways.

Author contributions

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Formal analysis: Minghui Li.
Investigation: Xue Gao.
Project administration: Tianai Miao.
Software: Tianai Miao.
Supervision: Hongfeng Sun.
Visualization: Xue Gao, Tianai Miao.
Writing – original draft: Minghui Li.
Writing – review & editing: Hongfeng Sun.

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