Exploring the mechanism of an active ingredient of ginger, dihydrocapsaicin, on triple negative breast cancer based on network pharmacology and *in vitro* experiments

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Abstract. To investigate the potential mechanism of ginger in the treatment of triple-negative breast cancer (TNBC) based on network pharmacology, molecular docking and *in vitro* cell experiments. The Traditional Chinese Medicine Systems Pharmacology Database And Analysis Platform, the Bioinformatics Analysis Tool For Molecular Mechanism Of Traditional Chinese Medicine and the HERB database and literature search were used to search for the main active compounds of ginger. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analyses were used to predict the possible molecular mechanism and signaling pathway of ginger in the treatment of triple negative breast cancer. The key core genes of ginger in the treatment of triple negative breast cancer were docked with the active ingredients of ginger on the Autodock platform, and the mechanism of ginger on triple negative breast cancer was further verified by *in vitro* cell experiments. As a result, 10 effective components, 27 potential targets and 10 Protein-Protein Interaction core genes were predicted in the treatment of triple negative breast cancer with ginger, involving 287 biological processes, 18 cellular components and 38 molecular functions. Ginger regulated the proliferation, migration and apoptosis of triple negative breast cancer cells by regulating TNF, IL-17, FoxO, MAPK, PI3K/AKT and other signaling pathways. The results of molecular docking showed that the lowest binding potential energy between dihydrocapsaicin (DHC) and EGFR protein was -7.70 kcal·mol-1, followed by that between 6-gingerol and EGFR protein was -7.30 kcal·mol-1 and that between DHC and CASP3 protein was -7.20 kcal·mol-1. *In vitro* cell experiments showed that ginger could inhibit the proliferation and migration of TNBC MDA-MB-231 cells, increase the mRNA expression of Caspase family CASP9 and the protein expression of CASP3 and BAX. Overall, based on the combination of network pharmacology and *in vitro* cell experiments, ginger has the characteristics of multi-target in the treatment of TNBC, which may play a regulatory role through the PI3K/AKT family. It provides a reference for the drug development of ginger and the clinical treatment of triple negative breast cancer.

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Introduction

Triple negative breast cancer (TNBC) is a heterogeneous disease, more common in young women, 15-20% of all breast cancers (1). In 2011, Lehmann classified 587 TNBC Gene expression profiling into six subtypes: BL1, BL2, IM,M, MSL and LAR (2). In 2015, Burstein further used RNA and DNA analysis to distinguish specific targets expressed by the immune system to subdivide TNBC into Lar, MES, Blis, and Blia, enabling specific targeted therapies through subtype molecular expression differences (3). With the development of modern biological science and technology, TNBC typing has been refined gradually, to a certain extent, improve the cure rate of clinical TNBC or prognosis of patients. At present, anthracycline and platinum is still the main clinical treatment. The survival rate of patients after clinical treatment is lower than that of non-TNBC patients, and the postoperative prognosis is poor, easy to relapse and metastasis (4,5). Studying new treatment methods has become an urgent problem to be solved in TNBC clinical practice. It may be a good idea to find Chinese medicine with fewer side effects as an alternative or complementary therapy for triple-negative breast cancer.

Ginger as a medicinal and edible Chinese medicine, not only as spices and vegetables edible, medicinal history is very long, in many classic prescriptions are recorded (6). Ginger is planted in all parts of China. The medicinal part is the fresh rhizome of Zingiberaceae plant ginger (7), which have the effects of relieving cold, warming and stopping vomiting, resolving phlegm and relieving cough. It contains gingerol, gingerol, polyphenol, turmeric ketone and other bioactive components, which have been reported to have anti-inflammatory and anti-tumor effects (8-11), paclitaxel synergist has also been studied to improve the clinical treatment of TNBC (12).

Ginger's pharmacological effects and edible characteristics highlight its different effects on cancer and normal cells, which has broad prospects in clinical application. However, the anti-tumor mechanism of ginger is still unclear, and the potential biological pathways include but not limited to cell cycle regulation, apoptosis and chromatin regulation related to DNA damage (13-15).

With the rapid development of network technology and bioinformatics, network pharmacology is becoming a new tool to elucidate molecular mechanisms and pharmacological effects. It can effectively establish a compound-protein/gene-disease network and systematically explain the material basis and mechanism of traditional Chinese medicine. Therefore, this study used network pharmacology methods to predict the mechanism of ginger in the treatment of triple-negative breast cancer and further explored the possible molecular mechanism and pathway of ginger in the treatment of triple-negative breast cancer through in vitro cell experiments. In order to provide scientific reference for the clinical treatment of triple negative breast cancer and the drug development of ginger.

Materials and methods

Drugs. Dihydrocapsaicin (DHC) was purchased from Guangzhou Qiyun Biotechnology (PCS0367) .

Cells. Triple negative breast cancer cell line MDA-MB-231 was purchased from Wuhan Fuheng Biological Co., Ltd.

Reagents. Reagents used in this study were Fetal bovine serum (S-FBS-SA-015, SERANA company), DMEM high glucose medium (batch number 12100, SOLAIBAO), thiazole blue powder (batch number 298-93-1, SOLAIBAO), RNA extraction kit (batch number ER501-01, Beijing all-gold biological). A two-step fluorescence quantitative PCR kit (batch number AUQ-01, Beijing all-gold biology) RIPA tissue/cell lysate (batch number R0010, Suolaibao), BCA protein detection kit (batch number PC0020, Suolaibao), CASP3 (batch number ET1608-64, HUABIO), BAX (batch number T40051F, Abmart), β-actin (batch number TA811000, ORIGENE), HRP-labeled rabbit anti-mouse antibody (batch number WLA024a, ten thousand kinds of biology), HRP-labeled goat anti-rabbit antibody (batch number HA1031, HUABIO), 4x protein loading buffer (including DTT) (batch number P1015, Suolaibao), skim milk powder (batch number D8340, Suolaibao), ECL ultrasensitive chemiluminescence solution I (batch number KF005. ECL Hypersensitive Chemiluminescence Solution II (lot number KF001, Solebold).

Instruments. CO_2 incubator (Binde, Germany), biological inverted microscope (SOPTOP), high-speed refrigerated centrifuge (Sigma, USA), one thousandth analytical balance (Sartorius, Germany), multi-function microplate reader (Thermo), PCR instrument (BD, USA), multi-function imager (analytikjena, Germany), electrophoresis apparatus and membrane transfer instrument (Beijing Liuyi Biotechnology Co., Ltd.), ultrasonic cell disruptor (Ningbo Scientz Biotechnology Co., Ltd.) were used.

Methods

Ginger active ingredient collection screening and target prediction. Ginger was used as the key word to collect the active ingredients of ginger in TCMSP (16), BATMAN-TCM (17), HERB (18) database and literature search. The 3D structure diagram of the ingredients was downloaded on PUBCHEM [PubChem (nih.gov)] and uploaded to the Swiss ADME (http://www.swissadme.ch/) for prediction and screening of pharmacokinetics (ADME) (19). Ingredients that meet the following conditions are considered to be active ingredients retained for subsequent experiments. 1 GI absorption (gastrointestinal absorption) is shown as 'high' in the pharmacokinetic parameters; 2 Druglikeness (drug-likeness screening) in three or more consistent. Finally, the active ingredient SDF format was uploaded to Pharmmapper (http://www.lilab-ecust.cn/pharmmapper/) (20) for target prediction to obtain the target of ginger.

Triple-negative breast cancer target collection. Through the Genecards, OMIM, TTD, Drugbank, DisGeNET and other disease databases, the disease targets were collected with triple negative breast cancer as the keyword, and the triple negative breast cancer related targets were obtained after deleting the repeated targets. The data information was uploaded to Cytoscape 3.7.1 software to draw the network diagram of ginger-active ingredient-target-disease , and the Venn diagram was obtained by intersecting the targets of ginger and triple negative breast cancer.

Construction of Protein-Protein Interaction (PPI) Network and Screening of Key Genes. The intersection target of ginger and triple negative breast cancer was uploaded to the STRING database [STRING: functional protein association networks (string-db.org)] for protein-protein interaction. After selecting the confidence level of 0.400, the tsv file was exported and the cytoNCA plug-in in Cytoscape 3.7.1 was used to calculate betweenness centrality (BC), closeness centrality (CC) and degree centrality (DC). Hub genes in PPI protein interaction network were screened out.

GO analysis and KEGG analysis. Metascape (21) was used to perform pathway enrichment analysis on the overlapping targets of ginger and triple-negative breast cancer. The platform integrates multiple authoritative functional databases such as GO and KEGG, supports batch gene or annotation, enriches and analyzes proteins. Metascape was updated once a month to ensure the reliability of the data. The results were saved and visualized with R software 3.6.1 and Cytoscape 3.7.1 was used to draw the network diagram of ginger-key component-core target-pathway.

Molecular docking. In order to further determine the credibility of the relationship between triple negative breast cancer targets and ginger core components, molecular docking analysis was performed between ginger active components and four important targets in PPI network. From the Protein Database (PDB; https://www.rcsb.org/) Download protein crystal structure in pdb format from the chemical composition database [Pubchem; PubChem (nih.gov)] obtained the MOL2 structure file of the active ingredients of ginger. Proteins

prepare docking macromolecules by dehydration, hydrogenation, calculation of charge, and distribution of atomic types. Ligands were prepared by hydrogenation, removing lone pair electrons and establishing special rotatable bonds. AutoDock software is then used to set the binding pocket for molecular docking, using the original ligand of each target as a reference. Finally, each target protein was docked to the ligand using AutoDock Vina and binding energy data were obtained for analysis. Molecular docking score <-4.52 kJ/mol indicates that the ligand and the target have binding activity, score <-5.0 kJ/mol indicates good matching activity, score <-7.0 kJ/mol indicates strong docking activity (22).

DHC inhibits proliferation of triple negative breast cancer cells. MTT assay was used to determine the inhibitory effect of ginger on the proliferation of human triple negative breast cancer cells (MDA-MB-231, Zhongqiao Xinzhou, ZQ0118) at different concentrations. Logarithmic phase cells were collected and seeded into 96-well plates ($5x10^3$ cells/well) at 200 μl per well. After 24 h of culture, cells in each group of 6 wells were treated with different concentrations of DHC solution (0, 100, 150, 200, 250 μmol/l) for 12,24,48 h. After that, 20 μl MTT (5 mg/ml) solution was added to the well, and the cell incubator continued to incubate for 4 h. The supernatant was carefully removed and 150 μl of dimethyl sulfoxide DMSO was added to each well to dissolve the blue-violet methyl thiazoline crystal. Finally, the absorbance (A) of the experimental hole was measured at 490 nm using a microplate reader to calculate cell viability. Cell viability (%)=[(experimental group ODs value-blank group ODw value)/(control group ODc value-blank group ODw] x100%, where ODw is the absorbance of the blank group, ODc is the absorbance of the control group, and ODs is the absorbance of the experimental group. Cell proliferation inhibition rate=[(A control hole-A experimental hole)/(A control hole-A blank hole)] x100%.

DHC inhibits the migration of triple negative breast cancer cells. MDA-MB-231 cells in the rapid growth phase were collected to carry out the scratch test. Five straight lines were drawn at the bottom of the six-well plate before the cell seed plate to reduce the experimental error. When the cell fusion degree ≥90%, three straight wounds were drawn perpendicular to the bottom of the six-hole plate. The injured monolayer was washed three times with 1x PBS to remove cell debris, and incubated with ginger administration group and blank control group for 24 h. Axivert 40CFL Zeiss microscope was used to capture photographs of wounds and scratch areas at 0 and 24 h. By taking 0 h as 100% empty area, the percentage of cell coverage area can be calculated by Image J image analysis software. All experiments were performed in three parts.

DHC increases caspase family CASP9 mRNA expression. When the cell seed plate grew to 70-80%, the drug was administered. After 24 h, the cells were collected and RNA was extracted using the RNA extraction kit. The RNA purity was measured when the A260/A280 range was 1.8-2.0. The RNA purity standard can be used for subsequent experiments. According to the instructions of PerfectStart® Uni RT & qPCR Kit, the collected RNA and the kit-related reagents were added to synthesize cDNA and remove gDNA. After the cDNA was obtained, the upstream and downstream primers were added and the qPCR SuperMix containing DNA polymerase and nucleotide was configured with the kit. The expression of CASP9(Forward primer: 5'-GCAGGCTCTGGATCT CGGC-3'; Reverse primer: 5'-GCTGCTTGCCTGTTAGTT CGC-3') mRNA was determined by PCR, β-Actin (Forward primer: 5'-CGTTGACATCCGTAAAGACC-3'; Reverse primer: Reverse: 5'-TAGAGCCACCAATCCACACA-3') expression was used as an internal reference to verify equal concentrations of cDNA in each sample. All processes and experimental consumables need to be sterile and enzyme-free. β-Actin expression was used as an internal reference to verify equal concentrations of cDNA in each sample.

DHC increases CASP3 and BAX protein expression in triple negative breast cancer cells. The cells were treated when the cell seed plate grew to 70-80%. After 24 h, the cells of each group were collected for lysis and protein extraction. The protein concentration was determined using the BCA protein assay kit and diluted to a uniform level. The protein structure is destroyed by adding protein loading buffer and high temperature boiling to denature the protein. Configure 12% concentration separation gel, select 90 V constant pressure running concentration gel, 120 V constant pressure running separation gel to disperse the protein bands. At the end of electrophoresis, the target protein and internal reference protein at different sites were retained according to the color Marker molecular weight cutting gel and transferred to PVDF membrane for membrane transfer. After the end of the membrane at room temperature, the membrane with 5% blocking site buffer (5% skim milk + TBST) soaked gently shake 60 min to block protein non-specific sites. Subsequently, the membrane was incubated with primary antibodies, including caspase-3 antibody (Cell Signaling Technology, CST #9662s; 1:1,000), caspase-9 antibody (CST #12827S; 1:1,000) CASPASE-8 Antibody (CST) #4790s; 1:1,000), overnight at 4°C. The second antibody was then added the next day and incubated for 60 min. After washing the membrane, the strips are visualized using Chemidoc TMXRS + (Bio-Rad, USA).

Statistical methods. The data were analyzed with GraphPad Prism 8.0.2, and the data were expressed as mean ± SEM of three independent biological replicates. The data between the two groups were compared by student's t-test. Multi-group experimental data, after one-way ANOVA, Dunnett method is used to compare multiple groups with a single common group, and Tukey method is used to multiple comparisons between different groups. IC50 value is calculated by fitting MTT measurement results and expressed as average value, 95% confidence interval (CI). And P<0.05 indicates that the differences are statistically significant.

Results

Results of network pharmacology analysis

Ginger active ingredients and potential target screening prediction. From TCMSP and other databases and literature search, 265 known components of ginger were found. After screening and prediction by Swiss ADME database, there were 10 potential active components of ginger, see Table I.

Table I. Ginger potential effective ingredient information.

			Drug-likeness				
MOL ID	Ingredient name	GI absorption	Lipinski	Ghose	Veber	Egan	Muegge
MOL002467	6-gingerol	High	Yes	Yes	Yes	Yes	Yes
MOL002495	6-shogaol	High	Yes	Yes	Yes	Yes	Yes
MOL002516	Vanillylacetone	High	Yes	Yes	Yes	Yes	No
MOL002459	10- gingerol	High	Yes	Yes	No	Yes	No
MOL008698	Dihydrocapsaicin	High	Yes	Yes	No	Yes	Yes
MOL000123	Geraniol	High	Yes	No	Yes	Yes	No
MOL000122	Eucalyptol	High	Yes	No	Yes	Yes	No
MOL000124	Citral	High	Yes	No	Yes	Yes	No
MOL000198	L-linalool	High	Yes	No	Yes	Yes	No
MOL003358	1,7-dihydroxyxanthone	High	Yes	Yes	Yes	Yes	Yes

When the gastrointestinal absorption of ginger components is strong and meets three or more of the five drug-like rules including the Lipinski rule, it is considered to be a potential active ingredient. The active ingredient SDF format was uploaded to Pharmmapper for target prediction, and 53 targets of ginger were obtained by deleting repeated targets.

Targets of triple negative breast cancer. Through the Genecards, OMIM, TTD, Drugbank, DisGeNET and other disease databases, the disease targets were collected with triple negative breast cancer as the keyword, and the triple negative breast cancer related targets were obtained after deleting the repeated targets. The intersection of ginger targets and triple-negative breast cancer-related targets was taken in Cytoscape 3.7.1 software to construct a ginger-active ingredient-target-disease network diagram, as shown in Fig. 1. Red is triple negative breast cancer disease, green is ginger and its 10 selected active ingredients, yellow is ginger and triple negative breast cancer common targets a total of 27. The node label color in the figure deepens as the degree value increases.

Ginger-triple negative breast cancer network interaction and key gene screening. The intersection target of ginger and triple negative breast cancer was imported into STRING database to obtain the protein interaction relationship. The network data tsv file was imported into Cytoscape 3.7.2 software to draw the protein interaction network diagram, as shown in Fig. 2A. The network consisted of 26 nodes (hidden unrelated node NQO2) and 144 edges (representing the interaction between targets). The average node degree was 10.7, the average local clustering coefficient was 0.737, and P<1.0x10-16, indicating that the interaction between these proteins played an important role in the network. Visualization in Cytoscape 3.7.1, the greater the node Degree, the larger the node, the darker the node color; the larger the edgeBetweenness (betweenness) of the node connection, the thicker the line and the darker the color. The cytoNCA plug-in in Cytoscape 3.7.1 was downloaded and screened by Degree ≥10, Betweenness ≥10, Closeness ≥0.7. Finally, 10 core genes were obtained as shown in Fig. 2B. The core genes in the Uniprot database search to get Table II.

GO and KEGG analysis. GO functional enrichment analysis and KEGG pathway analysis were performed on potential targets of ginger in the treatment of triple negative breast cancer using Metascape platform. Results A total of 343 GO terms were obtained, of which 287 were related to biological process (BP) (P<0.01), 18 to cellular component (CC) (P<0.01) and 38 to molecular function (MF) (P<0.01). Fig. 3 is drawn for the first 18 GO entries according to the number of gene enrichment, as shown in the figure. In the process of cell biology, the function of ginger in the treatment of triple negative breast cancer mainly focuses on the reaction of cells to organic cyclic compounds, cell lipid reaction, cell reaction to nitrogen compounds, reaction to inorganic substances, protein phosphorylation, tube morphogenesis, hormone reaction, kinase activity regulation and so on. The cell components were mainly enriched in vesicle cavity, secretory granule cavity, cytoplasmic vesicle cavity, membrane raft, membrane microdomain, granule cavity rich in ficolin-1 (fibronectin), endoplasmic reticulum cavity, extracellular matrix, etc. The molecular function part mainly regulates protein kinase activity, phosphotransferase activity, protein serine/threonine kinase activity, transcription factor binding, peptidase activity, MAP kinase activity, nuclear receptor activity, ligand-activated transcription factor activity, etc.

The KEGG enrichment analysis of ginger against triple-negative breast cancer involved a total of 93 signaling pathways, and a total of 93 pathways were screened under the condition of P<0.01. It mainly involves TNF signaling pathway, IL-17 signaling pathway, FoxO signaling pathway, prolactin signaling pathway, MAPK signaling pathway, apoptosis, etc., and is closely related to atherosclerosis, intestinal flora, breast cancer, prostate cancer, pancreatic cancer, bladder cancer, gastric cancer, liver cancer, colorectal cancer and other diseases, indicating that there are common characteristics in the pathogenesis of various malignant tumors. The first 20 functional enrichment related pathways of ginger in the treatment of triple negative breast cancer were drawn into a bubble diagram, as shown in Fig. 4.

A series of data such as the active ingredients of ginger, the common targets with triple-negative breast cancer, and

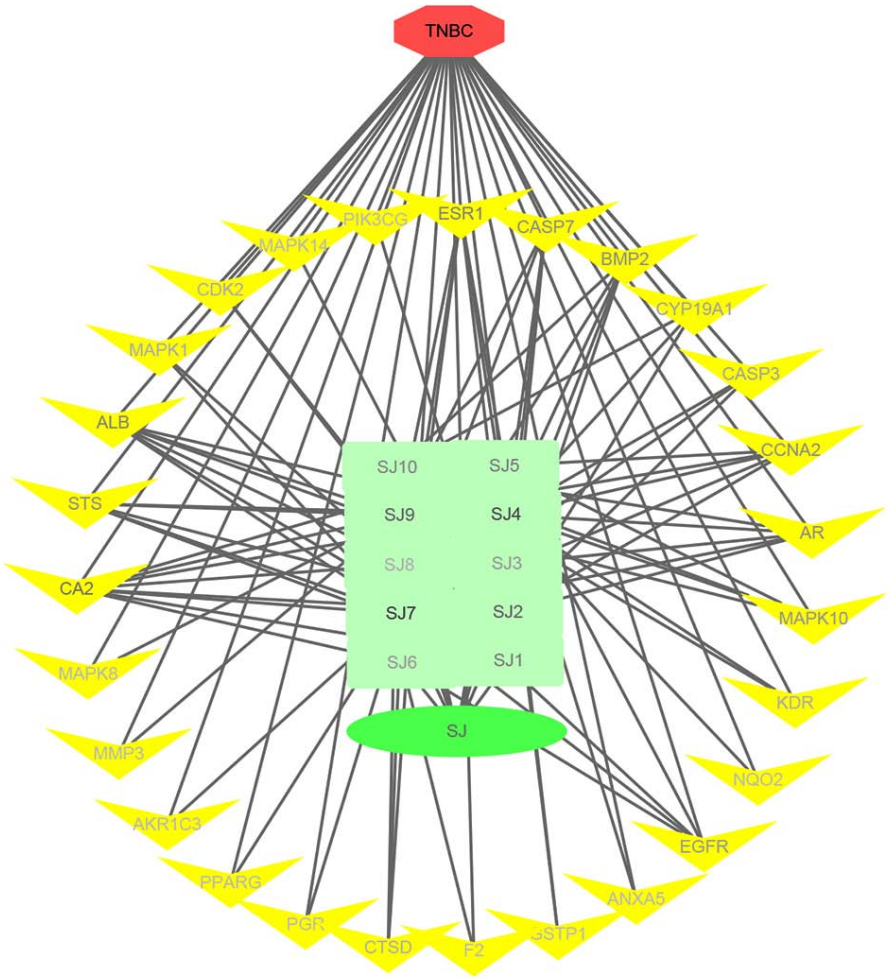

Figure 1. 'Ginger-active ingredients-targets-triple-negative breast cancer' network. A total of 10 effective components of ginger were screened by Swiss ADME database, the target of ginger action was predicted by Pharmaper and 27 common targets were obtained after intersection with TNBC target. Red is the TNBC target, green is ginger and its 10 screened active ingredients, and yellow is a total of 27 common targets between ginger and TNBC.

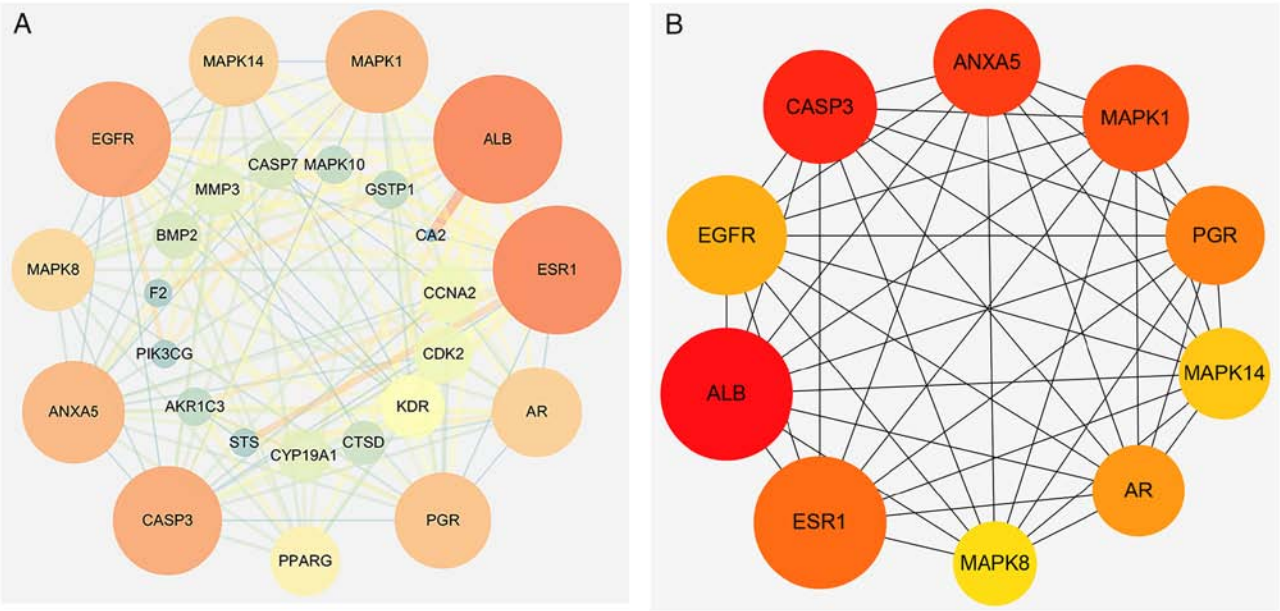

Figure 2. Ginger-TNBC network interaction map and core target map. (A) Ginger-TNBC protein-protein interaction analysis of potential targets genes. (B) A total of 10 core genes from the ginger-TNBC intersection target protein-protein interaction network. TNBC, triple-negative breast cancer; ALB, albumin; ESR1, estrogen receptor; EGFR, epidermal growth factor receptor; CASP3, caspase-3; ANXA5, annexin A5; MAPK, mitogen-activated protein kinase; PGR, progesterone receptor; AR, androgen receptor.

Table II. Core genes and details of ginger-triple negative breast cancer intersection target Protein-Protein Interaction network.

Gene	Protein full name	Degree	Betweenness	Closeness
ALB	Albumin	21.0	87.5	0.86
ESR1	Estrogen receptor	21.0	68.2	0.86
EGFR	Epidermal growth factor receptor	19.0	38.7	0.81
CASP3	Caspase-3	18.0	21.1	0.78
ANXA5	Annexin A5	17.0	20.1	0.76
MAPK1	Mitogen-activated protein kinase 1	17.0	29.6	0.76
PGR	Progesterone receptor	16.0	21.5	0.74
MAPK14	Mitogen-activated protein kinase 14	15.0	21.9	0.71
AR	Androgen receptor	15.0	23.7	0.71
MAPK8	Mitogen-activated protein kinase 8	14.0	11.2	0.69

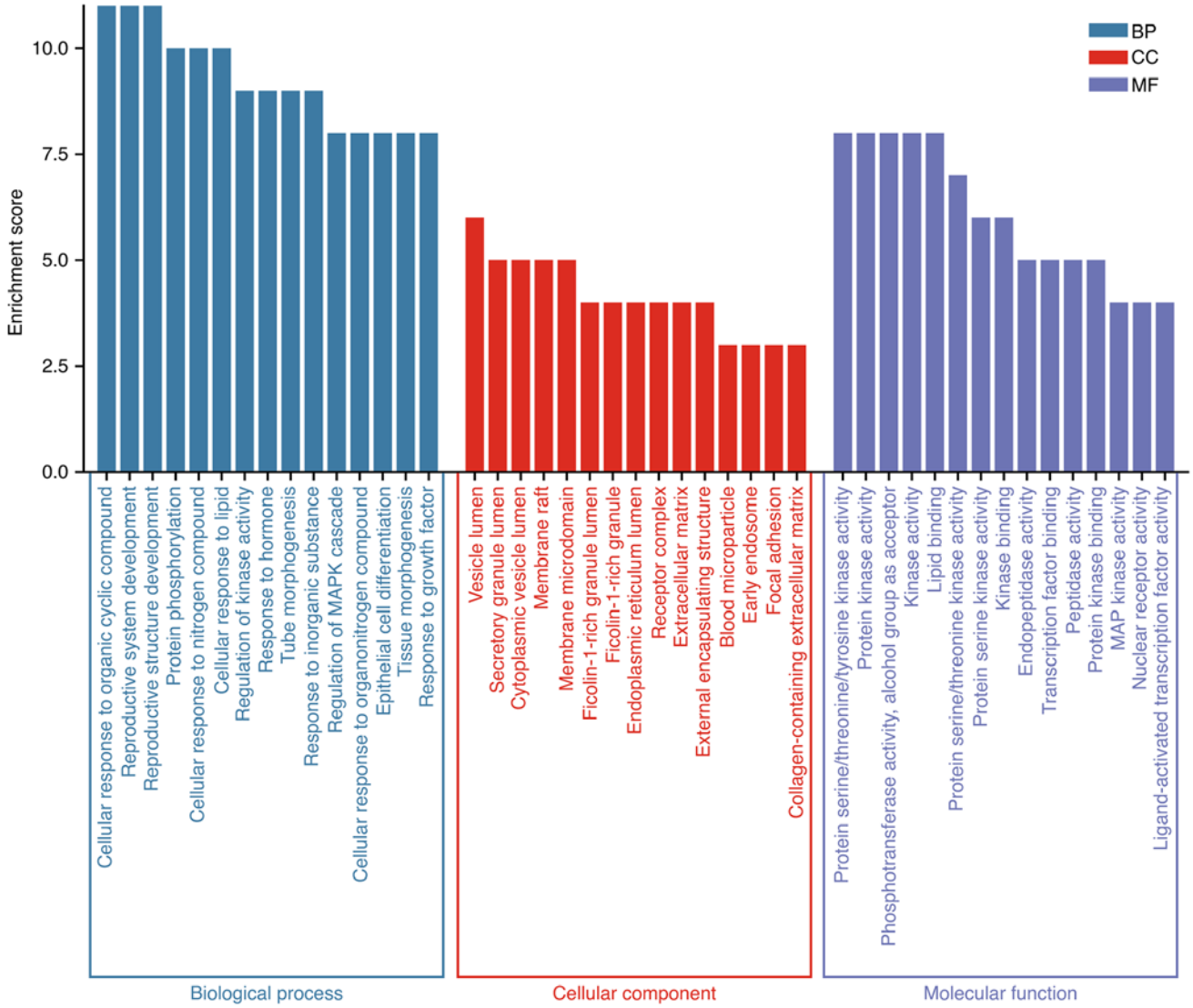

Figure 3. Gene Ontology biological function enrichment analysis of ginger-triple-negative breast cancer intersection targets. BP, biological processes; CC, cellular components; MF, molecular function.

the top 20 KEGG signaling pathways were used to construct a 'ginger-active ingredient-target-pathway' network through Cytoscape 3.7.21. Fig. 5 shows the pathway of ginger against triple-negative breast cancer. The network has 58 nodes and 223 edges, including 1 ginger drug node and 10 ginger active ingredient nodes (green), 27 common targets of ginger

and triple-negative breast cancer (red). The top 20 KEGG signaling pathways (blue), the greater the node degree value in the network, the greater the role in the network, the greater the node label. Ginger-active ingredient-target-pathway diagram clearly and intuitively reflects that ginger inhibits triple negative milk by acting on multiple targets of multiple components and multiple pathways.

Molecular docking. Molecular docking was performed between 10-gingerol, geraniol, citral, dihydrocapsaicin, L-linalool, 6-gingerol and the top 6 core targets in the PPI network. The lower the binding energy between the molecule and the target, the more stable the binding between the ligand and the receptor protein. Generally, the binding energy ≤-4.52 kcal/mol is used as the scoring standard, indicating that there is binding activity between the ligand and the receptor protein. When the binding energy is ≤-7.00 kcal/mol, it indicates that there is a strong binding activity between the ligand and the receptor protein (22-24). At the same time, the RMSD value of the docking results is less than 2 Å, indicating that the docking results deviate from the reference molecule to a low degree and the docking results are reliable. The above 2.1.2,2.1.3 screened out the core active ingredients of ginger in the treatment of triple negative breast cancer and the target protein docking data are shown in Table III.

The results showed that all docking binding free energies were less than -4.52 kcal·mol-1, in which the lowest binding potential energy of dihydrocapsaicin and EGFR protein was -7.70 kcal·mol-1, followed by the binding energy of 6-gingerol and EGFR protein was -7.30 kcal·mol-1, and the binding energy of dihydrocapsaicin and CASP3 protein was -7.20 kcal·mol-1, indicating that dihydrocapsaicin and 6-gingerol had high affinity with the core target, and the molecules could form stable conformation through interaction. The molecular docking results of the above three components with the lowest binding potential to their proteins were selected for visualization. The three-dimensional structure, two-dimensional structure diagram, detailed binding sites between active components and targets and detailed binding sites of each group were shown in Fig. 6. A, B, C purple structures are protein macromolecules; the yellow dotted line is the hydrogen bond of the ligand small molecule binding protein; the green structure is a protein-ligand binding residue. Dihydrocapsaicin was linked to the amino acid residues TYR-180 and ARG-150 of EGFR by hydrogen bonds with bond lengths of 2.3 and 2.5, respectively. 6-Gingerol and EGFR amino acid residues TYR-180, ALA-149 were linked by hydrogen bonds with bond length of 1.0, 2.0, 2.8, respectively. Dihydrocapsaicin was linked to the amino acid residues LYS-137 and ARG-164 of CASP3 by hydrogen bonds with bond lengths of 2.2 and 2.3, respectively. Further generating a 2D visualization, discovering dihydrocapsaicin-EGFR formed hydrophobic effects with amino acids such as Leu124, Tyr179 and Arg126. 6-Gingerol-EGFR forms hydrophobic effects with amino acids such as Tyr179, Arg126 and Arg150; Dihydrocapsaicin-CASP3 forms a hydrophobic effect with amino acids such as Lys137, Pro201 and Cys264. Dihydrocapsaicin was selected for subsequent cell experiments to verify the therapeutic effect of ginger on triple negative breast cancer.

In vitro cell experiment results

Inhibitory effect of DHC on proliferation of MDA-MB-231 cells. DHC had a significant inhibitory effect on the proliferation of MDA-MB-231 cells (IC_{50}: 125.4 μM, 95%CI: 114.1-137.3) (Fig. 7). With the increase of DHC concentration, the inhibitory effect on the proliferation of MDA-MB-231 cells was stronger. The effects of DHC concentration of 100, 150, 200, 250 μmol/l cultured cells for 12, 24, 48 h were detected by MTT method, as shown in Table IV. Compared with the control group, the DHC group had a certain inhibitory effect on the proliferation of MDA-MB-231 cells. When the concentration of DHC was 150, 200, 250 μmol/l, the inhibitory effect was more obvious, and with the prolongation of administration time, the inhibitory effect was enhanced.

Inhibitory effect of DHC on MDA-MB-231 cell migration. The MDA-MB-231 cells were treated with drugs after scratching. Compared with the blank group, the cell morphology of the high-dose DHC group changed significantly, and the cell scratch wound healing ability was significantly reduced (P<0.01). The cell scratch wound healing ability of high dose DHC group was significantly lower than that of low dose DHC group (P<0.05). The low dose DHC group and middle-dose DHC group cell migration ability has a downward trend, but compared with the blank group, the difference is not obvious, see Fig. 8A and B.

Effect of DHC on CASP9 mRNA expression in MDA-MB-231 cells. The effect of DHC on the expression of CASP9 in MDA-MB-231 triple negative breast cancer cells was detected by PCR. It was found that the expression of CASP9 in each dose group of DHC was increased to a certain extent compared with the solvent control group. There was no significant difference between the low dose group of DHC and the control group. The middle dose group and the high dose group of DHC were significantly different from the control group (P<0.05), as shown in Fig. 9.

Effect of DHC on CASP3 and BAX protein expression in MDA-MB-231 cells. Based on the above network pharmacology research, the apoptosis pathway was selected to verify the effect of ginger on MDA-MB-231 triple negative breast cancer cells by WB experiment. Compared with the normal group, the expression of CASP3 protein and BAX protein in DHC group and PI3K/AKT pathway inhibitor Wortmannin group increased significantly (P<0.05), as shown in Table V and Fig. 10.

Discussion

Breast cancer accounts for 31% of confirmed cancer cases in women in 2022, far higher than other common cancer diseases such as lung cancer and colorectal cancer, and is a major public health problem that threatens women's health (25). Triple negative breast cancer (TNBC) is a subtype of breast cancer that does not express estrogen receptor (ER) and progesterone receptor (PR), and does not overexpress human epidermal growth factor receptor 2 (ERBB2) of TNBC accounts for 15-20% of all breast cancers (26,27). At the molecular level, it is a highly heterogeneous disease. According to the gene

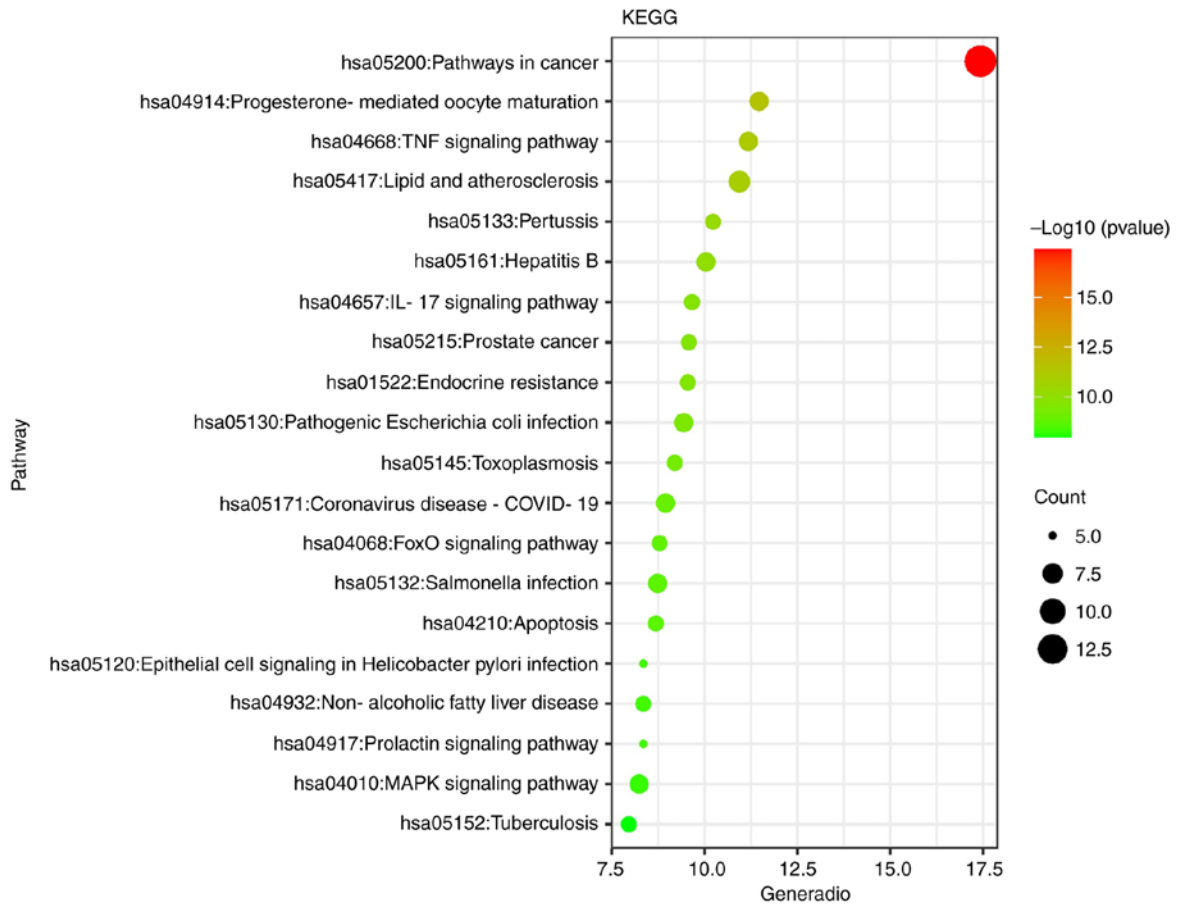

Figure 4. Enrichment analysis of KEGG pathway in ginger-triple-negative breast cancer. KEGG, Kyoto Encyclopedia of Genes and Genomes.

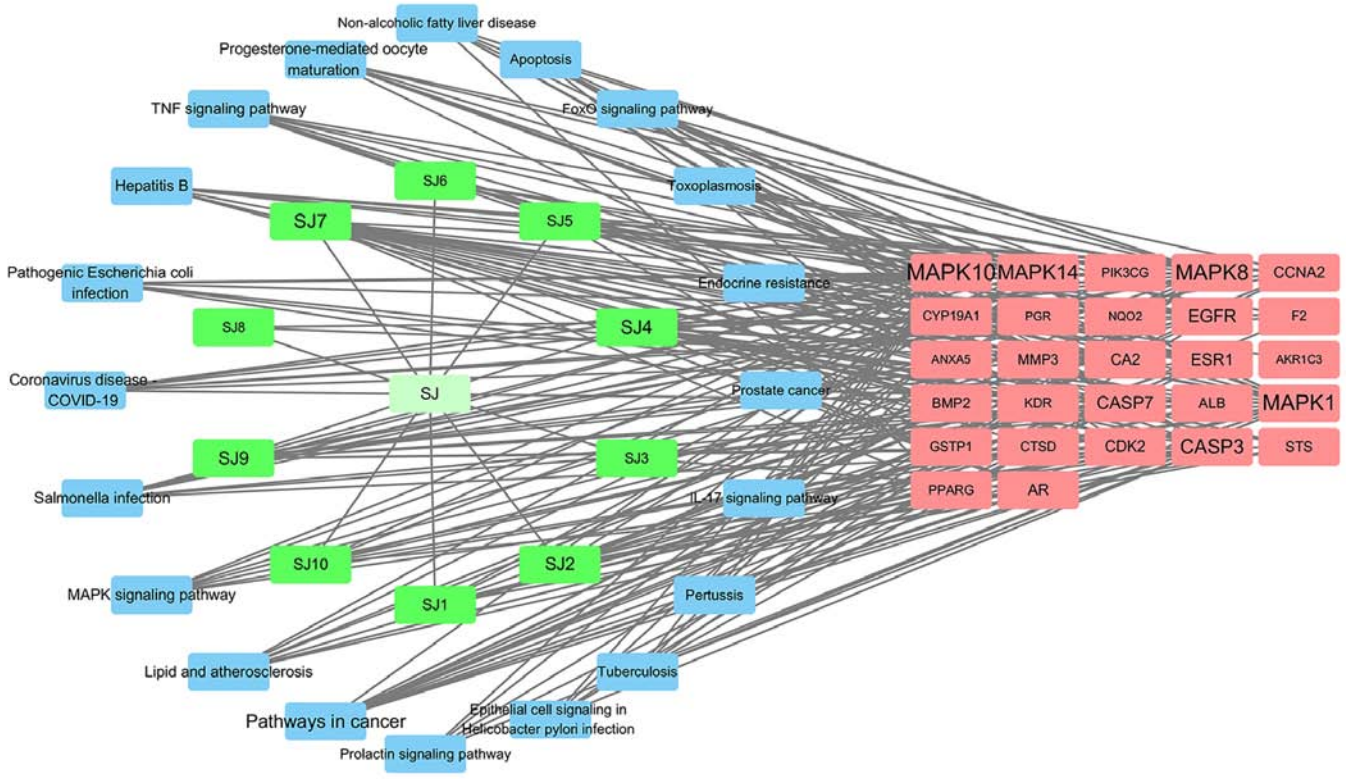

Figure 5. Network of compounds of Ginger-drug targets-TNBC targets-signaling pathways. It includes 1 ginger drug node and 10 ginger active ingredient nodes (green), 27 common targets (red) of ginger and triple negative breast cancer and the top 20 Kyoto Encyclopedia of Genes and Genomes signalling pathways (blue). The greater the degree value of nodes in the network, the greater the role they play in the network, and the larger the node label.

Table III. Ginger core component and the core target molecular docking results.

Active ingredient	Target protein	PDB ID	Binding energy/kcal·mol^{-1}	RMSD/Å
10-Gingerol	ALB	6EZQ	-5.60	1.659
	ESR1	6PSJ	-5.20	1.488
	EGFR	4RJ3	-6.80	1.917
	CASP3	7RN9	-6.10	1.721
	ANXA5	6K25	-6.50	1.605
	MAPK1	6G9J	-5.70	2.036
Geraniol	ALB	6EZQ	-5.30	1.949
	ESR1	6PSJ	-4.60	1.531
	EGFR	4RJ3	-5.50	0.721
	CASP3	7RN9	-5.30	0.576
	ANXA5	6K25	-5.50	1.387
	MAPK1	6G9J	-4.70	1.493
Citral	ALB	6EZQ	-5.50	0.549
	ESR1	6PSJ	-4.50	1.152
	EGFR	4RJ3	-5.70	0.573
	CASP3	7RN9	-5.20	0.827
	ANXA5	6K25	-4.90	0.792
	MAPK1	6G9J	-5.20	1.485
Dihydrocapsaicin	ALB	6EZQ	-6.80	0.992
	ESR1	6PSJ	-6.90	1.102
	EGFR	4RJ3	-7.70	1.777
	CASP3	7RN9	-7.20	1.554
	ANXA5	6K25	-7.00	1.235
	MAPK1	6G9J	-5.90	0.789
L-linalool	ALB	6EZQ	-5.50	1.762
	ESR1	6PSJ	-4.70	1.179
	EGFR	4RJ3	-5.80	1.107
	CASP3	7RN9	-4.90	1.041
	ANXA5	6K25	-5.10	1.532
	MAPK1	6G9J	-4.70	0.814
6-Gingerol	ALB	6EZQ	-6.00	1.688
	ESR1	6PSJ	-6.10	0.978
	EGFR	4RJ3	-7.30	1.356
	CASP3	7RN9	-6.70	1.482
	ANXA5	6K25	-5.90	0.927
	MAPK1	6G9J	-5.40	1.671

ALB, albumin; ESR1, estrogen receptor; EGFR, epidermal growth factor receptor; CASP3, caspase-3; ANXA5, annexin A5; MAPK1, mitogen-activated protein kinase 1.

expression profile, TNBC has six different subtypes, each subtype has unique gene expression characteristics. The clinical significance of these subtype differences is still under study (2). TNBC is also considered to be one of the most aggressive types of breast cancer, with rapid progression and low survival rate, mostly in young women (2,21,27). Compared with hormone receptor positive or ERBB2 positive subtypes, TNBC is more aggressive in clinical behavior and lacks molecular targets for treatment. Patients cannot use traditional hormone or ERBB2 targeted therapy. Chemotherapy is the main treatment option, and systemic chemotherapy has acute and chronic toxicity, which can cause nausea, vomiting and other adverse reactions. At the same time, TNBC has shorter progression-free survival, worse prognosis, higher recurrence, distant metastasis and mortality, especially in the first 5 years after diagnosis (21,28,29). Therefore, it is necessary to optimize current treatment strategies for triple-negative breast cancer patients.

Clinically many difficult 'Miscellaneous' use of modern medical methods cannot solve the use of traditional Chinese medicine may be relatively large breakthrough. A tumor is a mass of lumps formed by abnormal proliferation of local tissue

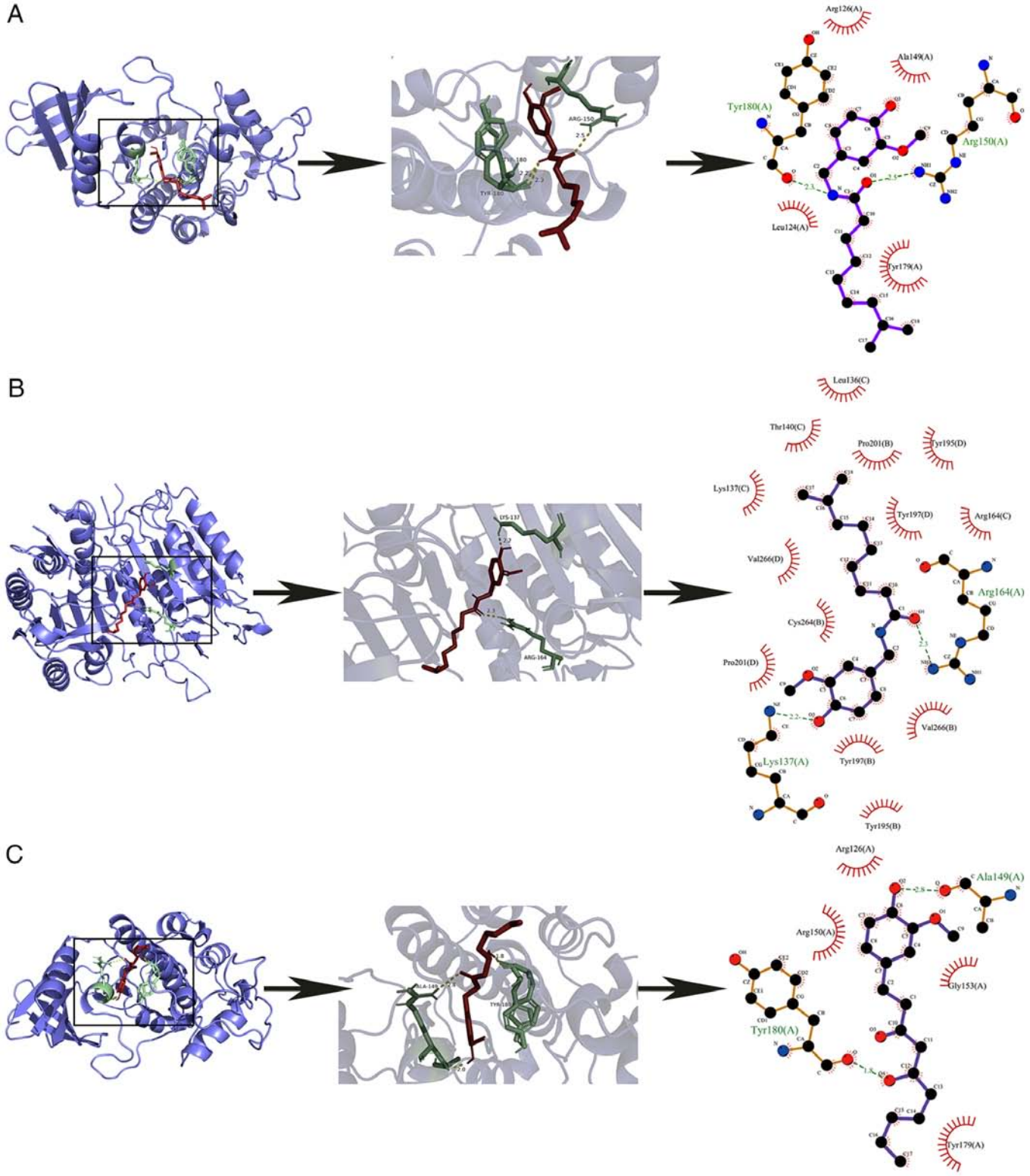

Figure 6. Visualization of docking of components and targets. (A) Dihydrocapsaicin-EGFR. (B) 6-gingerol-EGFR. (C) Dihydrocapsaicin-CASP3. EGFR, epidermal growth factor receptor.

cells. Traditional Chinese medicine attributes this morphological feature to 'accumulation'. 'Classic on medical problems. Fifty-five Classic on medical problems': 'Accumulation, Yin Qi also' (30). Sui dynasty 'Classic on medical problems. Fifty-five Classic on medical problems': 'Accumulators, born from the cold inside' (31). The close relationship between 'accumulation' and 'cold' can be seen in ancient Chinese classic medical books. Modern pharmacological studies have shown that aconite, cinnamon and ginger can regulate the expression of oncogenes, induce tumor cell apoptosis, inhibit tumor cell metastasis, block tumor cell cycle and other anti-tumor ways (31,32). Ginger is a typical edible traditional

Table IV. Inhibitory effect of DHC on the proliferation of MDA-MB-231 ($\bar{x} \pm s$; n=6).

Group	Dosage	Inhibitory effect, %		
		12	24	48
Control	-	0	0	0
DHC	100 μmol/l	18.1±2.3[a]	16.0±5.3[a]	1.6±3.6[a]
	150 μmol/l	40.9±7.7[a]	67.2±4.1[a]	52.3±6.9[a]
	200 μmol/l	63.5±4.6[a]	81.5±1.5[a]	96.9±1.0[a]
	250 μmol/l	76.2±4.4[a]	95.8±1.8[a]	99.2±0.3[a]

[a]P<0.01 compared with the control group at the same time. DHC, dihydrocapsaicin.

Table V. Effects of DHC on CASP3 and BAX protein expression in MDA-MB-231 ($\bar{x} \pm s$; n=3).

Group	Dosage	CASP3/β-actin	BAX/β-actin
Wortmannin	1 μmol/l	1.02±0.18[a]	1.00±0.36[a]
Control	-	0.38±0.01	0.55±0.22
DHC	200 μmol/l	1.00±0.01[a]	1.07±0.25[a]

[a]P<0.05 Compared with the control group. DHC, dihydrocapsaicin; CASP, caspase.

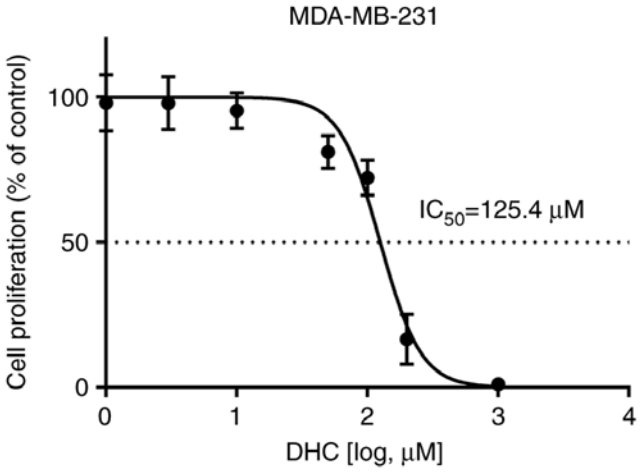

Figure 7. IC50 of DHC. The growth inhibitory effect of DHC on the triple-negative breast cancer cell line MDA-MB-231 was determined by MTT method. The IC50 value calculated using GraphPad Prism 8.0.2 is 125.4 μM (95% CI: 114.1-137.3). DHC, Dihydrocapsaicin.

Chinese medicine. It has been used for thousands of years as medicine and condiment.

Ginger has the effect of relieving cold, warming and stopping vomiting. Pharmacological effects include protecting liver and gallbladder, regulating immune system, anti-inflammatory and anti-allergic, anti-pathogenic microorganisms, anti-tumor and so on. The anti-tumor effect of ginger has been reported in many ways. Ling Zhou found that 6-shogaol inhibited the activation of TLR4/NF-κB signaling pathway, affected NF-κB signaling and down-regulated COX-2, cyclinD1, Bcl-2, MMP-9 and other key cytokines, thereby enhancing the anti-pancreatic cancer activity with gemcitabine (33). Kaewtunjai found that 60 days after lung cancer A549 cells were treated with a cytotoxic dose of crude ginger extract (ZOE), the protein expression of telomerase reverse transcriptase (hTERT) decreased, resulting in telomere shortening and cell senescence, thereby achieving the effect of crude ginger extract (ZOE) in inhibiting the activity and proliferation of lung cancer cells (34).

Ginger has many components and pharmacological activities. This study explored the mechanism of ginger in the treatment of triple negative breast by combining network pharmacology and *in vitro* cell experiments. After screening through database and literature search, ten potential active ingredients of ginger, 10-gingerol, geraniol, citral, dihydrocapsaicin, L-linalool, 6-gingerol, 1,7-dihydroxyxanthone, eucalyptol, 6-shogaol and zingerone, were obtained. These active ingredients have been reported to have anti-tumor effects. 10-gingerol was made into a loaded nanoemulsion to improve bioavailability. It was found to be cytotoxic to mouse and human TNBC cell lines 4T1 and MDA-MB-231 cells, arresting the cell cycle in the sub-G0 phase and inducing apoptosis (35). Liany used 6-gingerol-derived semi-synthetic compound SSi6 in a preclinical xenograft model to demonstrate that SSi6, as a single drug, has good anti-tumor activity *in vivo* without obvious side effects and can block the typical visceral organ metastasis of breast cancer, lymph node metastasis to the lungs and multiple organs (36). These documents and research results provide the basis and important reference for this study. In particular, network pharmacological docking results suggested that DHC binding activity to TNBC target proteins was particularly good, so we chose the DHC as the object of further study.

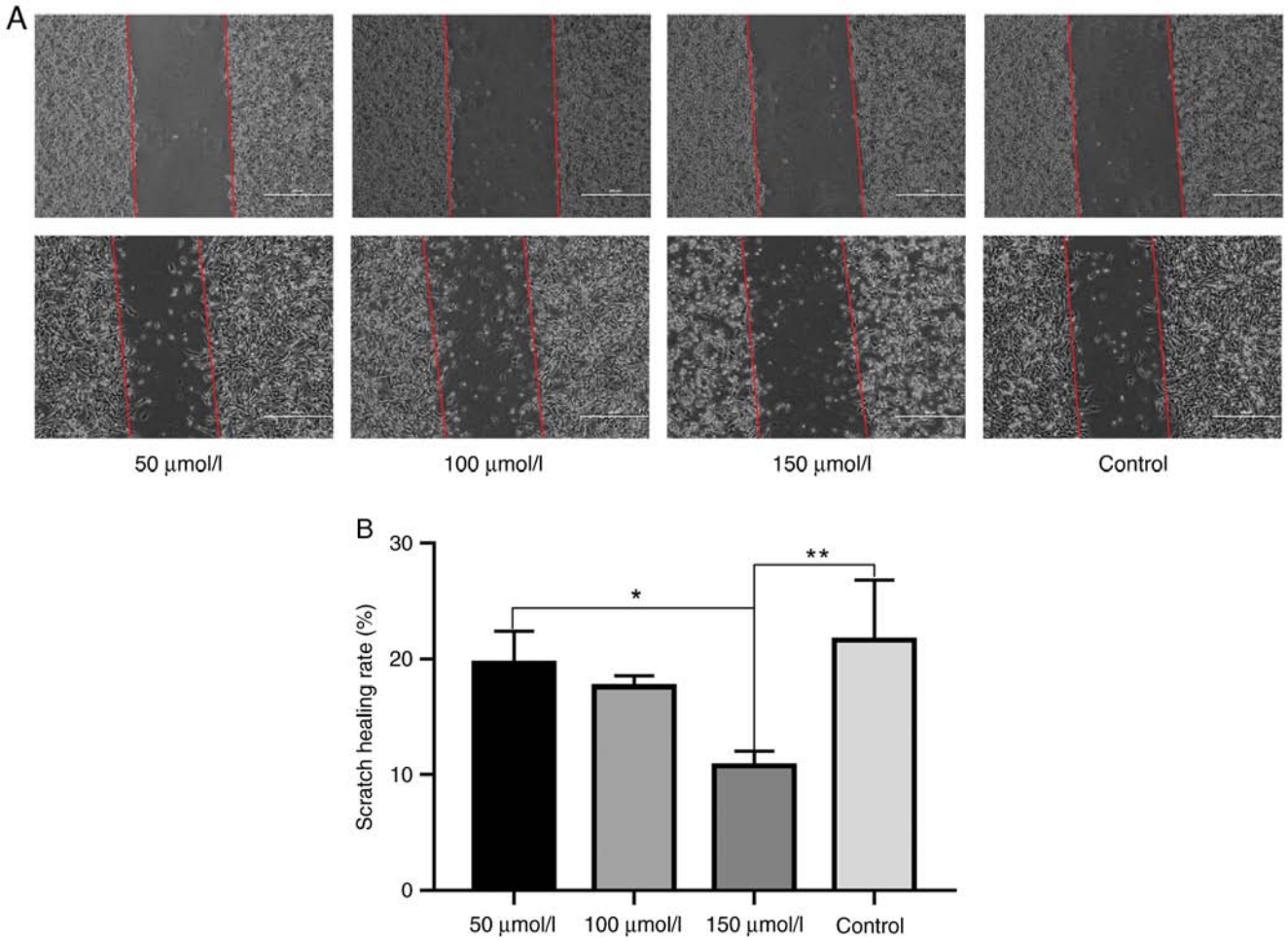

Figure 8. Comparison of cell migration capacity represented by scratch migration area of each group of cells. (A) Experimental images in each group using the scratch test at 0 h (as the control) and 24 h respectively, which was (B) quantified. The data were measured and expressed by the mean ± standard deviation. *P<0.05 and **P<0.01.

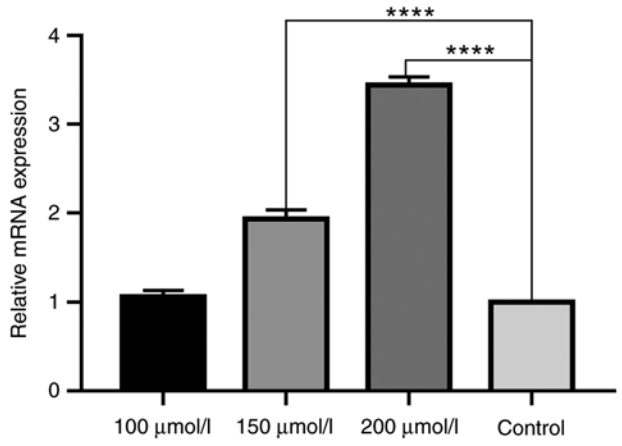

Figure 9. Effect of dihydrocapsaicin on caspase-9 mRNA expression in MDA-MB-231. ****P<0.0001.

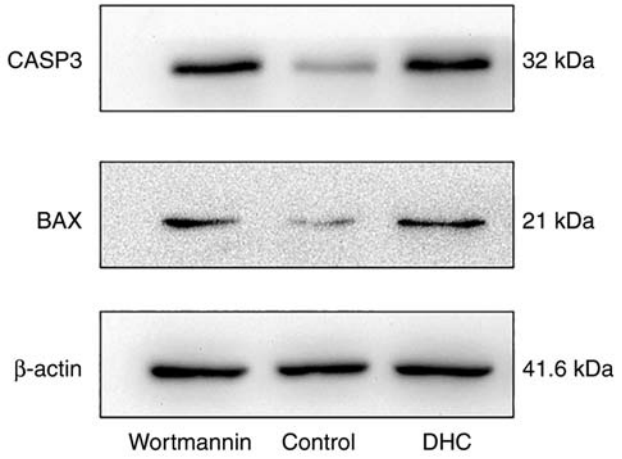

Figure 10. Electrophoresis of caspase-3 and BAX protein in MDA-MB-231.

PI3K/AKT signaling pathway is a star signaling pathway in cancer research. It plays an important role in regulating various cell functions (including metabolism, growth, proliferation, apoptosis, transcription and protein synthesis). PI3K, AKT and PTEN are important node proteins in the pathway and important factors regulating intracellular effects of downstream signaling pathways. The results of cell experiments showed that DHC group and PI3K/AKT inhibitor Wortmannin group could induce apoptosis and increase the protein expression of CASP3 and BAX. After the intervention of DHC and Wortmannin, the expression of apoptotic proteins increased. DHC may participate in the regulation

of PI3K/AKT signaling pathway by inhibiting the expression of PI3K and AKT proteins or promoting the expression of PTEN protein. The results of MTT assay showed that DHC had a good inhibitory effect on the proliferation of MDA-MB-231 cells at a dose concentration of 100, 150, 200 and 250 μmol/l. When the dose of DHC was 200 and 250 μmol/l, it showed dose-dependent and time-dependent. After 24 days of administration, the inhibition rate reached 80% or more, and the cells died in a large area. The results of cell scratch test showed that when the dose of DHC was 150 μmol/l, the cell migration was significantly inhibited. When the dose of DHC was 200 and 250 μmol/l, the scratch results of large area apoptosis could not be counted. The results of rt-PCR showed that DHC at 150 and 200 μmol/l could significantly reduce the mRNA expression of CASP9 in MDA-MB-231 cells.

The mechanism of DHC in treating triple negative breast cancer is closely related to Caspase family. The molecular docking results also confirmed the close relationship between the mechanism of DHC in the treatment of triple negative breast cancer and the Caspase family. The docking binding energy of CASP3 with the six core components of ginger was less than -4.25, and the average binding energy was -5.9, indicating that CASP3 was stably bound to the active ingredients of ginger.

This study explored the mechanism of ginger in the treatment of triple negative breast cancer by network pharmacology, molecular docking and *in vitro* cell experiments. The results showed that dihydrocapsaicin could inhibit the proliferation and migration of triple negative breast cancer MDA-MB-231 cells, and up-regulate the mRNA expression of Caspase family CASP9 and the protein expression of CASP3 and BAX. We will further explore other molecular mechanisms of DHC in the treatment of TNBC, including the use of different experimental methods such as flow cytometry to detect apoptosis, WB to detect the expression of signal protein in the treatment of TNBC pathway by DHC, and animal experiments to verify the therapeutic effect of DHC on TNBC *in vivo*. At the same time, other active components of ginger, such as 6-gingerol, were selected for the pharmacodynamic experiment to explore its therapeutic effect on TNBC. This study provides a direction for the treatment of triple negative breast cancer, and also provides an idea for the drug development of ginger.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

ZL, KS conceived and designed the study and helped to draft the manuscript. LL and YC are responsible for the collection of data. TH and LL participated in the statistical analyses. QM and YH made the figures, participated in data analysis, and were the main contributors to writing the manuscript. All authors read and approved the final version of the manuscript. QM and YH confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Animal studies were approved by the Committee on the Ethics of Animal Experiments of the Jiangxi University of Chinese Medicine (approval no. JZSYDWLL20200105).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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