Breast Cancer: Targets and Therapy

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ORIGINAL RESEARCH

Network Pharmacology-Oriented Identification of Key Proteins and Signaling Pathways Targeted by Xihuang Pill in the Treatment of Breast Cancer

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Purpose: The compound traditional Chinese medicine Xihuang pill (XHP) has been adopted to treat breast cancer (BC) for centuries, but its specific mechanism of action is unclear.

Materials and Methods: The active ingredients and related targets of XHP were screened using the TCMSP and TCMID databases. GSE139038 was downloaded from the GEO database, and differentially expressed genes (DEGs) were analyzed. The intersection of targets and DEGs were chosen to build an ingredients–target genes network. Protein–protein interaction network construction and functional enrichment analysis of target genes were conducted.

Results: A PPI network of 37 targets was constructed, and seven core nodes (FOS, MYC, JUN, PPARG, MMP9, PTGS2, SERPINE1) were identified. Functional enrichment analysis revealed that the aforementioned targets were mainly enriched in the IL-17, toll-like receptor, and tumor necrosis factor signaling pathways, which are deeply involved in the inflammatory microenvironment of tumors.

Conclusion: This network pharmacology study indicated that XHP can inhibit the development of BC by targeting a variety of proteins and signaling pathways involved in the inflammatory microenvironment.

Keywords: traditional Chinese medicine, Xihuang pill, breast cancer, network pharmacology

Introduction

Breast cancer (BC) is the most commonly diagnosed cancer among women worldwide.[1] Thanks to early detection, the mortality rate of BC has decreased even though its incidence has actually increased.[2] It is estimated that more than 90% of cancer-related deaths are caused by metastasis.[3–5] Metastasis involves both cancer cells and other cells (eg, stromal cells, immune cells) in the tumor microenvironment. These cells secrete various cytokines and/or growth factors, which in turn affect the progression of BC through various mechanisms.[6] Inflammation can influence the progression, metastasis, and outcome of BC by establishing favorable immune microenvironments.[7,8] Therefore, targeting inflammatory pathways could be helpful in the development of novel prevention and therapeutic strategies.

Xihuang pill (XHP) is a well-known compound traditional Chinese medicine (TCM) with anti-cancer activity. It has been used to treat BC, furunculosis, and scrofula and to relieve swelling and pain.[9] A meta-analysis revealed that XHP

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combined with chemotherapy could significantly enhance the tumor response and alleviate toxicity induced by chemotherapy in patients with BC.[10] Studies illustrated that XHP can inhibit growth and induce apoptosis in BC cells (MCF-7, MDA-MB-231).[9] XHP induced Bcl-2–independent apoptosis and S phase arrest in Hs578T cells.[11] XHP induced apoptosis in estrogen receptor-negative BC cells, possibly by upregulating the mRNA expression of TP53, which induced the expression of the apoptotic protein Bax.[12] XHP contains Niuhuang (Calculus Bovis), Shexiang (Moschus), Ruxiang (Olibanum), and Moyao (Myrrh). Modern pharmacological studies illustrated that Ruxiang and Moyao have strong anti-inflammatory effects.[13] Network pharmacology analysis indicated that XHP might target the IL-17, Toll-like receptors (TLRs), and tumor necrosis factor (TNF) signaling pathways. These signaling pathways are closely related to immunity and inflammation. Exploration of the molecular mechanism of XHP is of great significance for promoting its clinical application.

Materials and Methods

Identification of the Differentially Expressed Genes (DEGs) of BC

The gene expression dataset GSE139038 was downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). GSE139038 was based on the GPL27630 Print_1437 platform. GSE139038 contains 65 samples including 41 breast tumors (24 early stage, 17 locally advanced), 18 adjacent normal tissues (paired normal), and 6 apparently normal tissues from breasts in patients who underwent surgery for non-malignant conditions. R packages ("limma" and "pheatmap") were used to analyze GSE139038. DEGs between normal tissue and breast tumors were detected on the basis of the criteria adjusted P-value < 0.05 and |logFC| > 1.

Screening the Chemical Ingredients and Related Targets of XHP

The ingredients of XHP and related targets were screened using the TCMSP (http://tcmspw.com/index.php) and TCMID databases (http://119.3.41.228:8000/tcmid/). Ingredients meeting the criteria oral bioactivity (OB) ≥30% and drug-likeness (DL) ≥0.18 were selected to identify related targets.

Ingredients–Targets Network Construction

The common genes between ingredients-related targets and the DEGs of BC were collected and sorted. The ingredients–targets network was visualized using Cytoscape software (www.cytoscape.org/).[14]

Protein–Protein Interaction (PPI) Network Construction and Analysis

The STRING database (http://string-db.org/) is designed to analyze PPI information. To evaluate potential PPIs, DEGs were uploaded to the STRING database. The PPI pairs were extracted with a combined score >0.4. Subsequently, the PPI network was visualized using Cytoscape software. Nodes with a higher degree of connectivity tend to be more essential in maintaining the stability of the entire network. CytoHubba, an app plugin in Cytoscape, was used to calculate the degree of each protein node.[15] In our study, degree, betweenness, and closeness were used to determine the degree of connectivity. The intersections of the top 10 genes in each method were regarded as core proteins.

Functional Enrichment Analysis

R packages ("clusterProfiler," "org.Hs.eg.db," "enrichplot," and "ggplot2") were used to conduct biological process (BP), cellular component (CC), molecular function (MF), and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses (p-value cutoff = 0.05, q-value cutoff = 0.05).

Results

Identification of the DEGs of BC

The GSE139038 samples were separated into normal and tumor group (24 and 41 samples, respectively). In total, 1464 DEGs were identified, including 990 downregulated and 474 upregulated genes, as presented in the volcano map (Figure 1) and heatmap (Figure 2).

Screening of the Active Ingredients and Targets of XHP

In total, 46 ingredients and 209 targets (Table S1) of XHP were identified using the TCMSP and TCMID databases. Targets and DEGs were integrated to form an ingredients–targets network using Cytoscape software. The integrated network contained 37 molecules and 37 target proteins (Figure 3).

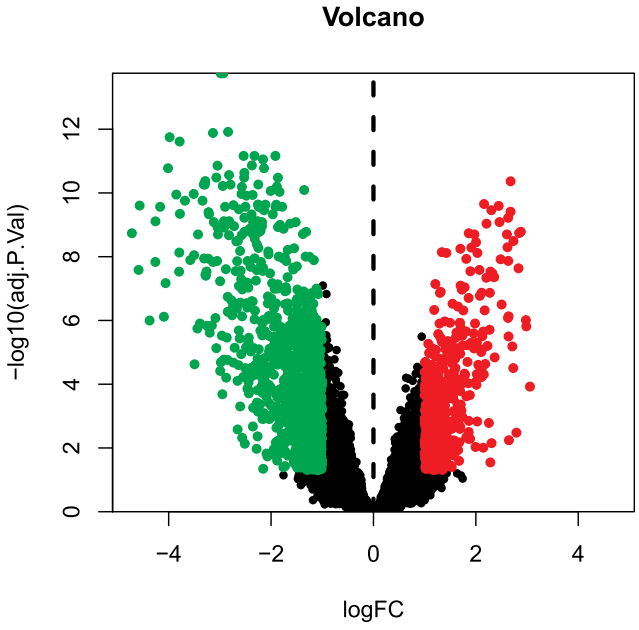

Figure 1 Volcano map of differentially expressed genes (DEGs). The red and green dots indicate DEGs that were significantly upregulated (474 genes) and downregulated (990 genes), respectively (adjusted p-value < 0.05, and |logFC| > 1).

PPI Network Construction

The aforementioned 37 targets were mapped to the STRING database to construct the PPI network (Figure 4). The PPI pairs were extracted with a combined score >0.4, and disconnected nodes in the network were deleted. The network contained 36 nodes and 151 edges, and the PPI enrichment p-value was <1.0e−16. The CytoHubba plugin was used to located hub nodes according to degree, betweenness, and closeness (Tables 1–3). The intersections of the top 10 nodes in each method were identified as core targets (FOS, MYC, JUN, PPARG, MMP9, PTGS2, SERPINE1). The PPI network (Figure 5) of core targets has significantly more interactions than expected (enrichment p-value = 1.13e−07).

Functional Enrichment Analysis

R packages were adopted to conduct functional enrichment analysis of the 37 targets. BP analysis indicated that targets were significantly enriched in response to lipopolysaccharide (GO: 0032496), response to molecule of bacterial origin (GO: 0002237), and response to purine-containing compound pathways (GO: 0014074; Figure 6). CC analysis revealed that the targets were mainly enriched in RNA polymerase II transcription factor complex (GO: 0090575), nuclear transcription factor complex (GO: 0044798), and apical plasma membrane pathways (GO: 0016324; Figure 7). MF analysis indicated that targets were significantly enriched in transcription factor activity, RNA polymerase II proximal promoter sequence-specific DNA binding (GO: 0000982), chemokine receptor binding (GO: 0042379), and activating transcription factor binding pathways (GO: 0033613; Figure 8). To further reveal the potential mechanism of action of XHP in the treatment of BC, KEGG pathway enrichment analysis was conducted.

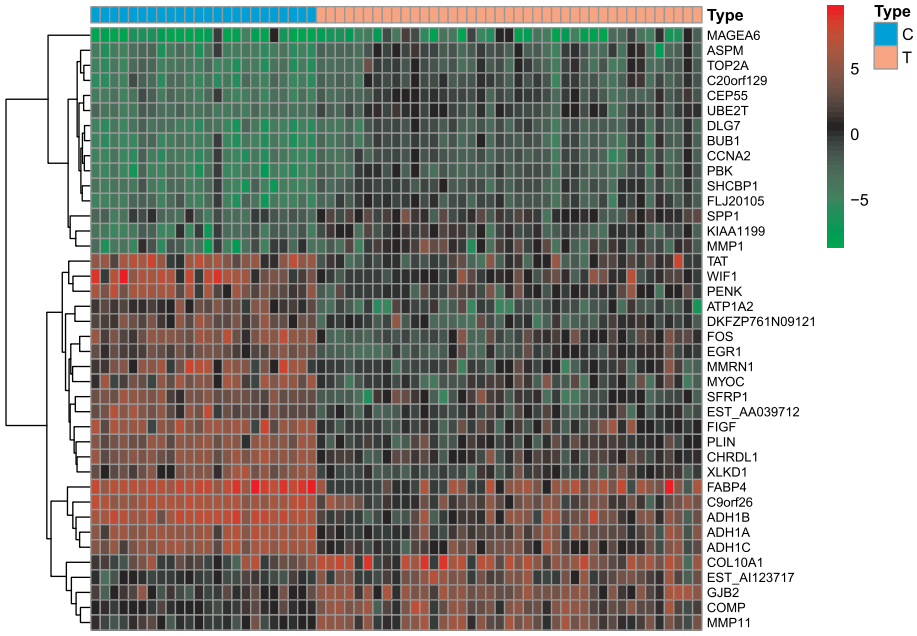

Figure 2 Heatmap of differentially expressed genes. Red indicates that the gene is highly expressed in the sample, and green indicates that the gene has lower expression in the sample.

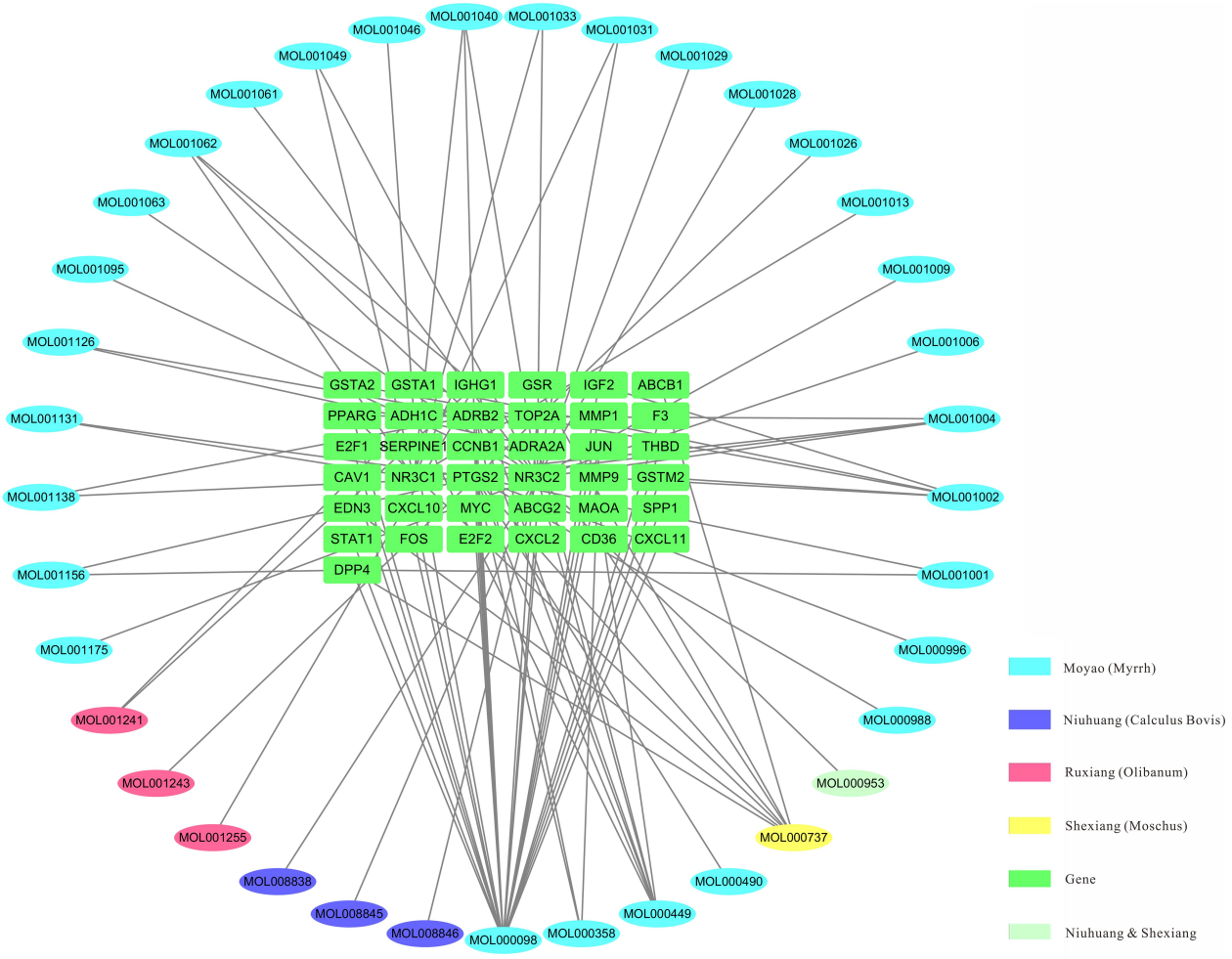

Figure 3 Ingredients–target genes network of Xihuang pill. Ellipse, chemical compounds from different herbs; Green rectangle, target genes.

The IL-17 signaling pathway (hsa04657), TLR signaling pathway (hsa04620), TNF signaling pathway (hsa04668), and BC (hsa05224) were screened using q-value <0.05 (Figure 9). The interaction network between the KEGG pathways and targets is presented in Figure 10.

Discussion

Compound TCMs have the characteristics of "multi-component, multi-pathway and multi-target" synergistic effects, and they have unique advantages in the treatment of a series of complex diseases such as cancer, diabetes, and stroke.[16–18] Network pharmacology has integral and systematic characteristics, and thus, it can effectively explore the basis of medicinal substance and mechanism of action, which has great significance for improving the therapeutic application of compound Chinese medicines.

In recent years, the inhibitory effects of TCM on tumor recurrence and metastasis have attracted increasing attention.[19,20] Relevant studies discovered that TCMs can improve the quality of life of patients with cancer, reduce the risks of tumor recurrence and metastasis, and prolong survival.[21–23] Although a few reports revealed that XHP can inhibit proliferation and induce apoptosis in BC cells,[9,11,12] the mechanism of action is unclear.

We screened 37 chemical molecules in XHP and 37 target proteins. Some molecules targeted multiple proteins, among which seven proteins (FOS, MYC, JUN, PPARG, MMP9, PTGS2, SERPINE1) were identified as core nodes. FOS forms a tight complex (non-covalently) with the transcription factor JUN/AP-1, which plays important roles in the development of BC.[24,25] A recent study also found XHP could regulate the MEKK1/SEK1/JNK1/AP-1 pathway, which confirmed the possible interaction between XHP and the FOS/JUN/AP-1 complex.[26] Chen et al discovered that the overexpression of MMP9 stimulated the invasiveness of BC cell-formed spheroids in vitro and DCIS in vivo, whereas

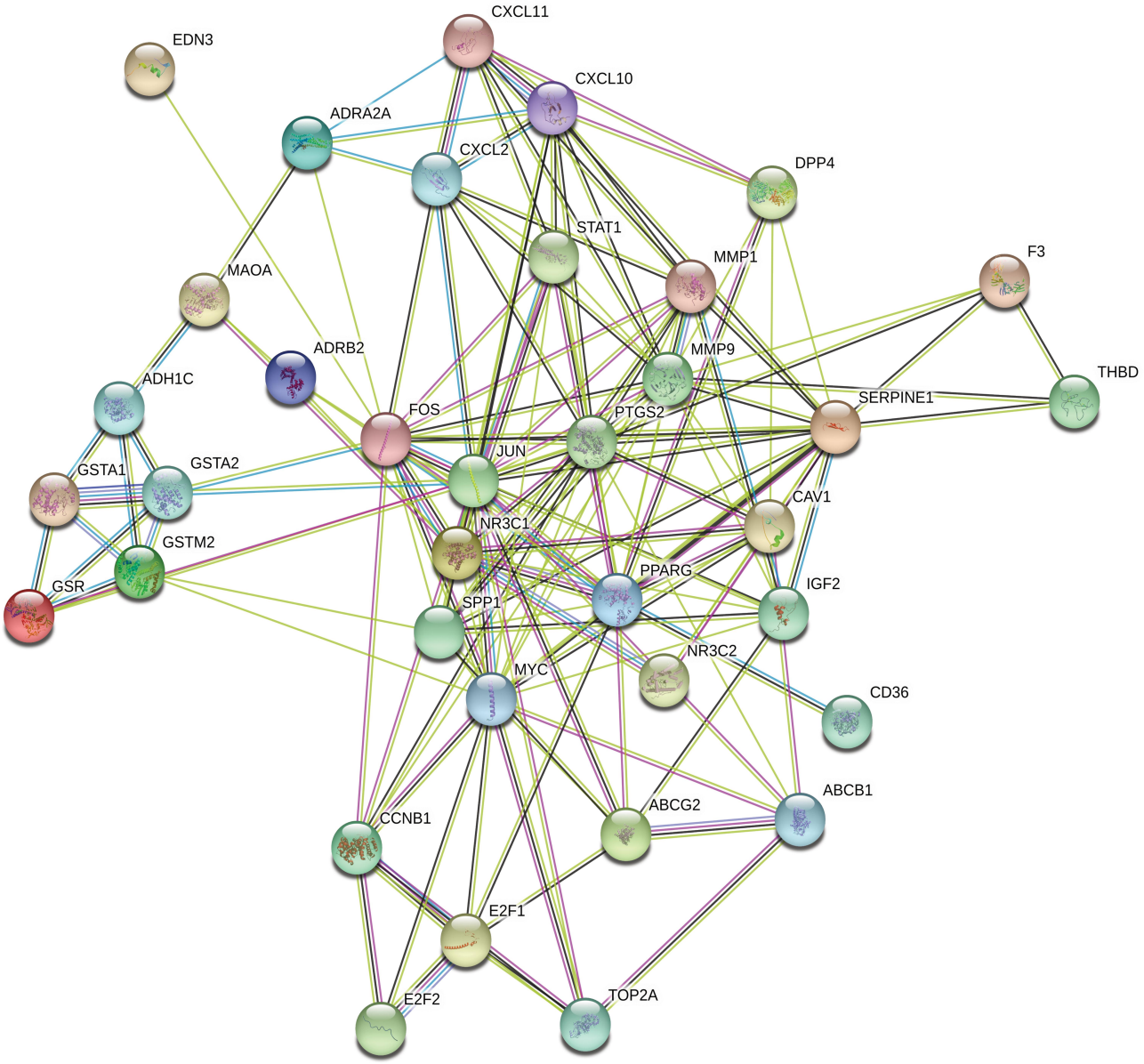

Figure 4 Protein–protein interaction (PPI) network of target genes. The network featured 36 nodes and 151 edges with a combined score >0.4 and PPI enrichment p-value < 1.0e−16.

depletion of MMP9 inhibited their invasiveness.[27] Upregulation of PTGS2 is also associated with increased cell adhesion, phenotypic changes, anti-apoptosis, and tumor angiogenesis.[28] In cancer cells, PTGS2 plays key roles in the production of prostaglandin E2 (PGE2) and development of tumors.[29] A study by Bos et al indicated that COX2 (also known as PTGS2) and other genes mediate BC metastasis to the brain.[30] Nandi et al found that PGE2 promotes lymphangiogenesis associated with BC by activating EP4 receptors on lymphatic endothelial cells.[31] Kochel et al discovered that multidrug resistance-related protein can export PGE2 and promote metastasis in basal/triple-negative BC.[32] SERPINE1 (also known as plasmin activator inhibitor-1) has been demonstrated to be highly expressed in various types of tumor biopsies.[33] More importantly, SERPINE1 and urokinase-type plasminogen activator have been identified as prognostic factors for disease progression and relapse in BC.[33,34]

Tumor cells have the ability to produce cytokines and chemokines by activating transcription factors such as nuclear factor-κB (NF-κB) and interferon regulatory factors (IRFs).[35] These cytokines and chemokines induce the mobilization and reprogramming of immune cells and activate stromal cells in the extracellular matrix adjacent to the

Table 1 Top 10 Genes in the Network Ranked by Degree

Rank	Name	Score
1	JUN	19
1	MYC	19
3	MMP9	18
4	PTGS2	17
4	FOS	17
6	PPARG	16
7	SERPINE1	15
8	SPP1	12
8	MMP1	12
10	CXCL10	11

Table 2 Top 10 Genes in the Network Ranked by Betweenness

Rank	Name	Score
1	FOS	167.0219
2	MYC	161.2401
3	JUN	138.6833
4	PPARG	132.3155
5	MMP9	98.98592
6	PTGS2	88.6258
7	SERPINE1	81.44932
8	NR3C1	72.60143
9	GSTM2	47.24919
10	GSTA2	46.27636

Table 3 Top 10 Genes in the Network Ranked by Closeness

Rank	Name	Score
1	JUN	27
1	MYC	27
3	FOS	26
4	MMP9	26
5	PTGS2	25.83333
6	PPARG	25.16667
7	SERPINE1	24.5
8	SPP1	23.33333
9	MMP1	23
10	NR3C1	22.66667

tumor. These cells in turn produce more cytokines and chemokines, thereby amplifying the entire process. Cytokines and/or growth factors secreted by these immune or stromal cells further regulate the activity of recruited immune cells, leading to the sustenance of a tumor-friendly inflammatory microenvironment.[7,36] Therefore, cytokine and chemokine pathways and the inflammatory tumor microenvironment could be therapeutic targets with great potential. Su et al found that XHP might promote Treg cell apoptosis in the

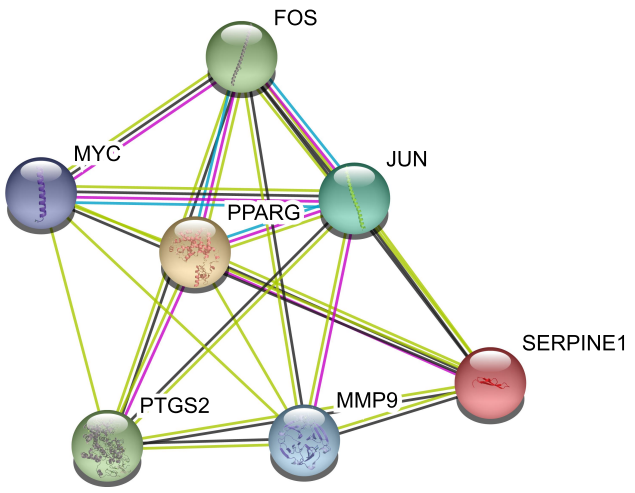

Figure 5 Protein–protein interaction network of core proteins. Seven genes were found in the intersections of the top 10 genes according to degree, betweenness, and closeness.

tumor microenvironment and further inhibit the tumor growth of 4T1 mouse breast cancer cells.[26] Functional enrichment analyses indicated that target proteins were significantly enriched in the IL-17, TLR, and TNF signaling pathways. These pathways are well known to play significant roles in inflammation and tumor development.[35,37–43] It has been reported that IL-17 activates the Src/PI3K/Akt/NF-κB, MAPK, Stat3, and COX-2 pathways, which play significant roles in tumorigenesis, angiogenesis, and metastasis.[44,45] IL-17 is overexpressed in the intratumoral stromal cells of triple-negative BC. Upon overexpression, IL-17 can activate tumor microangiogenesis through its signal transduction pathways, resulting in increased tumor secretion of VEGFA, thereby promoting tumor progression.[46] TLRs trigger multiple signaling pathways involving nuclear factor B, IRFs, and MAPKs to produce various cytokines with important roles in diseases such as cancer.[47] Inhibition of TLR signaling can suppress human BC cell viability, invasion, and migration.[48,49] TNF-α is involved in all stages of BC progression, participating in cell proliferation, survival, and motility; inflammation maintenance; acquisition of stemness; and resistance to chemotherapy.[43,50] TNF-α levels at the tumor site or in plasma/serum in patients with BC are correlated with the clinical status and outcome.[51]

Conclusion

This network pharmacology study indicated that XHP can inhibit the development of BC by targeting a variety of proteins and signaling pathways involved in the inflammatory microenvironment. Additional research is needed to

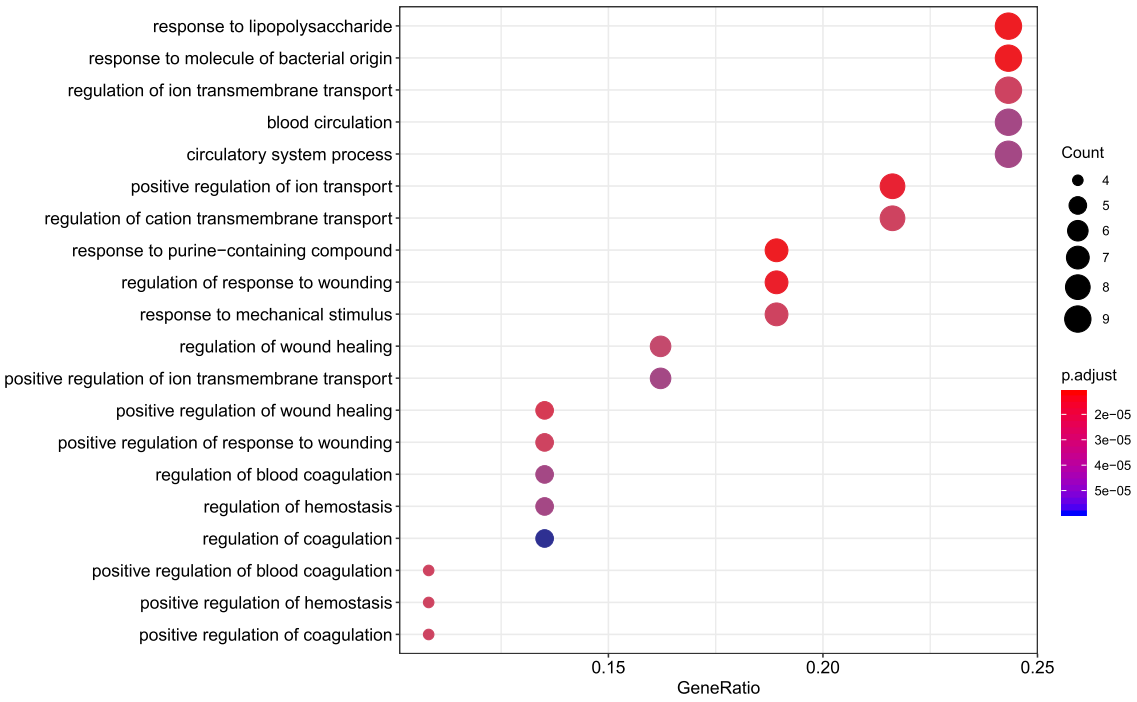

Figure 6 Biological process analysis of the target genes. The top 20 biological processes enriched in Gene Ontology analysis are presented. The bubble size indicates the gene count, and the color indicates the adjusted p-value.

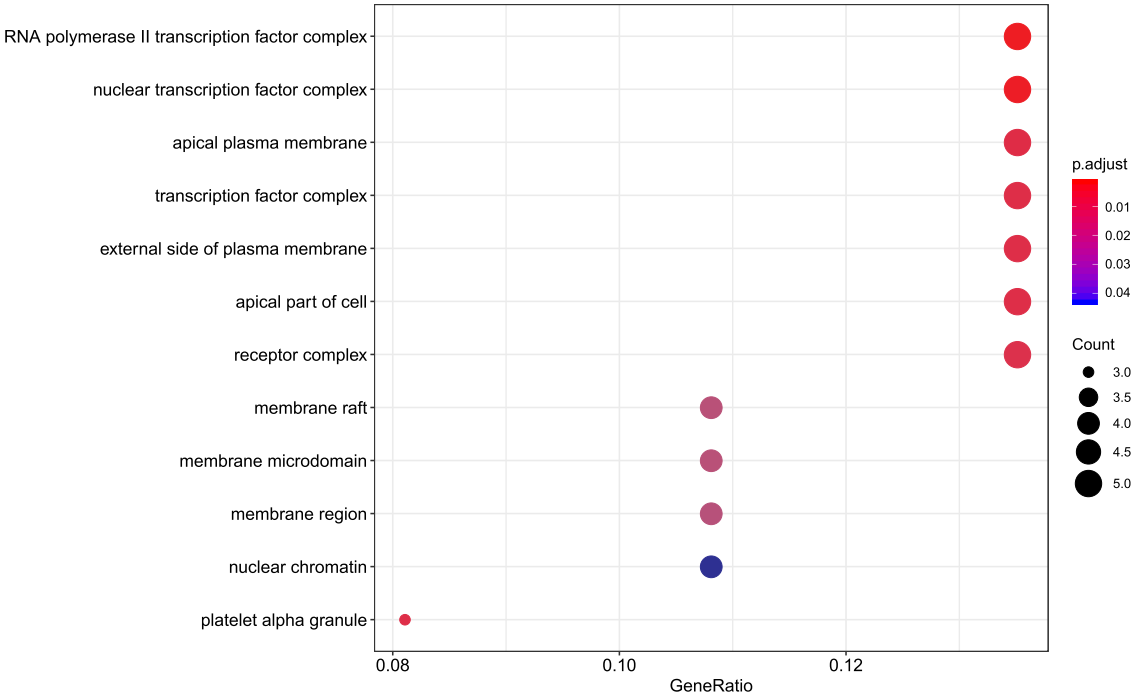

Figure 7 Cellular component analysis of the target genes. The top 20 cellular components enriched in Gene Ontology analysis are presented. The bubble size indicates the gene count, and the color indicates the adjusted p-value.

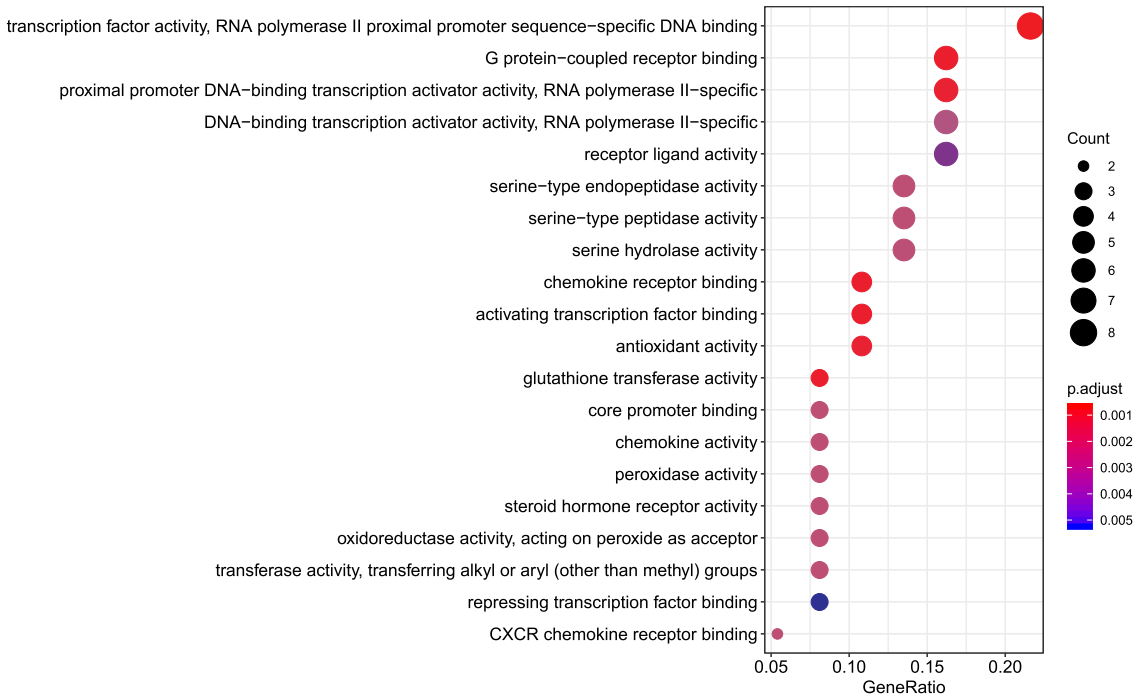

Figure 8 Molecular function analysis of the target genes. The top 20 molecular functions enriched in Gene Ontology analysis are presented. The bubble size indicates the gene count, and the color indicates the adjusted p-value.

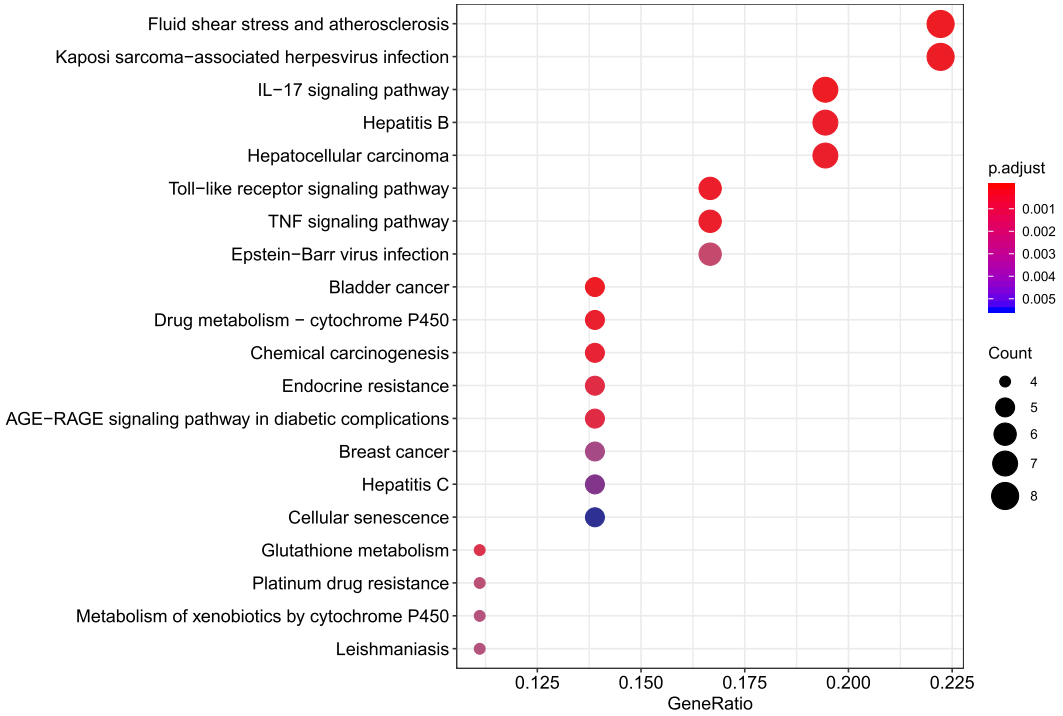

Figure 9 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the target genes. The top 20 enriched KEGG pathways are presented.

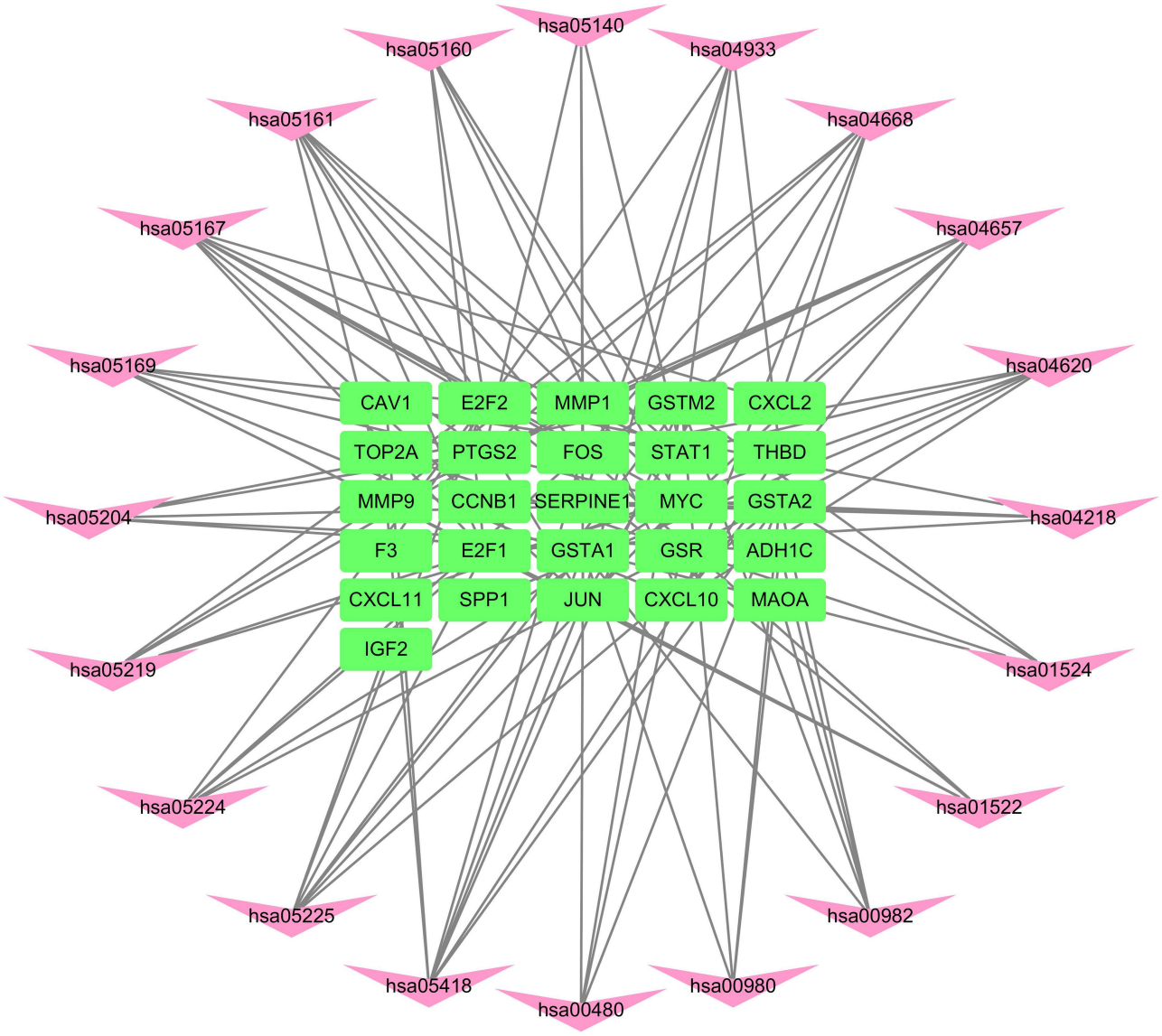

Figure 10 Network of the target genes–Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The pink darts indicate KEGG pathways, and the green rectangles indicate target genes.

clarify the effects of XHP on different molecular subtypes of BC.

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Disclosure

All authors declare no conflicts of interest.

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