Breast Cancer: Targets and Therapy

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ORIGINAL RESEARCH

Exploring the Mechanism of Lianqiao Jinbei Decoction Inhibiting HER2-Positive Breast Cancer Based on Network Pharmacology and Experimental Verification

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Purpose: Through network pharmacological prediction and in vitro experimental verification, the mechanism of action of Lianqiao Jinbei Decoction (LJD) in inhibiting HER2-positive breast cancer cells was clarified, providing experimental evidence for its treatment of HER2-positive breast cancer.

Methods: Network pharmacology method was used to construct the potential target network of LJD in the treatment of HER2+ breast cancer. After cell culture in vitro, the proliferation of HER2+ SK-BR3 breast cancer cells was investigated using CCK-8 technique. The apoptotic potential of SK-BR3 cells was detected by flow cytometry, and the migration of SK-BR3 cells was detected by cell scratch assay. The expression of HER2 protein in SK-BR3 breast cancer cells was detected by ELISA.

Results: HER2 was identified as the central gene and quercetin, β-sitosterol, and luteolin were the primary active ingredients using network pharmacology analysis. Serum-containing LJD medication can stop SK-BR3 cells from proliferating ($P<0.05$). Serum-containing LJD drugs at high, medium, and low concentrations may induce SK-BR3 cell death ($P<0.05$). LJD serum at high, medium, and low concentrations reduced the migration of SK-BR3 cells ($P<0.05$). The expression of HER2 protein was decreased by LJD high, medium, and low concentration drug-containing serum ($P<0.05$).

Conclusion: Regarding treating HER2-positive breast cancer, LJD has a multi-component, multi-target, and multi-pathway mode of action. The primary target of LJD's activity is the HER2 protein. Serum-containing LJD medication can prevent SK-BR3 cells from proliferating and migrating while encouraging their apoptosis. This effect may be attained by preventing HER2 protein expression.

Keywords: Lianqiao Jinbei Decoction, HER2-positive breast cancer, network pharmacology, mechanism study

Introduction

Breast cancer results from a breakdown of normal gene regulation, which leads to the malignant proliferation and growth of breast duct epithelial cells. Among malignant cancers in women, its morbidity and mortality rates are the highest, and it has a younger age trend.[1] It is currently one of the health issues that the public is generally concerned about. In the

Received: 13 February 2025
Accepted: 23 June 2025
Published: 14 July 2025

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early stages of the disease, hard breast masses, breast discharge, axillary lymph node enlargement, and other symptoms are common; in the later stages, the disease spreads to other organs and systems of the body as a result of metastasis. Breast cancer is far less common in developing and impoverished countries than in developed ones, and when it is discovered, it is often in the middle or late stages. This means that patients may not have access to timely treatment or surgery, which can have a serious negative impact on their physical and mental well-being, increase the strain on their families, and impede social development.

Human epidermal growth factor receptor-2 (HER2/ERBB2), as a tyrosine kinase receptor, is involved in the proliferation and survival of cells. The occurrence of HER2-positive breast cancer is closely related to the amplification or overexpression of HER2 gene.[2] In all types of breast cancer, HER2-positive breast cancer has the worst prognosis, highest death rate, shortest survival time, and highest recurrence rate when left untreated.[3,4] Even though there are numerous treatments available for HER2-positive breast cancer, a significant obstacle to current treatment is the persistence of multi-drug resistance, metastasis and recurrence, heart damage, and other problems. Traditional Chinese medicine, a national treasure, offers special benefits and prowess in the treatment of breast cancer. It can alleviate symptoms, stop tumor growth, lower the risk of complications, enhance patients' quality of life, and enhance patients' prognosis. The combined application of traditional Chinese medicine and Western medicine as adjuvant therapy can effectively reduce toxicity and improve therapeutic effect.[5] Breast cancer is classified as "Ruyan" in traditional Chinese medicine, which also holds that heat toxicity, blood stasis, and phlegm turbidity are the primary causes of the disease and that "Tandu Yujie" are the fundamental pathways leading to breast cancer.[6] The main goals of treatment are to "Jieduquyu, Huatansanjie". As a representative of this treatment method, Lianqiao Jinbei Decoction (LJD) has shown good tumor suppression effect in our clinical practice. "Jingyuequanshu" published LJD for the first time. It is made up of Weeping forsythia (*Forsythia suspensa* (Thunb). Vahl), Honeysuckle flower (*Lonicera japonica* Thunb.), Sargentgloryvine stem *(Sargentodoxa cuneata* (Oliv.) Rehd. & E. H. Wilson in C. S. Sargent), Dandelion *(Taraxacum mongolicum* Hand.-Mazz.), *Prunella vulgaris* (*Prunella vulgaris* L.), and Bolbostemma rhizoma (*Bolbostemma paniculatum* (Maxim.) Franquet). Its effects include dissolving, lowering edema, eliminating blood stasis, and detoxifying. However, the mechanism underlying its therapeutic effectiveness in the treatment of breast cancer remains unknown. Based on the theory of systems biology, network pharmacology is an emerging discipline that explores and discovers new therapeutic targets and drugs through network topology analysis of comprehensive biological information such as genes, targets, and signaling pathways. The rise of the field of network pharmacology provides new methods and concepts for the study of the mechanism of action of traditional Chinese medicine components in many kinds of diseases, including cancer, and also accelerates the discovery of new therapeutic targets and the process of drug development.[7,8]

This study used network pharmacology to investigate the molecular mechanism of LJD in the treatment of HER2-positive breast cancer. Based on the findings of network pharmacology, cell experiments were used to confirm the role of LJD in the proliferation and apoptosis of HER2-positive breast cancer cells, thereby providing a theoretical foundation for clinical application.

Materials and Methods

Network Pharmacological Analysis

LJD Effective Compounds—Target Screening

The active ingredients of *Weeping forsythia* (https://www.worldfloraonline.org/taxon/wfo-4000014961), *Honeysuckle flower* (https://www.worldfloraonline.org/taxon/wfo-0000359579), *Sargentgloryvine stem* (https://mpns.science.kew.org/mpns-portal/plantDetail?plantId=517495&query=Sargentgloryvine+Stem&filter=&fuzzy=false&nameType=all&dbs=wcs), *Bolbostemma rhizoma* (https://www.worldfloraonline.org/search?query=Bolbostemmatis+Rhizoma), and *Prunella vulgaris* (https://mpns.science.kew.org/mpns-portal/plantDetail?plantId=166020&query=Prunella+vulgaris+L&filter=&fuzzy=false&nameType=all&dbs=wcs) *were* searched in the TCMSP database, and the active ingredients of dandelion were screened in the TCMID database. At the same time, the action targets of the active ingredients of the related drugs were collected in the TCMSP database. The results were selected according to the criteria of oral bioavailability (OB)≥30% and drug-likeness (DL)≥0.18 and supplemented according to the literature.

Discovery of Disease Targets for HER2-Positive Breast Cancer

The term "HER2-positive breast cancer" was used to look for genes linked to HER2-positive breast cancer using the HERB, TTD, GeneCards, and Drugbank databases. After consolidation, the target of HER2-positive breast cancer was obtained by removing duplicates. According to items 1 and 2 of article 32 of the Measures for "Ethical Review of Life Science and Medical Research Involving Human Subjects" (issued in China on February 18, 2023): (1) Research utilizing legally obtained public data, or data generated through observation without interfering with public behavior; (2) Research conducted using anonymized information data; Our study meets the above conditions and is therefore exempt from approval.

Construction of Drug-Active Ingredient–Target Interaction Network for LJD Treatment of HER2-Positive Breast Cancer

The intersection of HER2-positive breast cancer disease targets and LJD action targets is the target of LJD treatment of HER2-positive breast cancer, as determined by the online Venny program (v2.1). Following the elimination of combined weight, the Cytoscape software (v3.7) was used to construct the drug-active component–target interaction network of LJD in the treatment of HER2-positive breast cancer. The mechanism of action of LJD in the treatment of HER2-positive breast cancer was then explored.

Analysis of Protein–Protein Interaction (PPI)

To create a PPI network of core targets, intersection targets were chosen, and core targets were vetted by importing Cytoscape (v3.8.2) into the STRING database.

GO and KEGG Enrichment Analysis

To perform enrichment analysis of biological processes (BP), cell components (CC), molecular functions (MF), and KEGG pathways, intersection targets were added to the DAVID database. The results of the analysis for BP, CC, MF, and KEGG were derived by utilizing the Microbioinformation database.

Cell Experiment Verification

Cells and Animals

The HER2-positive breast cancer cell line SKBR3 was kindly donated by the First Affiliated Hospital of Chongqing Medical University (approved by the Ethics Committee of Hebei University of Chinese Medicine). The Laboratory Animal Center of Hebei Medical University provided 20 SPF-grade SD female rats (200 ± 20g) for purchase (Certificate number: SCXK (Hebei) 2022–001). 20 SPF-grade SD female rats were used and raised in a standardized environment at the Experimental Animal Center of Hebei University of Chinese Medicine (temperature 22 ± 2°C, humidity 50 ± 10%, 12-hour light and dark cycle). They were raised in separate cages and provided with sterile bedding and adequate diet. Before the experiment, they were adaptively raised for one week and randomly divided into the negative control group and the LJD group (10 rats in each group). The sample size was based on the effect size analysis of the pre-experiment and conformed to the "3R principle". Anesthesia was performed by intraperitoneal injection of pentobarbital sodium solution before blood collection. The experimental plan was approved by the Ethics Committee of Hebei University of Chinese Medicine and followed the animal welfare guidelines throughout the process. This study was approved by the Ethics Committee of Hebei University of Chinese Medicine (Approval No. DWLL202303034). Animal experiments were conducted in accordance with ethical guidelines, and cell experiments utilized certified cell lines from authorized sources, complying with ethical standards.

Drugs, Reagents, and Devices

The composition and dosage of LJD were purchased from the Pharmacy of Traditional Chinese Medicine of the First Affiliated Hospital of Hebei University of Chinese Medicine (Shenwei Granules). The composition and dosage of LJD were as follows: *Weeping forsythia* 15g (Batch number: 230220P1), Honeysuckle flower 9g (Batch number: 230102Q4), Bolbostemma rhizoma 9g (Batch number: 22010551), Dandelion 9g (Batch number: 22010551). 230108A5), *Prunella vulgaris* 9g (Batch number: 230225L1), Sargentgloryvine stem 20g (Batch number: 230526K2). The formula particles

were dissolved in pure water to prepare a medicated solution with a concentration of 2g/mL and stored in a refrigerator at 4°C. Besides, the following equipments were used in the experiment: 25T Corning flask; Culture plates (Costar, NEST); Sterile straw (NEST); 1.5mL centrifuge tube (Biologix); 15 mL centrifuge tube (Solarbio); Sterile suction head (Biologix); DMSO (Biomol); Chloropystreptomycin mixture (100X) for cell Culture (Solarbio); Trypsin EDTA digestive solution (0.25%) containing Solarbio; 1XPBS buffer (pH7.2–7.4) (Solarbio); RPMI Medium 1640 (Gibco); Blood cell counting plate (Skjylean); Frozen storage tube (Solarbio); Sealing film (Parafilm); CCK-8 reagent (MCE); HER2 ELISA Kit (Xinbosheng Company); Enzyme labeling instrument (Flash); Cell freezing liquid ammonia tank (Haier); 4°C refrigerator (Haier); Inverted microscope (Leica); Centrifuge (Shandong Baiou Medical Technology Co., LTD).; Cell ultra-clean workbench (Thermo Field); Flow cytometry Instruments, software and reagents (Beckman); Pipette (Byand).

Preparation and Grouping of LJD Drug-Containing Serum

Following one week of normal diet, the rats were randomly assigned to the LJD group and the negative control group, with ten rats in each group, for the duration of the trial. Rats in the LJD group received a daily dose of 12.6 g/kg via the equivalent dose conversion coefficient method, whereas the negative control group received the same volume of clean water intragastrically twice a day for three days in a row. After two hours of intragastric administration on the fourth day, femoral arterial blood was drawn, refrigerated at 4°C for two hours, centrifuged at 1000 rpm for twenty minutes, and the supernatant was extracted.

Cell Culture and Grouping

The SK-BR3 cell line was grown in a unique culture and maintained at 37 °C in an incubator with 5% CO_2. Based on IC50 data, the drug concentration was established, and SK-BR3 cells were divided into LJD high-dose, medium-dose, and low-dose groups. Additionally, negative control and positive control groups were established. In the positive control group, 2 μL trastuzumab + 1 mL RPMI 1640 medium (mass concentration: 22 mg/mL) was included.[9]

Cell Proliferation Assay

SK-BR3 cells at the logarithmic growth phase were digested with 0.25% trypsin-EDTA and resuspended in complete RPMI 1640 medium. The cell suspension was adjusted to a density of 4×10^3 cells/well and seeded into 96-well plates (100 μL/well), with five replicate wells per group. Peripheral wells were filled with 100 μL PBS to minimize edge effects. After overnight incubation (37°C, 5% CO_2) to allow cell adhesion, the original medium was aspirated. For IC50 determination, cells were treated with LJD drug-containing serum at concentrations of 0%, 1.25%, 2.5%, 5%, 10%, 20%, and 30% for 24 hours. The medium was then replaced with 100 μL fresh medium containing 10% CCK-8 reagent, followed by incubation for 2 hours at 37°C. Absorbance (A) at 450 nm was measured using a microplate reader. The proliferation rate (%) is equal to [(A control-A blank) - (An experiment-A blank)] ×100%.

Proliferation activity detection was used to determine the ideal drug concentration, and IC50 values of 1/4 and 1/2 were used to establish low and medium-dose groups. Using the CCK-8 method described above, the OD values of SK-BR3 cells were detected after 24 hours, 48 hours, and 72 hours of intervention by LJD in high, medium, and low dose groups. The associated cell activity was then estimated to monitor LJD's impact on SK-BR3 cell growth.

Cell Apoptosis Assay

The cells were in the logarithmic development stage when they were put in the six-well plate. The drug-containing culture medium (three multiple holes) was replaced when 50–80% of the cells had adhered to the wall, and the cells were then collected for detection following the appropriate treatment period. The culture media for the cells was removed and placed into a suitable centrifuge tube. The cells were then washed once with 1 mL of PBS and digested for 2 minutes with 1 mL of EDTA-free pancreatic enzyme. After being added, the collected cell culture fluid was moved into the centrifuge tube. After five minutes of centrifuging 1000 g, remove the supernatant, gently resuspension the cells in PBS, and count. After taking 50,000 to 100,000 suspended cells and centrifuging the supernatant, the cells were gently re-suspended in 195 μL of Annexin V-FITC binding solution. Add 10μL of propyl iodide staining solution and 5μL of Annexin V-FITC. Incubate for 10–20 minutes at room temperature and out of direct light. Finally, put the mixture in an ice bath. Once the reaction is finished, run it through a 200-mesh filter screen and test the machine right away.

Cell Scratch Assay

To ensure consistent scratch positioning and reliable quantification in the scratch assay, the bottom surface of the six-well plate is pre-marked with three parallel horizontal lines spaced approximately 0.5 cm apart using a sterile marker and ruler. Cells are then seeded at a density of 7×10^5 cells per well and incubated for 24 hours at 37°C with 5% CO_2 until they reach 100% confluency. Once the cells have grown over, use a ruler to draw two vertical lines that are perpendicular to the marked line and the hole plate. This will allow the marked line and the scratch to intersect several times, forming a fixed detection point from these crossing points.

HER2 Protein Detection

Cells in the logarithmic growth phase were seeded in 6-well plates at a density of 5×10^5 cells per well. After reaching 70–80% confluence, the cells were further incubated for 24 hours, and the supernatant was collected. The HER2 protein content in the supernatant was then measured according to the ELISA kit instructions.

Data Statistics and Analysis

In statistical analysis, data were expressed as mean ± standard deviation ($\bar{x} \pm s$) and one-way analysis of variance (ANOVA) was used to compare the differences among groups. The significance level was set as $P<0.05$. The statistical analyses were all carried out using the SPSS software (v26.0).

Results

Active Components and Targets of LJD

After the initial screening in the TCMSP and TCMID databases, a total of 75 chemical components were obtained; the invalid active components were then deleted, and 60 active components, or 281 targets, were obtained following the addition of literature and the deletion of chemical components about non-human targets. The UniProt database was used to standardize the target names.

HER2-Positive Breast Cancer Target Mining

The linked targets of HER2-positive breast cancer were screened and combined using the term "HER2-positive breast cancer" from the HERB, TTD, GeneCards, and Drugbank databases. A total of 1125 relevant targets were screened, including 1082 in the GeneCards database (relevance score >33), 14 in the HERB database, 5 in the TTD database, and 24 in the Drugbank database. A total of 1099 targets were obtained after taking their intersection to remove duplicate targets.

Construction of LJD Drug-Active Ingredient–Target Interaction Network

Using Cytoscape software, the intersection of HER2-positive breast cancer disease targets and LJD action targets was created (Figure 1). There are 1024 edges and 202 nodes in the network. The importance increases with the degree value. The nodes PTGS2, NCOA2, PTGS1, AR, and HSP90 have bigger degrees.

Key Targets of the PPI Network

After data was introduced into Cytoscape software (v3.8.2), CytoNCA was used to perform topological analysis after the PPI protein network interaction map of LJD in the therapy of breast cancer was created. The core goals were nodes with sizes greater than the median of double degree value, greater than the median of intermediate centrality, and almost equal to the median of centrality. After screening a total of eighteen targets, the top five core targets were, among others, TP53, AKT1, STAT3, CASP3, and HSP90AA1. The core target also includes the HER2 gene that was investigated in this work. To create the core target's PPI network, the core target is loaded into the STRING database (Figure 2). These targets might be crucial in how LJD treats HER2-positive breast cancer.

GO Functional Enrichment Analysis and KEGG Signaling Pathway Enrichment Analysis

Among the GO functional enrichment analysis results, the biological process analysis targets include response to estradiol, positive regulation of RNA polymerase ii promoter transcription, and regulation of gene expression. Targets

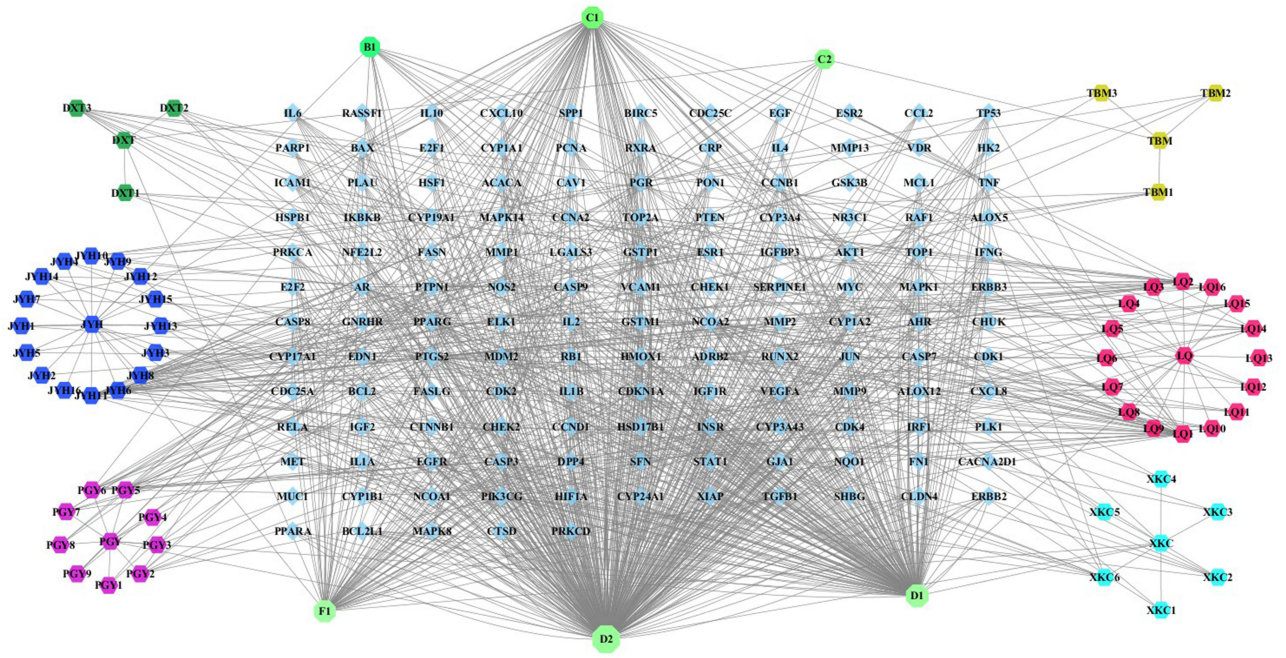

Figure 1 LJD drug-active ingredient-target interaction network.

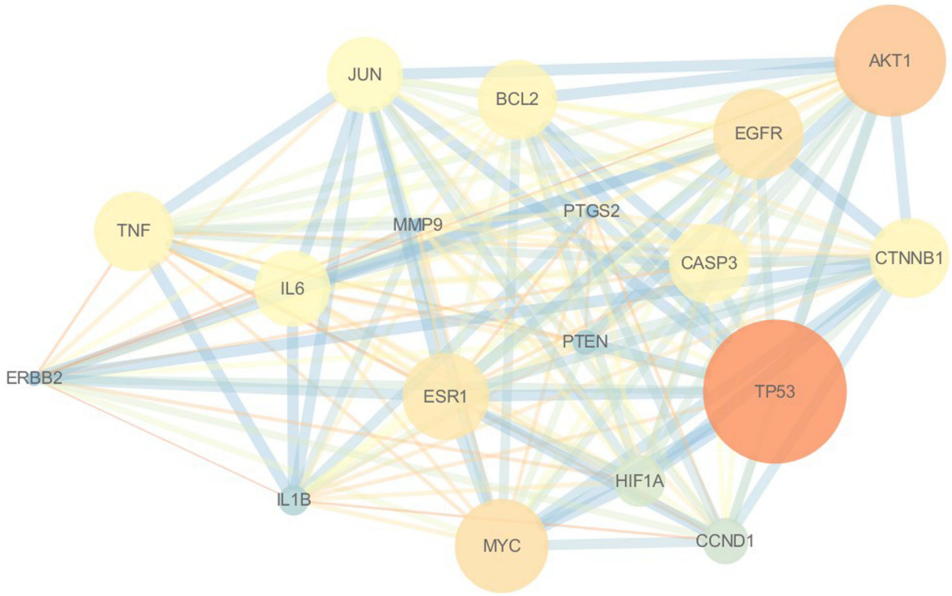

Figure 2 Core target of PPI protein network interaction map in LJD treatment of breast cancer.

for cell component analysis include the cytoplasm, nucleus, and molecular complexes. Targets in molecular function analysis primarily include protein kinase binding, enzyme binding, and identical protein binding. One hundred and seventy signaling pathways were found by KEGG signaling pathway enrichment analysis, with the majority of these being associated with tumors, prostate cancer, Phosphatidylinositol-3-hydroxykinase/Threonine kinase (PI3K/Akt) pathways, and other conditions. Bar charts were created using the top 10 results in the BP, CC, and MF analysis, respectively (Figure 3). The top 10 KEGG signaling pathways were plotted as bubble plots (Figure 4).

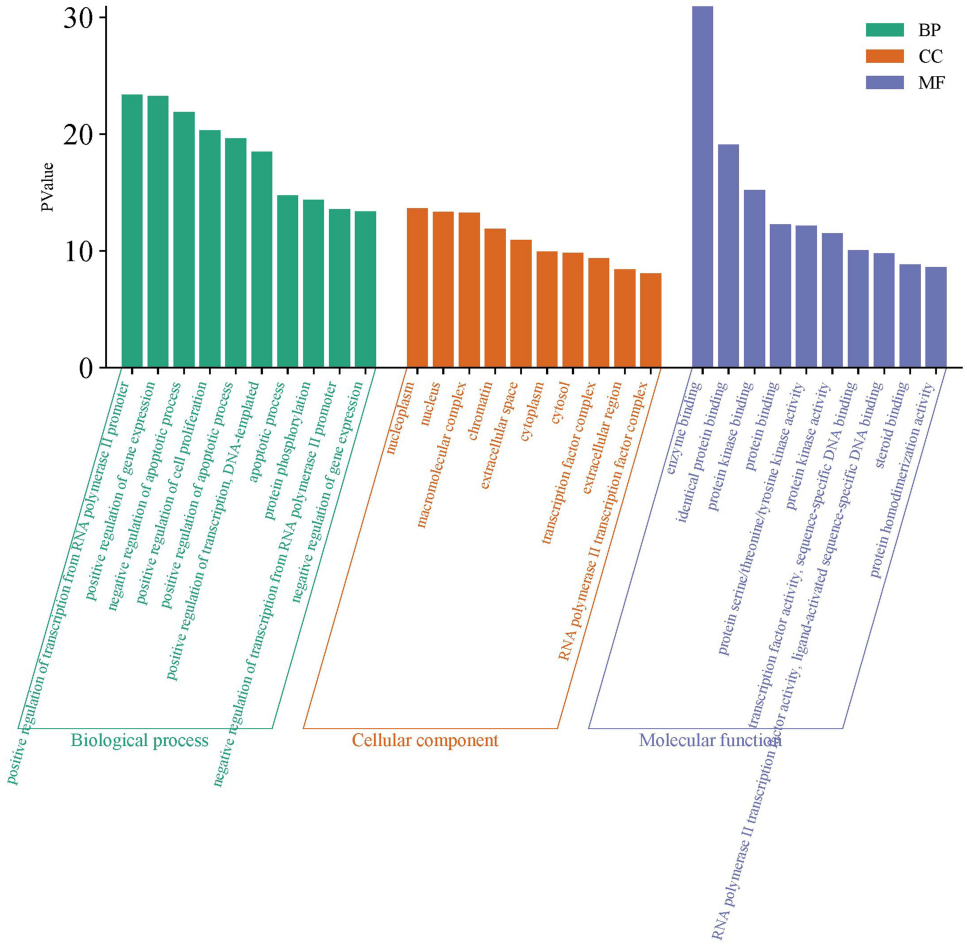

Figure 3 GO enrichment analysis of the core targets among the drug-active component-target interaction network.

Effect of LJD Drug-Containing Serum on Proliferation of SK-BR3 Cells

In this investigation, the IC50 value of serum containing LJD medication was screened using the CCK-8 technique. The concentration of the LJD drug-containing serum was set at 1.25%, 2.5%, 5%, 10%, 20%, and 30% based on prior experience and pertinent data, and absorbance was observed in SK-BR3 cells after 24 hours of drug intervention. The findings demonstrated that as the drug-containing serum concentration of LJD increased, the proliferative activity of SK-BR3 gradually decreased in comparison to the control group. The cell semi-inhibitory rate (IC) was around 50% when the drug-containing blood concentration of LJD was 5.109 ± 0.162% (CI = 95%) (Figure 5). To conduct follow-up studies, dose groups of 5%, 2.5%, and 1.25% were chosen as high, medium, and low doses, respectively.

The proliferative activity of SK-BR3 cells in each group was then measured using the CCK-8 technique after 24, 48, and 72 hours. The outcome is as follows (Figure 6): Serum containing high, medium, and low doses of LJD medication may significantly (P<0.05) inhibit the proliferative activity of SK-BR3 in a dose- and time-dependent manner when compared to the negative control group. The proliferative activity of HER2 could be significantly reduced (P<0.05) in the positive control group, while the effect was not as strong as in the high serum dose group. P<0.05 indicated that the difference was statistically significant.

Effect of LJD Drug-Containing Serum on Apoptosis of SK-BR3 Cells

The negative control group was able to induce SK-BR3 cell apoptosis in comparison to the blank control group (Figure 7 and Table 1), but this difference was not statistically significant. The LJD serum groups at high, medium, and low doses, as well as the positive control group, all demonstrated a dosage dependence that was statistically significant (P<0.05) in

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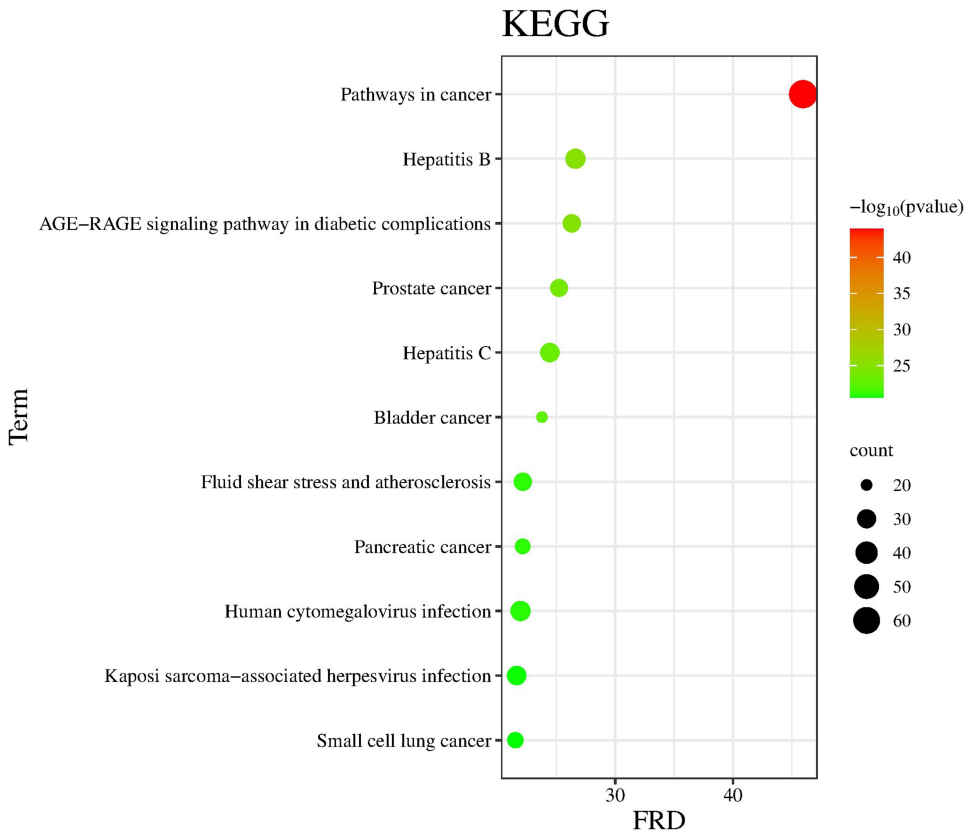

Figure 4 KEGG enrichment analysis of the core targets among the drug-active component-target interaction network.

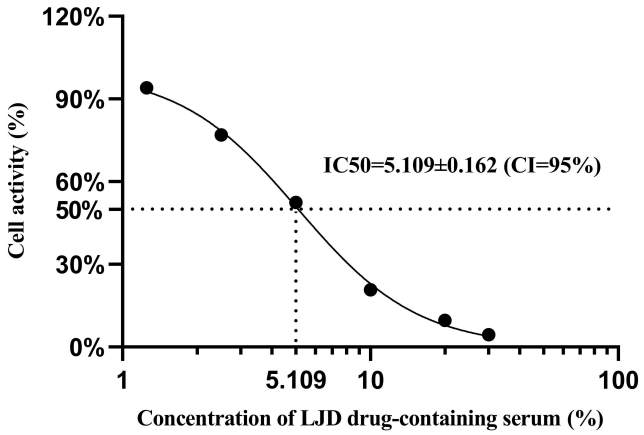

Figure 5 IC50 curve of the effect of LJD drug-containing serum on SK-BR3 cells.

promoting the apoptosis of SK-BR3 cells. The LJD drug-containing serum high, medium, and low dose groups as well as the positive control group were able to induce apoptosis in SK-BR3 cells in comparison to the negative control group. The positive control group was able to do this most effectively, and the difference was statistically significant ($P<0.05$).

Effect of LJD Drug-Containing Serum on SK-BR3 Cell Migration

Compared with the blank control group (Figure 8), LJD drug-containing serum had an inhibitory effect on the migration of SK-BR3 cells, and the higher the concentration, the more obvious the inhibitory effect was. The negative control

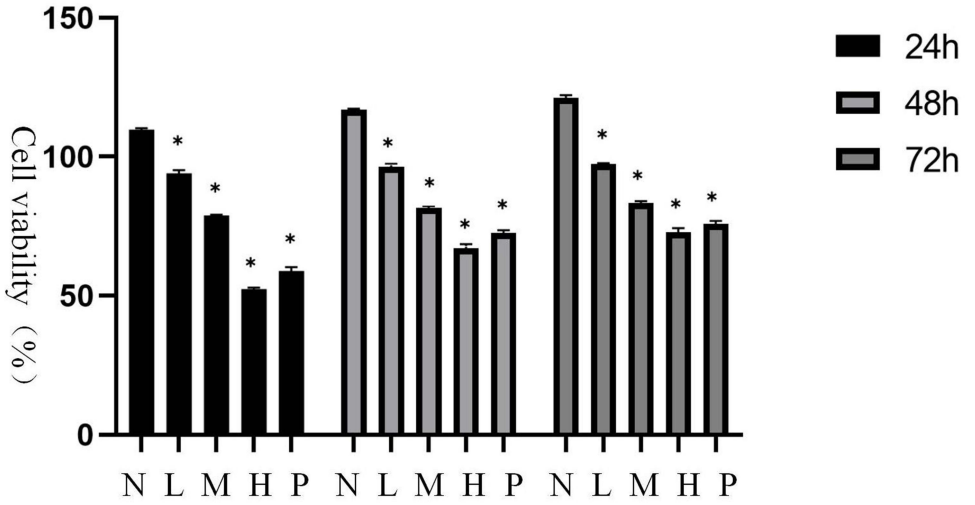

Figure 6 Effect of different concentrations of LJD drug-containing serum on proliferation activity of SK-BR3 cells. The * represents the P value less than 0.05 compared with the negative control group.
Notes: N: Negative control group; L: Low-dose group; M: Medium-dose group; H: High-dose group; P: Positive control group.

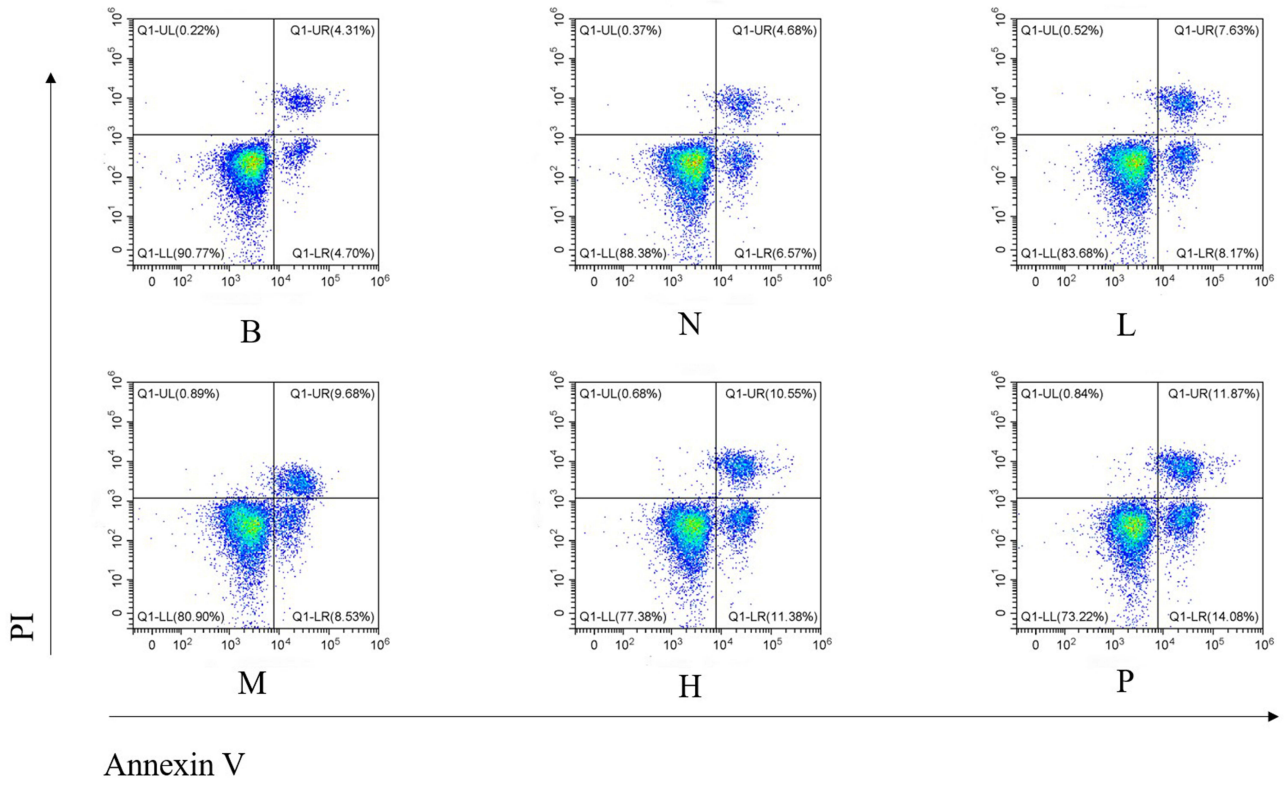

Figure 7 Effect of different concentrations of LJD drug-containing serum on apoptosis of SK-BR3 cells.

group inhibited the migration of SK-BR3 cells. The positive control group had the best inhibitory effect on the migration of SK-BR3 cells, and the difference was statistically significant ($P<0.05$).

Effect of SK-BR3 Cells in LJD Drug-Containing Serum on the Expression of HER2 Protein

After a 24-hour intervention, ELISA was used to measure the expression of HER2 protein in SK-BR3 cells in the blank control group, negative control group, drug-containing serum, and positive control group (Figure 9). The findings

https://doi.org/10.2147/BCTT.S522528

Table 1 Cell Apoptosis Rates in Each Group

Group	Early Apoptosis Rate (%)	Late Apoptosis Rate (%)	Total Apoptosis Rate (%)
Blank control group (B)	4.40±0.43	5.01±0.50	9.40±0.91
Negative control group (N)	4.18±0.46	6.75±0.31	10.93±0.50
LQJBJ low dose group (L)	7.50±0.28	8.28±0.24	15.78±0.48*#
LQJBJ medium dose group (M)	9.99±0.41	8.56 ±0.54	18.56±0.90*#
LQJBJ high dose group (H)	10.74±0.19	11.25 ±0.45	21.99±0.36*#
Positive control group (P)	12.17±0.75	14.66±0.67	26.82±0.82*#

Notes: The * sign represents the P value less than 0.05 compared with the blank group, while the # sign represents the P value less than 0.05 compared with the negative control group.

demonstrated that the negative control group's HER2 protein expression level was lower than that of the blank control group; however, this difference was not statistically significant. In serum high, intermediate, and low-dose groups receiving LJD medication, there was a substantial decrease in HER2 protein expression, indicating a statistically significant dose-dependent effect ($P<0.05$).

Discussion

Breast cancer is the malignant tumor with the highest incidence in women, and its occurrence and development often go through the continuous stage of *normal epithelial cells → simple hyperplasia → precancerous lesions → tumor → metastasis*.[10] Overexpression and mutation of the HER2 gene have been identified in many cancers and are key influencers of cancer progression and therapeutic efficacy.[11] Targeted therapy and immunotherapy often achieve higher benefits in HER2-positive breast cancer patients.[12,13] In clinical treatment, the optimal course of treatment is usually selected based on the patient's HER2 status, tumor size, lymph node metastasis and other prognostic variables. The humanized anti-HER2 monoclonal antibody trastuzumab, followed by several other targeted drugs such as lapatinib, pertuzumab and Trastuzumab emtansine (T-DM1), have been widely used in clinical practice to reduce the risk of early disease recurrence and significantly improve the survival rate of patients with metastasis disease.[14] In addition, new therapeutic targets such as WNT5A, UCHL1, PPARG, HIF-1α and a variety of miRNAs have also been found to

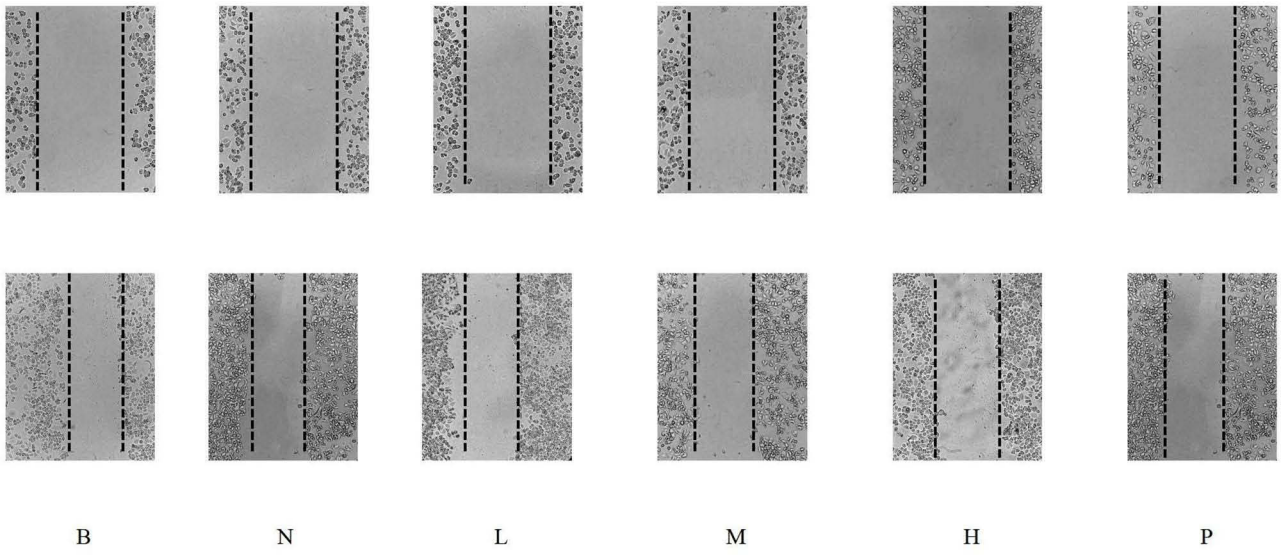

Figure 8 Effect of different concentrations of LJD drug-containing serum on migration of SK-BR3 cells.

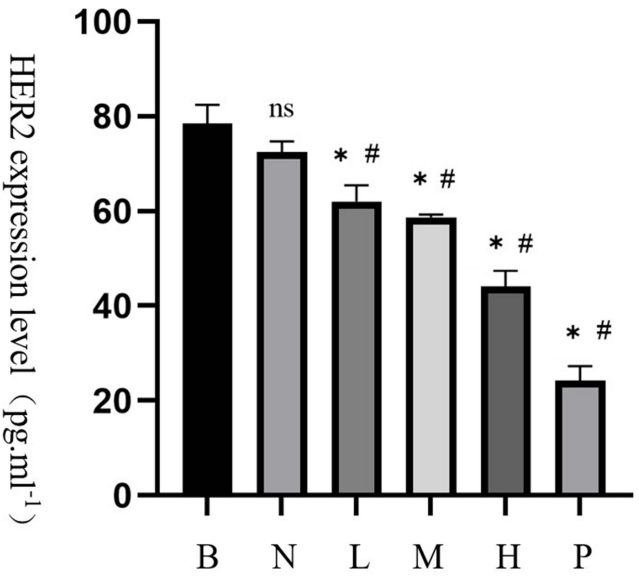

Figure 9 Expression of HER2 protein in SK-BR3 cells cultured with different concentrations of LJD drug-containing serum. The * represents the P value less than 0.05 compared with the blank group, and the # represents the P value less than 0.05 compared with the negative control group.

effectively inhibit the progression of breast cancer.[15–19] However, despite the improvement in efficacy, these risks pose new challenges for the clinical treatment of HER2-positive breast cancer due to its associated drug-related side effects and the fact that some patients will relapse and metastasize after treatment.

Due to the unique syndrome differentiation and treatment theory and the holistic concept of TCM, by regulating the body's qi and blood, dredging meridians, strengthening the body's health and eliminating pathogenic factors, it can regulate the patient's immune function and relieve the symptoms of the disease and has unique advantages in inhibiting the occurrence, development and treatment of breast cancer.[20–22] At present, traditional Chinese medicine treatment for breast cancer mainly includes Chinese herbs oral decoction and application, external application, acupuncture and acupoint injection, etc. Many herbs and natural drugs have been proved to have good anti-tumor activity, and certain progress has been made in the development of new drugs and tumor therapy.[23–25] In clinical treatment, breast cancer patients experience surgery, radiotherapy, chemotherapy, physical weakness, and poor mood. Traditional Chinese medicine can improve patients' tumor-related symptoms, reduce patients' adverse reactions, and improve their quality of life.[26,27] In terms of microscopic mechanism, TCM can affect cell proliferation, apoptosis and metabolism, regulate related gene pathways and immune response. According to traditional Chinese medicine theory, the pathogenesis of breast cancer involves the liver, spleen, kidney, and stomach.[28] "Tandu Yujie" is the core pathogenesis of breast cancer, and "Jiedu Quyu, Huatan Sanjie" are the basic corresponding treatment.[29,30] A variety of Chinese medicinal formula have shown good effects in inhibiting HER2-positive breast cancer. For example, the results of the study by Li et al showed that Xianling Lianxia formula could enhance the inhibitory effects of trastuzumab on the proliferation, colony formation ability, migration, and invasion of HER2-positive BC cells and promotes apoptosis.[31] The mechanism may be the regulation of Janus kinase (JAK)/Signal transducer and activator of transcription (STAT) pathway, Nuclear factor kappa-B (NF-κB) pathway, and other pathways, and affect cytokines related to immune function. Liu et al found that Yanghe Huayan decoction could significantly inhibit the invasion and angiogenesis of HER2-positive breast cancer cells by regulating the expression of p-Akt signaling, while the study led by Zeng et al highlighted the therapeutic potential of Yanghe decoction for HER2-positive breast cancer.[32,33] LJD is also a representative prescription of relevant TCM treatment methods and principles and has good clinical efficacy. Therefore, we selected LJD for our research of HER2-positive breast cancer.

Through network pharmacology analysis, we constructed a PPI protein network interaction map of LJD in the therapy of HER2-positive breast cancer, and performed KEGG and GO analyses. We found that HER2 was closely related to TP53, MYC, JUN, BCL2, AKT1, PTEN, EGFR, IL1B and other key genes among biological behavior of tumor cell

proliferation and apoptosis (Figures 1 and 2), which was also confirmed by the results of several previous studies. For example, Gu et al found that HER2 inhibits the pro-apoptotic function of p53 by activating the Phosphatidylinositol 3′-kinase (PI3K)/Akt pathway in gastric cancer, while TP53 mutation could impair DNA repair ability and lead to increased genomic instability, affecting the malignant progression of tumors.[34] Meanwhile, in HER2-positive breast cancer, TP53 mutation often coexists with HER2 amplification, promotes the characteristics of tumor stem cells, induces the immunosuppressive phenotype of tumor associated macrophages (TAM) by regulating the expression of a variety of cytokines, and reduces the anti-tumor activity of $CD8^{+}$T cells.[35] HER2 can also activate MYC through the MAPK pathway, promote the expression of Cyclin family genes, and accelerate the proliferation of HER2-positive breast cancer cells.[36] In addition to the above, we focused on the possible relationship between HER2 overexpression and signaling pathway regulation and biological behavior of breast cancer cells. In the pathway related to HER2-positive breast cancer, the HER2 gene is located the upstream of PI3K/Akt signaling pathway, and up to 70% of breast cancer cases involve the PI3K/Akt signaling pathway being activated. As for the results of GO analysis (Figure 3), the biological process part mainly reflects that HER2 has a regulatory effect on cell proliferation and apoptosis. The molecular function reflects that HER2 also affects enzyme binding and protein kinase binding and activity. On the one hand, it is due to HER2 itself as a member of the epidermal growth factor receptor family and its conformation as a tyrosine kinase. On the other hand, according to KEGG analysis (Figure 4), three main pathways—tumor-related, PI3K/Akt, and breast cancer-related—were used by LJD to treat HER2-positive breast cancer. As its key downstream signaling pathway, HER2 amplification has been shown to positively enhance PI3K/AKT/mTOR signaling pathway, which not only plays a key role in promoting cell proliferation and inhibiting cell death but also mediates drug resistance in breast cancer.[37] For example, patients with PTEN loss in HER2-positive breast cancer have reduced sensitivity to trastuzumab because HER2 activation of AKT1 promotes cell survival, whereas PTEN loss amplicates AKT signaling, leading to treatment resistance.[38] In the study by Yuan et al, the role of various natural drugs and monomeric agents including flavonoids, polyphenols, alkaloids, etc. in the clinical trials of breast cancer treatment by targeting PI3K/AKT signaling pathway was also discussed in depth.[39] Therefore, the combination of HER2-targeted therapy and AKT pathway inhibitors may increase and reduce the drug toxicity related to HER2-targeted therapy, provide more effective and safe treatment options for cancer patients, and further improve the prognosis of patients.

Weeping forsythia, Honeysuckle flower, and dandelion have the effect of cleansing and heat-clearing, while *Prunella vulgaris* and Bolbostemma rhizoma could dissipate phlegm and disperse accumulation; Sargentgloryvine stem plays the role of detoxifying and eliminating blood stasis, clearing phlegm, and dispersing. It also removes stasis and lessens pain, strengthening the ability to reduce swelling and disperse accumulation. This core pathophysiology is located close to the "Tandu Yujie". LJD herbal extracts have been shown to offer therapeutic benefits against breast cancer in recent investigations. Triterpenoids, which are the primary constituents of Weeping forsythia, can impede the growth of cancer cells in the breast, liver, and colon; in fact, half of the rate of inhibition of breast cancer cells can reach 6.29 mg/L.[40] Isochlorogenic acid C, the main active component of Honeysuckle flower, could reverse epithelial–mesenchymal transformation in breast cancer cells through the EGFR pathway.[41] Dandelion is widely used in the clinical treatment of breast cancer. Modern pharmacological studies have found that dandelion can play a role in regulating the cell cycle, inhibiting proliferation, promoting apoptosis, anti-invasion, metastasis, regulating autophagy, affecting estrogen, and regulating immunity.[42] The extract of Sargentgloryvine stem has an obvious inhibitory effect on breast cancer cells, and the inhibitory rate can reach 61.54% at 100 mg/L.[43] The main active component of Bolbostemma rhizoma induces the expression of Egr1 and p21 through the Egr1/p21 signaling pathway, affects the cell cycle, and promotes apoptosis.[44] Prunella sinensis contains various anti-tumor active ingredients, which can regulate apoptosis-related proteins through various pathways to promote the apoptosis of breast cancer cells and inhibit the metastasis of breast cancer cells.[45]

The TCMSP and TCMID databases provided 281 LJD targets and 60 active compounds for this investigation. One thousand ninety-nine HER2-positive targets for breast cancer were retrieved from the databases of HERB, TTD, GeneCards, and Drugbank databases. Besides, an additional 137 targets were collected from the junction of these databases. Quercetin, β-sitosterol, and luteolin were the primary active components of LJD. Largely present flavonoid quercetin has been used extensively in the treatment of several diseases. It can influence the course of breast cancer by influencing immune drug resistance, angiogenesis, invasion and metastasis, chemotherapeutic sensitization, and cell proliferation and apoptosis.[46]

Quercetin can effectively prevent and treat HER2-positive breast cancer through multiple targets and pathways, such as PI3K/AKT and JNK signaling pathways, GAS5/Notch1 signaling pathways, and EGFR/AKT/mTOR signaling pathways.[47–49] Luteolin can not only promote tumor cell apoptosis and inhibit tumor cell migration but also inhibit breast cancer stemness and enhance chemotherapy sensitivity through Nrf2-mediated pathway.[50,51] Similar to cholesterol, β-sitosterol is a plant-based chemical found in fruits, vegetables, nuts, seeds, soybean, and cottonseed oils. It can lower cholesterol, relieve cough, expectorate phlegm, inhibit tumor growth, and heal tissues. Through estrogen receptors, it can influence the expression of cyclin D1 and mRNA and control the growth and death of breast cancer cells.[52]

One of the most significant biological traits of tumor cells is their proliferation, which is crucial to the development, invasion, metastasis, and recurrence of tumors. The foundation of investigating tumor growth is the study of cell proliferation. It is also a hub for research on anti-tumor drugs at the same time. Many academics think that one of the most significant and active elements in the growth of malignant tumors is the proliferation of tumor cells. To better prevent and treat malignant tumors, research on the mechanism of proliferation will aid in understanding the mechanism underlying the incidence and growth of these tumors. It will also offer new concepts and targets for the creation of anti-tumor medications.

When an organism develops, a combination of genetic and environmental factors can lead to apoptosis, a process of cell death in which DNA is the primary genetic material. The process of planned cell death known as apoptosis aids in preserving tissue balance and homeostasis.[53] Apoptosis can aid in the removal of aberrant cells from the body and lessen the build-up of possible cancerous cells in the tumor. Apoptosis also contributes significantly to the treatment of tumors at the same time. These days, apoptosis is linked to several anticancer medications and therapies. Conversely, an unchecked apoptotic process will disrupt the body's typical structure and physiological processes.

At the same time, tumor development and metastasis are significantly influenced by tumor cell movement.[54] Tumor cells can infect lymphatic or blood veins and generate metastases elsewhere in the body thanks to their capacity to travel, which causes metastasis and the tumor to spread. A crucial stage in the spread of tumors is tumor cell migration.[55] Numerous molecular and cellular processes, such as intercellular adhesion, cell invasion and movement, cell–matrix interaction, and so on, are involved in the process of tumor cell migration. In addition to illuminating the mechanism of tumor spread, research into these mechanisms can yield fresh concepts for creating novel therapeutic approaches. To stop tumor metastasis, it is crucial to understand the mechanism and regulatory system governing tumor cell movement.

The CCK-8 technique was used in this work to determine the impact of LJD on the proliferation of SK-BR3 cells. The findings demonstrated that LJD inhibited the activity of SK-BR3 cells, with the inhibitory effect becoming stronger with increasing concentration. The SK-BR3 cells' apoptosis experiment results demonstrated that LJD can induce apoptosis; the greater the concentration, the greater the effect of LJD on apoptosis. The results of cell scratch assay showed that LJD could block and prolong the migration time of SK-BR3 cells, and the effect depended on the concentration. Beyond that, LJD drug-containing serum could also cause SK-BR3 cells to proliferate less, migrate less, and undergo apoptosis.

In our research, compared with the control group, the expression of HER2 protein was decreased in high, medium and low dose groups. The LJD drug-containing serum group exhibited dosage-dependent HER2 protein expression, with the highest expression of HER2 protein in the high-dose group. It was discovered that by encouraging the lysosomal degradation of HER2, the novel flexible heterolascomasin SL-1-39 suppressed the growth of HER2-positive breast cancer cells.[56] Through an endocrine mechanism, galangal can inhibit the HER2 gene and cause breast cancer cells to undergo apoptosis.[57] In conjunction with the previously mentioned findings on migration, apoptosis, and proliferation, we conjectured that the mechanism preventing the serum containing weeping forsythia from proliferating, migrating, and encouraging apoptosis is connected to preventing the expression of HER2.

In conclusion, the expression of HER2 protein is closely related to the proliferation, apoptosis and migration of HER2 breast cancer cells, which can be regulated by targeting HER2 protein expression. LJD can inhibit the proliferation and migration of HER2-positive breast cancer cells and promote their apoptosis. Subsequently, we further found that LJD inhibited HER2 protein expression in a dose-dependent manner. Therefore, we speculate that the mechanism of LJD inhibiting proliferation, migration and promoting apoptosis is related to its inhibition of HER2 protein expression, but its microscopic regulation mechanism still needs to be further explored.

However, this study has several limitations. First, the SKBR3 cell line was selected as the research model for HER2-positive breast cancer due to its high expression of HER2. Nevertheless, the results from in vitro experiments based on

a single cell line may not fully reflect the heterogeneity of clinical tumors. Second, the absence of key molecular techniques such as qRT-PCR and Western blot limited the in-depth analysis of HER2 regulatory mechanisms. Although the network pharmacology predictions and in vitro experimental results corroborated each other, demonstrating LJD's potential to inhibit tumor cell proliferation and migration while inducing apoptosis through multi-target effects, its precise mechanism of action —particularly the specific regulatory pathways (transcriptional or post-translational levels) underlying HER2 downregulation —requires further elucidation. Future studies will integrate multiple cell lines, animal experiments, agonist/inhibitor interventions, and multi-omics technologies to systematically investigate the in vivo efficacy and molecular mechanisms of LJD. This will provide more comprehensive experimental evidence for developing novel HER2-targeted therapeutic strategies and advance the translational application of traditional Chinese medicine in precision breast cancer treatment.

Conclusions

Network pharmacology results suggest that LJD has a multi-component, multi-target, and multi-pathway mode of action in the treatment of HER2-positive breast cancer, with the core target being the HER2 protein. In vitro cell experiments confirm that LJD can inhibit the proliferation and migration of SK-BR3 cells and promote their apoptosis. The mechanism may be related to the regulation of HER2 protein expression. Therefore, LJD is a potential decoction for the treatment of HER2-positive breast cancer with high research space.

Data Sharing Statement

Data supporting the findings of this study can be available from the corresponding author upon a reasonable request.

Ethical Statement

This study was approved by the Ethics Committee of Hebei University of Chinese Medicine (Approval No. DWLL202303034). Animal experiments strictly adhered to international and national ethical guidelines, including the 3R principles (Replacement, Reduction, Refinement), the World Organisation for Animal Health (OIE) Animal Welfare Standards, China's Animal Management Regulations and Guidelines for the Humane Treatment of Laboratory Animals, as well as the Helsinki Declaration for biomedical research involving animals and cell experiments utilized certified cell lines from authorized sources, complying with ethical standards.

Network Pharmacology Analysis: This study utilized publicly available data from databases such as HERB, TTD, GeneCards, and DrugBank for analysis. According to items 1 and 2 of article 32 of the Measures for "Ethical Review of Life Science and Medical Research Involving Human Subjects" (issued in China on February 18, 2023): Ethical review can be waived for human life science and medical research that uses human information data or biological samples in the following situations, provided that no harm is caused to the human body and no sensitive personal information or commercial interests are involved.

This waiver aims to reduce the unnecessary burden on researchers and facilitate research in life sciences and medical studies involving human subjects.

(1) Research utilizing legally obtained public data, or data generated through observation without interfering with public behavior;

(2) Research conducted using anonymized information data;

Our study meets the above conditions and is therefore exempt from approval.

Funding

This research was supported by the National Natural Science Foundation of China (Grants No. 81603412); Hebei Natural Science Foundation (Grants No. H2024423105); Scientific Research Project of Hebei Administration of Traditional Chinese Medicine (Grants No. 2023045, 2024023); Hebei Province "three three three talent project" funded project (Grants No. A202002008); Scientific Research Project of Health Commission of Hebei Province (Grants No. 20220962, 20240282); Hebei Institute of Traditional Chinese Medicine Pharmaceutical Preparation Industry Technology Special Project (Grants No. YJY2024006); General Projects for Improving Scientific Research Capacity of Hebei College of Traditional Chinese Medicine (Grants No. KTY2019009).

Disclosure

The authors declare no conflicts of interest in this work.

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