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Network pharmacology to unveil the biological basis of gy ii in breast cancer treatment

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ABSTRACT

Gubenyiliu II, GY II for short is a representative type of Traditional Chinese Medicine (TCM) widely used for breast cancer treatment with good outcomes in China for several decades.

However, the biological basis of GY II remains to be holistically elucidated. In this study, an experimental method of network pharmacology and experiments *in vitro* were proposed to explore more exact efficacy and potential mechanisms of GYII. First, corresponding potential target genes both for GII components and breast cancer were extracted from established databases. 205 compounds in13 herbs of GY II and 265 antitumor or immune-related genes were investigated based on network-based, large-scale target prediction. Furthermore, with intersection genes of GYII and breast cancer were mapped, the Protein-Protein Internetwork (PPI) network of shared genes was constructed. Then Kyoto Encyclopaedia of Genes and Genomes (KEGG) functional annotation clusters were acquired and presented as top 25 pathways.

Meanwhile 3 typical ingredients, such as kaempferol, luteolin and quercetin of all the components, were verified by high performance liquid chromatography (HPLC). At last, the cell viability, proliferation, migration, cycles, apoptosis, the changes of cell morphology, molecular mechanism of TCM inhibiting tumor cell in vitro experiments were carried out for efficiency verification. In conclusion, this study suggested great potentials in tumor immune microenvironment regulation and tumor prevention of GY II herbs generally, and provided a systematic strategy for unveiling the commonness in the biological basis of GY II in breast cancer treatment.

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Introduction

The breast cancer is a prevalent case that is recurrent and long-distance metastasis. It is one of the most serious threatening diseases for women all over the world and usually leads to decreased qualities of life. China has a long history of using traditional Chinese medicine (TCM) for treating breast cancer. With increasing scientific evidence in medical research and clinical trials, the use of TCM in cancer treatment is gradually being recognized as a complementary and alternative therapy all over the world.[1-3] GY II is a representative type of TCM widely used for breast cancer treatment in China for several decades in Beijing Hospital of Traditional Chinese Medicine. A large amount of medication experience and clinical cases have proved the effectiveness of GY II, which can improve patients' 5-year survival rate and quality of life.

Traditionally, TCM adopts a relative and holistic point of view in breast cancer treatment. In TCM, breast cancer is caused by Qi Deficiency and Blood Stasis, while Toxin is considered the primitive motivation.[4-8] The GY II is used to treat breast

cancer under the basic principle "Tonifying Qi, Activating Blood Circulation and Detoxification" and applied clinic oncology doctors in our hospital for more than fifties years. In GY II, the herbs are divided into three categories according to function, health-strengthening herbs such as Codonopsis Radix, Poria Cocos(Schw.) Wolf., Atractylodes Macrocephala Koidz., Fructus Ligustri Lucidi, Lycii Fructus, Epimrdii Herba and Hedysarum Multijugum Maxim, blood-activating herbs such as Chuanxiong Rhizoma, Spatholobus Suberectus Dunn and Curcumae Rhizoma, and toxicity-reducing herbs such as Fritillariae Thunbrgii Bulbus, Sophorae Flavescentis Radix and Herba Sarcandrae.

The treatment strategy by strengthening health reflects the classical therapeutic theory that "pathogenic-qi cannot invade the body if health-qi remains strong" in the Canon of Internal Medicine (Huangdi Neijing).[9] Health-strengthening prescriptions are widely applied in cancer treatment, such as Sijunzi(consisted of Codonopsis Radix, Poria Cocos(Schw.) Wolf., Atractylodes Macrocephala Koidz. and Glycyrrhiza uralensis Fisch.) decoction in colorectal cancer,[10] and Shenqi Fuzheng injection(consisted of Codonopsis Radix and Hedysarum Multijugum Maxim.) in colorectalcancer and breast cancer.[11,12] Additionally, blood-activating herbs were proved to have a good effect on cancer treatment in many experimental studies, such as new Isoflavanes from Spatholobus suberectus were found to have significant cytotoxicity against both MCF-7 and MDA-MB-231 cell lines.[13] And panax notoginseng was proved to inhibit tumor growth through activating macrophage to M1 polarization.[14] The reducing toxicity herbs are also believed to have a good cancer treatment. For example, it is be certified that iRadix Sophorae Flavescentis can induce apoptosis through by caspase, MAPK Activation and ROS signaling pathways in 5637 human bladder cancer cells.[15]

A large amount of research effort has been put into the studies on the biological basis of TCM from a variety of perspectives, such as their immune, metabolic regulatory and inhibit tumor effects. However, the understanding of the antitumor mechanism of TCM is not clear enough,[16,17] which is constrained by the following three interrelated factors: the complex composition, the lack of target information, and the complex biological system involved in cancer development.

The advent of the big data era, Network pharmacology approach was proposed, which changed the current research mode of "single target" and provided a potential research strategy for analyzing the biological basis of TCM. The concept is consistent with the multitarget characteristic of TCM and makes it suitable for studying the complex mechanism of TCM,[18,19] which provides a new research paradigm for translating TCM from an experience-based medicine to an evidence-based medicine system. It guides the discovery of new active ingredients in TCM from the perspective of networks. For instance, the pharmacological mechanism of Yanghe Decoction on HER2-positive breast cancer were explored by a network pharmacology approach, which set up 4 networks and found some potential anti-cancer compounds, including HER2-positive breast cancer network, compound-compound target network of YHD, YHD-HER2-positive breast cancer network and compound-known target-HER2-positive breast cancer network. [20]

To further expound the application of TCM in the treatment of cancer from a holistic point of view, in this study, we took the GY II as an example to explore the therapeutic effects in their biological basis of in breast cancer. In this research, 205 TCM compounds and 265 gene targets were found with network pharmacology prediction methods. And the 48 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways involved in the antitumor or immunological activities of the TCM compounds are identified.[21] Despite the need for further investigation, it was indicated that GY II may provide researchers with a valuable candidate library for tumor immune regulatory and tumor preventive drug development.

Material and methods

TCM Compounds Data Preparation and Target Prediction

We collected compound information of 13 TCM herbs in GY II that are widely used for breast cancer treatment for several decades in Beijing Hospital of Traditional Chinese Medicine. The 13 TCM herbs include different categories, such as health-strengthening herbs (qi-tonifying (Yi-Qi) herbs), blood-activating herbs(stasis dissolving (Huo-Xue-Hua-Yu) herbs) and toxicity-reducing herbs(heat clearing and detoxifying (Qing-Re-Jie-Du) herbs), according to the traditional classification based on TCM efficacy. The obtained ingredients are entered into the TCMSP platform (http://tcmspw.com/tcmsp.php) matching information, and the condition for screening active medicinal ingredients is oral bioavailability (OB)≥30% and drug-likeness (DL)≥0.18[22]. The molecular structure of the active pharmaceutical ingredients sdf were obtained on the TCMSP platform, and its potential targets were predicted on the phammapper platform[23]. Together, 205 ingredients of herbs in GY II were identified and canonical SMILES for each ingredient was searched in TCMSP, with which as ingredient ID, corresponding targets for each ingredient ID were predicted in TCMSP database. And 265 targets of active components were collected and scanned in UniProt(http://www.uniprot.org/), which originated from "Human" and prepared for the following study work.

Target Prediction for the TCM Compounds Applied in Cancer Treatment

Major significant breast cancer target genes were obtained from Therapeutic Target Disease (TTD, http://db.idrblab.org/ttd/), Drug Bank(http://www.drugbank.ca/), and Disease Gene NET (DisGeNET, http://www.disgenet.org/web/DisGeNET/menu/home).[24-26] Totally, 422 targets were found and scanned in UniPro, which originated from "Human". Taking advantage of the prediction and assay results, we compared the common targets in active components and breast cancer, and scanned the targets in in the

upstream in a KEGG pathway of the biomolecules. The indirect relationship was established if the predicted target is same or in the upstream in a KEGG pathway of the biomolecules, or if they are related via PPI[27]. The cover rate was calculated as |The predicted targets related to the reported targets (or DEGs)/The predicted targets| × 100%.

Literature Mining

We obtained the related biomolecules for each TCM ingredient and to compare the results with the predicted targets in PubMed database. The co-occurrence of compound and target appearing in one or more abstracts was used to define the association of them. The results from the literature mining were then verified via a manual examination by deleting the false positive responses.

Analysis Workflow Based on Network Pharmacology

In this study, we proposed an approach based on network pharmacology to study the antitumor mechanisms of herbs in GY II. The "active ingredient-target" and cancer targets network is mainly constructed and analyzed by software Cytoscape 3.7.1[28]and applied for predicting the potential targets of the TCM compounds and to visualize the analysis results as networks in this manuscript. Among them, "node" is used to indicate the component or target, and "edge" is used to indicate the relationship between them. The the network characteristics, including Degree, Betweeness, and Closeness, were used to study the more important components and targets of the relationship.

KEGG Pathway Enrichment Analysis

We performed the KEGG pathway enrichment analysis for the predicted targets of the TCM compounds applied in cancer therapy in order to identify their biological functions. We used a hypergeometric test for enrichment analysis. We performed target enrichment under the background of 13388 human proteins and checked the p-values of the pathways to see if they were significantly enriched[9]. The enrichment p-values of 48 pathways from cancer hallmarks and immune-related pathways in the KEGG database were examined. The significantly enriched KEGG pathways ($p < 0.05$, fdr adjusted) were retained for further research.

Content determined by High Performance Liquid Chromatography (HPLC) Preparation of GY II, reagents and Apparatus 13 herbs of GY II, such as Codonopsis Radix, Poria Cocos(Schw.) Wolf., Atractylodes Macrocephala Koidz., Fructus Ligustri Lucidi, Lycii Fructus, Epimrdii Herba, Hedysa*rum Multijugum Maxim, Chuanxiong Rhizoma, Spatholobus Suberectus Dunn, Curcumae Rhizoma, Fritillariae Thunbrgii Bulbus, Sophorae Flavescentis Radix* and *Herba Sarcandrae*, all of which were purchased by prescription dose as ready-to-use Yinpian from Beijing Hospital of Traditional Chinese Medicine, Capital Medical University. Yinpian of GY II herbs were made into decoction formulation before transferred into Vacuum cryogenic freeze dryer (Epsilon 2-4LSC, Chris, German). GY II was kept in closed container with silica gel to keep dry. The kaempferol, luteolin and quercetin were provided by the Chinese Food and Drug Inspection Institute (97.0%, 93.2%,98%, Beijing, China). HPLC grade acetonitrile was purchased from the ANPEL Scientific Instrument Co., Ltd.(Shanghai, China). Methyl alcohol was from Shanghai Lingfeng Chemical Regent Co., Ltd. (Shanghai, China), and phosphoric acid was from Sinopharm Chemical Reagent Co., Ltd. Other chemicals were all analytical grade. Water was purified by Milli- Q System (Millipore, Merck, USA). HPLC analysis was conducted with an LC-2010HT HPLC System (SHIMADZU, Japan). The thermostatic bath was from Tianjin Taisite Co., Ltd. 98-1-B Series with 220 V, 50 Hz. The analytical balance from Shanghai Jingketianmei Co., Ltd. was used for sample weighing.

High-performance liquid chromatography conditions

The column was a TC- C18 column (4.6 mm×250 mm, 5μm), and the mobile phase was a mixture of acetonitrile(A) and 1.0 g/L phosphoric acid solution(B) in a gradient elution mode. The gradient procedure was set as follows: 5%–95% B in 0–80min; 95%–5% B in 80–90min. The flow rate was 1 mL/min, the column temperature was 35°C, the detection wavelength was 254 nm, and the injection volume was 20 μL.

Cell Culture

The MDA-MB-231 human breast cancer cell lines (Cell Center of the Medical Research Institute of the Chinese Academy of Medical Sciences, Beijing, China) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco®) supplemented with 10% fetal bovine serum (FBS) (Gibco®) and 1% penicillin-streptomycin (Hyclone, Logan, UT, USA). All cells were cultured at 37 ◦C in a humidified incubator (Sanyo, Osaka, Japan) with a 5% CO2 atmosphere.

Cell Viability Assay

Inhibition effect of traditional Chinese medicine and its monomer and chemotherapy drugs on tumor cells. The cell viability was determined by bromination method (MTT) of 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazole. The cells were seeded into 96-well plates (Corning, New York, USA) at a density of 3 × 103cells/well (9.375 × 103cells/cm2), incubated overnight at 37 C and 5% carbon dioxide, and were exposed to various concentrations of drugs, of which the GYII drug concentrations were 1, 2, 4, and 8 (mg/ mL); Kaempferol (K) drug concentration of 20, 40, 80, 160 (unit: umol); Luteolin (L) drug concentration of 20, 40, 80, 160(unit: umol); Quercetin (Q) drug concentration of 20, 40, 80, 160 (unit: umol); Epirubicin (E) 20, 40, 80, 160 (unit: ug/ml), the cultivation of 24 hours, respectively. After adding 15 μL of 5 mg/mL MTT (Sigma-Aldrich, St. Louis, MO, USA) solution, the cells were cultured for 4 h. After removal of the solution, the cells were lysed in 150 μL DMSO and absorbance was measured at 570 nm using a microplate reader (Thermo Scientific, Waltham, MA, USA). The

optical density (OD) was used to calculate the cell viability after treatment with different concentrations of a variety of drugs in different concentrations.

Migration Assay

First, lay the six-hole plate with marker behind the six-hole plate, compare it with a ruler, and draw horizontal lines evenly, about every 1cm to cross the hole. MDA-MB-231 cells were added into the 6-well plate.The second day after full, with a spear than the ruler, as for the orifice diameter horizontal scratch, washing cells with PBS 3 times, than join the drug-containing medium, including drug concentration of GYII, the monomer and the chemotherapy drugs respectively(concentration: IC50 alue concentration), 5% CO2 incubator in 37 degrees, develop photos after 24 hours, observe the cell migration.

TCM interferes with tumor cell cycle, apoptosis and the changes of cell morphology

Detection of tumor cell cycle

Cell lines treated with different drugs for 24h were collected and digested with 0.125% trypsin for 2min. Digestion was stopped with complete medium and centrifuged at 1500rpm for 5min. Resuspend with 2-3mlPBS, wash once, and centrifuge at 1500rpm for 5min.The supernatant was discarded, the cells were gently flipped open, and the pre-existing 70% ethanol at -20□ was added for fixation for 24h. Centrifuge at 1500rpm for 5min, discard the supernatant, add 2-3mlPBS to resuspend, wash once, and centrifuge at 1500rpm for 5min. The cells were resuspended with 500μLPBS, 2μL RNase A (5mg/mL), and incubated at 37□ for 45min.(final concentration of RNase A was 20μg/mL). Add 1mg/ mL PI 30-50μl and incubate at 4□ for 1 hour.(The final concentration of PI reached 50μL/mL).Loss cytometry.

Detection of apoptosis, SSC, FSC

Cell lines treated with different drugs for 24h were collected and digested with 0.125% trypsin for 2min. Digestion was stopped with complete medium and centrifuged at 1500rpm for 5min. Wash the cells twice with PBS (scatter the cells with fingers and gently blow them with a gun for mixing). Wash the resuspend cells for the second time and collect them into a 1.5MLEP tube. After centrifugation, discard the supernatant, add 500uL Binding Buffer, suspend the cells and place them on ice. After adding 5uL Annexin V-FITC mixture, 5uLPI was added and gently mixed. The reaction was carried out at room temperature and protected from light for 15min. The cells were filtered through a 300-mesh filter into the upper sample tube of flow cytometry, than detected Cell apoptosis, cell size and organelle complexity by flow cytometry.

At the early stage of apoptosis, due to the exposure of phosphatidylserine and the intact cell membrane, the cells showed positive Annexin V-FITC staining and negative PI staining. The necrotic or late apoptotic cells were positive for Annexin V-FITC staining and positive for PI staining. Flow chart shows early apoptosis on the lower right and late apoptosis on the upper right. Necrotic cells on the upper left and living cells on the lower right.

Molecular mechanism of Chinese medicine inhibiting tumor cell proliferation after 24 h exposure to the drug (the drug concentration was GY II, the monomer concentration, and the IC50 value concentration of the chemotherapeutic drug), 231 cells were collected and lysed with RIPA lysis buffer (Beyotime Biotechnology, Beijing, China) containing a protease inhibitor (Calbiochem, San Diego, CA, USA) and a phosphatase inhibitor. The cell lysate was centrifuged at 12000 RPM at 4□ for 15 min, and the supernatant was collected. Pierce BCA assay kit (Thermo Scientific, Rockford, IL, USA) was used to determine the protein concentration. The same amount of total protein (45 g/lane) was separated by 12% SDS-PAGE and transferred to PVDF membrane. The membrane was sealed with 5% BSA (Amresco Solon, oh, USA) at room temperature for 1 h and incubated with a primary antibody at 4 □ for 24h. Antibody was anti-ERK/p-ERK (1:1000). The membrane was rinsed in TRIS buffer brine (TBST) containing 0.1% Tween-20 and detected at room temperature for 1 h with 1:10,000 secondary antibodies. These signals were detected using the Odyssey Infrared Imaging System (Li-COR Biosciences, Lincoln, NE, USA). The relative density of protein bands was determined by Odyssey Version 3.0 software (Li-Cor Biosciences). The experiment was repeated three times.

Statistical Analysis

Data are shown as the means ± standard deviation (SD) and median ± interquartile range. Multiple types of data were used in the manuscript and various statistical analysis methods were applied. Statistical analysis was performed using Kolmogorov-Smirnov (KS) test, Wilcoxon rank sum test and Student t-tests. The significance levels were set at * $p < 0.05$, ** $p < 0.01$.

Results

The Prediction and Examination of Potential Targets of GYII by network pharmacology analysis due to the complex composition of TCM and the lack of corresponding target records, the potential target lists of TCM compounds were obtained by the TCMSP platform and predicted on the phammapper platform. For each investigated TCM compound, we searched the PubMed database by its name in the abstracts and selected the compounds with adequate related reports (total item number ranging from 500 to 1000) for the verification of target prediction results. The results from literature mining were then verified via manual examination by deleting the false positive responses.

Together, 205 ingredients of herbs in GY II were identified and canonical SMILES for each ingredient was searched in TCMSP, with which as ingredient ID, corresponding targets for each ingredient ID were predicted in TCMSP database. And 265 targets of active components were collected and scanned in

Table1 Top 26 pathways enriched from the shared target genes.

UniPro, which originated from 'Human' and prepared for the following study work. Target Prediction for the TCM Compounds Applied in Cancer Treatment Major significant breast cancer target genes were obtained from Therapeutic Target Disease(TTD), Drug Bank, and Disease Gene NET (DisGeNET,). Totally, 442 targets were found and scanned in UniPro, which originated from 'Human'. Taking advantage of the prediction and assay results, we compared the common targets in active components and breast cancer, and scanned the targets in in the upstream in a KEGG pathway of the biomolecules. The enrichment p-values of 48 pathways from cancer hallmarks and immune-related pathways in the KEGG database were examined. The significantly enriched KEGG pathways ($p < 0.05$, fdr adjusted) were retained for further research.

Using network pharmacology analysis, we discovered that 4 findings, as Fig.1:

a. Functionally, the predicted targets of compounds from GY II were enriched in both immune-related and antitumor pathways. And some ingredients are common to many herbs, such as quercetin, luteolin, β-sitosterol, formononetin, Stigmasterol, kaempferol, glycitein, Calycosin, Anhydroicaritin, 8-Isopentenyl-kaempferol. This phenomenon explains the synergistic effect between herbs and herbs to some extent(Xiang Xu Xiang Shi).

b. Moreover, the same target can be treated with multiple components, which can be found in one or more herbs, such as Androgen receptor, Apoptosis regulator Bcl-2, Aryl hydrocarbo receptor, ATP-binding cassette sub-family G member 2, Beta-2 adrenergic receptor, Carbonic anhydrase II, Cyclin-dependent kinase inhibitor 1, DNA topoisomerase 1, DNA topoisomerase 2-alpha, DNA topoisomerase II, Epidermal growth factor receptor, Estrogen receptor, Estrogen receptor beta, Neuronal acetylcholine receptor protein, alpha-7 chain, RAC-alpha serine/ threonine-protein kinase, Transforming growth factor beta-1, Tubulin beta-1 chain and Vascular endothelial growth factor A. This phenomenon further explained the synergistic effect between herbs and herbs(Xiang Xu Xiang Shi).

c. Furthermore, the same component can act on multiple target genes. According to statistics,

there are 47 such components, of which 31 can act on more than three gene targets. For example, the quercetin has 13 target genes such as Androgen receptor, Apoptosis regulator Bcl-2, Aryl hydrocarbon receptor. The luteolin has 7 target genes such as. The licochalcone A has 6. And kaempferol, 7-Methoxy-2-methyl isoflavone, Anhydroicaritin each have 5 target genes. To some extent, it explains the multi-target problem of TCM, except that one herb has multiple components.

d. Compounds in the same herb may exhibit the same or distinguished mechanisms in breast cancer treatment, which was demonstrated as the compounds influence pathway gene expressions in the same or opposite directions.

Together, this study suggested great potentials in tumor immune microenvironment regulation and tumor prevention of GY II herbs generally, and provided a systematic strategy for unveiling the commonness in the biological basis of GY II in breast cancer treatment.

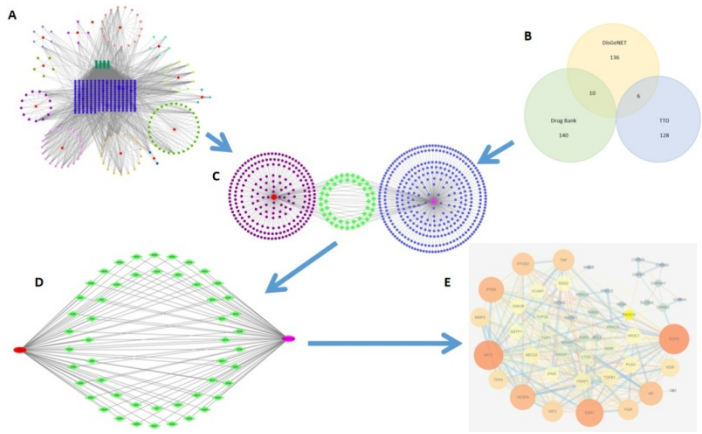

Fig.1. Network of GYII and breast cancer. (A) Targets of GYII ingredients. (B) Venn for breast cancer target genes from three most commonly used databases. (C) Target genes mapping for GYII and breast cancer. (D)Inserted genes shared by GYII and breast cancer. (E) PPI network constructed with shared target genes.

The content of kaempferol, luteolin and quercetin determined by High Performance Liquid Chromatography (HPLC)

Method validation

As shown in Table 2, the relative standard deviations (RSDs) of precision test ranged from 0.15%–0.80% (n = 6). The RSD of repeatability test varied from 1.02% to 1.15% (n = 6). The stability of the solutions was determined at 0, 2, 4, 8, 12, 24, and 48 h, and the RSD of the stability teat ranged from 0.58% to 1.15%. The average recovery rate of the kaempferol, luteolin and quercetin were 96.54% ± 0.03% and 105.16% ± 0.02%, respectively. Moreover, the RSD varied from 0.91% to 2.18% (n = 6). The above results revealed the repeatability and accuracy.

Table1 Top 26 pathways enriched from the shared target genes			
KEGG Term Description	**Gene Count**	**Background**	**FDR**
Pathways in cancer	18	515	2.16E-14
Estrogen signaling pathway	10	133	1.26E-10
Fluid shear stress and atherosclerosis	10	133	1.26E-10
Proteoglycans in cancer	10	195	1.53E-09
PI3K-Akt signaling pathway	10	348	1.71E-07
AGE-RAGE signaling			
pathway in diabetic	9	98	1.73E-10
Complications			
Cholinergic synapse	9	111	3.95E-10
MicroRNAs in cancer	9	149	3.39E-09
Hepatocellular carcinoma	8	163	1.21E-07
Neuroactive ligand-receptor			
8		272	3.35E-06
Interaction EGFR tyrosine kinase inhibitor resistance	7	78	3.04E-08
HIF-1 signaling pathway	7	98	1.12E-07
Prostate cancer	7	97	1.12E-07
Breast cancer	7	147	1.10E-06
Calcium signaling pathway	7	179	3.29E-06

Table2 Method validation for the determination of 3 compounds by HPLC.

Peak No.	Compounds	Regression Equation	Test range (μg/ml)	Precision experiment		Repeatability experiment		Stability experiment		Recovery experiment	
				Area of peak	RSD (%)	Area of peak	RSD (%)	Area of peak	RSD (%)	Average recovery rate(%)	RSD (%)
18	Luteolin	y=7.6074x+24.417 R^2=0.9997	5-500	153.02	0.49	143.02	1.10	142.96	1.01	96.54	0.93
20	Quercetin	y=10.957x-19.351 R^2=0.9993	5-500	175.06	0.80	165.06	1.15	184.66	1.15	105.16	2.18
24	kaempferol,	y=8.907x-17.351 R^2=0.9992	5-500	504.06	0.15	484.06	1.02	569.66	0.58	93.54	0.91

Note: Each value represented in tables are means±SD (n=6). Three compounds were identified by their retention times (min): Luteolin (23.5, peak 18), Quercetin (26, Peak 20), Kaempferol (28.6, peak24)

Discussion

This should explore the significance of the results of the work.

HPLC fingerprint analysis and determination of kaempferol, luteolin and quercetin to further confirm the composition of the GY II, we constructed a standard HPLC fingerprint.

We selected and marked 28 common peaks as characteristic peaks. Compared retention time and ultraviolet spectrum with standard references, peak18,20 and 24 were in conformity with luteolin, quercetin and kaempferol. The determination of luteolin, quercetin and kaempferol in samples were analyzed by HPLC according to China Pharmacopoeia (2015 edition). By comparing the retention time with standard substances, peaks 18, 20 and 24 were identified as luteolin, quercetin and kaempferol, respectively [Figure 2]. The contents of luteolin, quercetin and kaempferol are 0.28% (g/g) , 0.25% (g/g) and 0.63% (g/g), respectively.

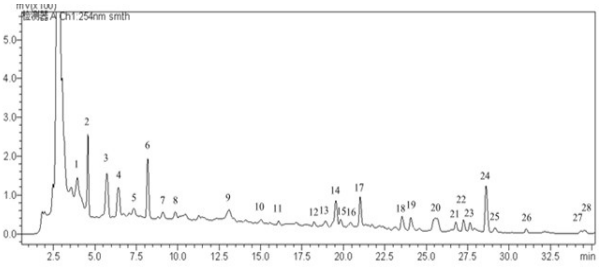

Fig. 2. HPLC chromatograms of GY II

The number of peaks in the HPLC chromatograms: Luteolin (23.5, peak 18), Quercetin (26, Peak 20), Kaempferol (28.6, peak24).

Inhibition of GYII on proliferation and migration of triple negative breast cancer cells we conducted a series of in vitro experiments to investigate the effects of GYII and three monomers on the proliferation of breast cancer cells and compared them with the chemotherapeutic drug epirubicin. First, the viability of MDA-MB-231 breast cancer cells was detected by 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazole-2 bromination (MTT) assay. Cells cultured with different concentrations of GYII, monomer and chemotherapeutic drug intervention showed dose-dependent inhibition (Figure 3A). Inhibition is rapid and persistent.In contrast, GYII had a mild inhibitory effect on MDA-MB-231 cells (Fig. 3A).Because Kaempferol (K), Luteolin (L), Quercetin (Q) and Epirubicin (E) had high GYII sensitivity to MDA-MB-231 cells. (IC50) were g/mL, respectively, for subsequent studies.

Differential Inhibitory Effects of GYII and Its Decomposed monomer on 231 Cell Migration the scratch assay was used to investigate the effects of GYII, its three monomers and chemotherapeutic agents on breast cancer cell migration.Under the intervention of half inhibitory concentration of GYII, monomer and chemotherapeutic drugs, cultured cells for 24h, the same pharmacodynamic concentration of GYII and monomer significantly inhibited cell migration, and the chemotherapeutic drugs also had a corresponding effect on cell migration, but not obvious Fig3B,C.

Effects of GYII and Its Decomposed monomer on cell morphology and size, cell cycle and organelle complexity Flow cytometry was used to detect the effects of GYII, its three monomers and chemotherapy drugs on the cell morphology, size and cell cycle of breast cancer cells. GYII in half inhibitory concentration, monomer and effective drug intervention culture cell under 24 h, the cells were collected for the corresponding cell cycle process（Fig4A,B）, each cycle of cells, cell size and cell organelle complexity has carried on the statistical analysis, under the same drug concentration of

GYII, monomer to not significantly affect the cell morphology and organelles complexity,

（ Fig3D,E ） on the contrary, chemotherapy drugs can cause cell volume increased obviously, intracellular organelles is more complicated.

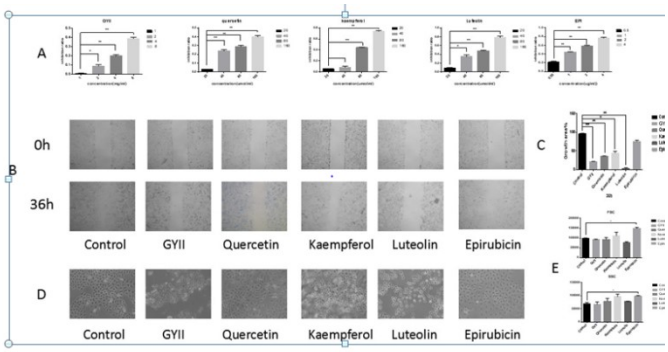

Fig.3. GYII effectS on proliferation, migration and morphologic changes of tumor cells. (A) The inhibition rate of the drug to the tumor. (B) (C)Inhibition of tumor migration by the drug for 24 hours. (D)The effect of drugs on the morphology of tumor cells. (E) changes of cell size and organelle complexity after drug treatment for 24h by flow cytometry.

The effect of GYII and Its Decomposed monomer on cell apoptosis Similarly, flow cytometry was used to detect the effects of GYII, its three monomers and chemotherapy drugs on apoptosis of breast cancer cells.The cells were cultured for 24h with half inhibitory concentration of GYII, monomer and chemotherapeutic drugs, and the cells were collected for corresponding apoptosis treatment. GYII at the same pharmacological concentration could significantly promote cell apoptosis, whereas Kaempferol (K), Luteolin (L), Quercetin (Q) and chemotherapy drug epirudoxorucin had no significant effect on cell apoptosis.（Fig4C,D）

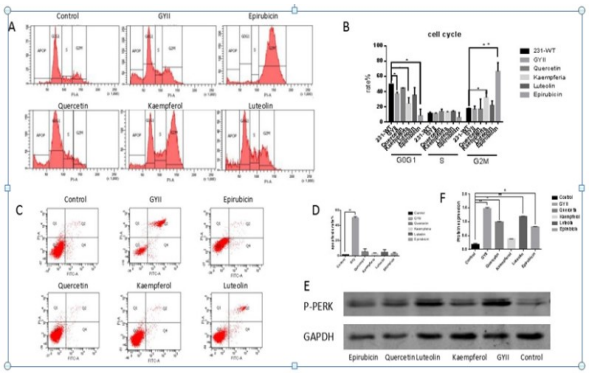

Fig.4. Effects of GYII,each monomer and chemotherapy drug on tumor cell cycle, apoptosis and protein pathway. (A)(B) Cell cycle changes in tumor cells after drug intervention for 24h. (C)(D)Proportion of apoptosis of tumor cells after drug intervention for 24h. (E)(F) GYII and its monomer regulated the extracellular signal-regulated kinase (ERK) pathways in 231 cells. Cells were treated with the test drugs and the levels of p-ERK were detected by Western blotting. Data are presented as the mean ± SD and normalized to GAPDH (* $p < 0.05$,** $p < 0.01$). Effects of GYII and Its Decomposed monomer on the ERK Signaling Pathways ERK pathway, including ERK1 and ERK2, is the key to transduction of signals from surface receptors to the nucleus. On the one hand, ERK pathway is involved in tumor cell proliferation and differentiation to promote tumor proliferation; on the other hand, it is involved in a variety of biological reactions such as cell morphology maintenance and cell apoptosis. GYII and its monomer can overexpress phosphorylated ERK in tumor cells after 24 h treatment.（Fig4E, F）

Discussion

Breast cancer is an important cause of female death. Currently, breast cancer therapeutic schedule include radiotherapy and chemotherapy, endocrine therapy, targeted therapy, etc., all of which are more or less limited by cellular toxicity, systemic symptoms, therapeutic resistance, etc. In this paper, the integrality and potential therapeutic targets of TCM in the treatment of breast cancer were explored through network pharmacology and TCM theory, so as to better guide clinical medication.

GYII is the classic prescription which is strictly compatible, with the basic principle “Tonifying Qi, Activating Blood Circulation and Detoxification”. The network pharmacology showed that the active components in GYII, such as quercetin, luteolin, kaempferol, astragaloside IV, etc., were extremely rich in targets, which could regulate cell cycle, apoptosis, transporters, angiogenesis, tumor microenvironment (cytokines, extracellular vesicles, autophagosomes, etc.).Traditional Chinese medicine prescription is a complex body with multiple components and multiple targets, and the mechanism of anti-tumor action on breast cancer is also the result of the joint action of multiple pathways.Pharmacology showed that quercetin has anti-inflammatory, anti-oxidation, can inhibit tumor cell proliferation, angiogenesis, promote apoptosis and so on. The small molecule quercetin were confirmed that Lef1 inhibition,decreased the expression of Lef1 and resensitized cells to docetaxel. Meanwhile the Lef1 inhibition also downregulated ABCG2, Vim, and Cav1 expression and equally decreased Smad-dependent TGF-β signaling pathway activation.[29] It is found that the flavonoid luteolin exerts a significant cytotoxic effect on the colon cancer cell line HCT116 and the breast adenocarcinoma cell line MDA-MB231, by inducing both apoptotic and necrotic cell death, and that this effect is not impaired by HIF-1 activation.Interestingly, luteolin induces a decrease in HIF-1 transcriptional activity. This is accompanied by a decrease in the levels of protein markers of stemness and invasion, and by a reduction of migratory capacity of the cells.[30] Kaempferol

displays several pharmacological properties, among them antimicrobial, anti-inflammatory, antioxidant, antitumor, cardioprotective, neuroprotective, and antidiabetic activities, and is being applied in cancer chemotherapy. Specifically, kaempferol-rich food has been linked to a decrease in the risk of developing some types of cancers, including skin, liver, and colon. The mechanisms of action include apoptosis, cell cycle arrest at the G2/M phase, downregulation of epithelial-mesenchymal transition (EMT)-related markers, and phosphoinositide 3-kinase/protein kinase B signaling pathways.[31] Molecular mechanics based MM-GBSA was used to validate docking results. The analyses of the docking showed a favorable interaction between kaempferol and the catalytic-important aminoacyl residues, especially GLU396, LEU398 and ASP458 in the ATP-binding site of PAK4 when compared with what was obtained in the 4T6-PAK4 complex.[32]

In our earlier study, we investigated the molecular mechanisms underlying the beneficial effects of GYII on murine breast cancer models. GYII showed significant inhibitory effects on tumor growth and metastasis in the murine breast cancer model. A better inhibitory effect on 4T1 cell proliferation and migration was found in the decomposed recipes (DR) of GYII. Moreover, heparanase expression and the degree of angiogenesis were reduced in tumor tissues. we decreased expression of heparanase and growth factors in the cells treated with GYII and its decomposed recipes (DR2 and DR3), and thereby a reduction in the phosphorylation of extracellular signal-regulated kinase (ERK) and serine-threonine kinase (AKT). These results suggest that GYII exerts anti-tumor growth and anti-metastatic effects in the murine breast cancer model. The anti-tumor activity of GYII and its decomposed recipes is, at least partly, associated with decreased heparanase and growth factor expression, which subsequently suppressed the activation of the ERK and AKT pathways.[33] It also can interfere tumor proliferation by activating autophagy to unblock collaterals and inhibiting angiogenesis.[34]

Although TCM prescriptions have a long history and remarkable clinical effects, the complexity of their components has hindered the further study of TCM. An important finding of this study is that Chinese medicine compound, monomer and chemotherapy drugs can inhibit tumor proliferation. Compared with chemotherapeutic drugs, the tumor cells increased significantly under the action of epirubicin at the same concentration of drug (IC50 value), and the intracellular organelles became very complex.On the contrary, the morphology of cells and the complexity of organelles did not change significantly under the treatment of TCM and monomer. Moreover, Chinese medicine compound and monomer can inhibit tumor migration more effectively. Combined with the network pharmacological targets, it is suggested that Chinese herbal compounds may contain anti-cancer and anti-metastasis components, and the mechanism of tumor metastasis is complex, including metastasis genes、epithelial mesenchymal transformation,and the cellular microenvironment includes the delivery of exosomes ect. The anti-metastasis mechanism of GYII deserves further study. In cell cycle and apoptosis experiments, we found that the mechanism of action of chemotherapeutic drugs is to interfere with cell cycle, but not affect apoptosis., Kaempferol can also block the cell cycle in the G2M phase. The main way of TCM compound inhibiting tumor is apoptosis, especially late apoptosis, which has no obvious effect on cell cycle。

The activation of ERK pathway in tumor proliferation of tumor biology functions such as the stability of cell morphology and cell apoptosis in joint action, this experiment, GYII has obvious evidence of tumor suppression and promote cell apoptosis, shows that the activation of ERK pathway is mainly promoted the apoptosis and maintain cell morphology.The downstream ERK pathway proteins that promote tumor proliferation need to be further verified.And to optimize the addition and subtraction of the compound and prescription, the research group will carry out the next optimization to guide the clinical more reasonable and effective.

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