Echinacoside Suppresses the Progression of Breast Cancer by Downregulating the Expression of miR-4306 and miR-4508

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Abstract
The main treatment of breast cancer includes surgical resection, radiotherapy, chemotherapy, endocrine therapy, and molecular targeted therapy, but the outcomes remain unsatisfactory. Previous studies demonstrated that echinacoside, microRNA (miRNA/miR)-4306 and miR-4508 were associated with lymph node metastasis, chemoresistance and self-renewal capability in breast cancer, but in-depth studies on the underlying mechanism of their anticancer effects have not been performed to date. In order to identify the role of miR-4306 and miR-4508, and the mechanism of the antitumor effect of echinacoside in breast cancer, the present study first examined the expression of miR-4306 and miR-4508 in breast cancer tissues to examine their possible role in the development of breast cancer, then evaluated the effect of echinacoside on the expression of miR-4306 and miR-4508 on the viability, apoptosis, cell cycle, migration, and invasion abilities of breast cancer cells to explore the anti-cancer effect of echinacoside and the involvement of miR-4306 and miR-4508. Finally, the breast cancer cells and mice bearing breast cancer xenografts were treated with echinacoside and inhibitors of miR-4508 or miR-4306 to confirm their role on the anticancer effect of echinacoside. The results showed that miR-4306 and miR-4508 were decreased in breast cancer tissues and cells. Echinacoside inhibited cell proliferation, invasion and migration, and promoted the apoptosis of breast cancer cells by downregulating the expression of miR-4306 and miR-4508. In conclusion, this is the first study to show the association between echinacoside and miRNAs in cancer. The present study elucidates an underlying molecular mechanism of the antitumor effect of echinacoside on breast cancer, and thus may contribute to preventive and therapeutic strategies for breast cancer.

Keywords
echinacoside, breast cancer, miR-4306, miR-4508, proliferation, invasion, migration, apoptosis

Introduction
Breast cancer seriously threatens the survival and life quality of patients.¹ Breast cancer can be divided into 4 types according to the expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and Ki-67 index.²³ Breast cancer alone accounts for 30% of all female cancer cases, and its incidence rate is annually increasing by ~0.3%.⁴ The main treatment of breast cancer includes surgical resection, radiotherapy, adjuvant chemotherapy, endocrine therapy, immunology therapy, and molecular targeted therapy,⁵ but the outcomes of these treatments remain unsatisfactory. Although breast cancer can be treated, 35% to 40% of patients still experience postoperative recurrence and metastasis.⁶ Surgery and chemotherapy are effective in the early stage of breast cancer. However, the majority of patients present with tumor metastasis at the time of diagnosis; thus, early diagnosis directly affects the treatment and prognosis of breast cancer.⁷ Therefore, an in-depth study of the molecular mechanism of occurrence, development, invasion, and metastasis of breast cancer will contribute to the early detection, early treatment and prognosis of breast cancer. It is of great importance for patients with breast cancer.
Moreover, Ye et al. found that echinacoside can increase the oxidation of 8-oxoguanine in tumor cells. It can also induce tumor invasion and metastasis by regulating metastasis-related genes and signal transduction pathways. Previous studies have shown that miRNA-based therapies have potential for controlling tumor metastasis. However, the identification of specific miRNAs that can regulate tumor progression and metastasis depends on the in-depth understanding of the metastasis process and its mechanisms. A previous study demonstrated that low miR-4306 expression is strongly associated with lymph node metastasis and poor survival in triple-negative breast cancer. Another study indicated that miR-4508 showed a potential association with breast cancer chemoresistance and self-renewal capability. However, an in-depth study on the anticancer effect of these 2 miRNAs has not been performed thus far.

Echinacoside is a natural phenylethanoid glycoside which was first isolated from Echinacea root 60 years ago. Echinacoside can also be extracted from Cistanche, which has been widely used in the treatment of the common cold in Europe. Cistanche (Rou Cong Rong in Chinese) are perennial parasite herbs growing in arid lands and warm deserts, whose chemical constituents include volatile oils, echinacoside, iridoids, lignans, alditols, oligosaccharides, and polysaccharides. Some studies have shown that echinacoside has the following functions: neuroprotective, anti-aging, scavenging free radicals, protecting vascular endothelial cells, and promoting hematopoiesis. It was also reported that echinacoside induced the apoptosis of pancreatic cancer and other tumors. Wang et al. reported that echinacoside could induce the production of reactive oxygen species in pancreatic cancer cells, disturb the mitochondrial membrane potential and cause cell apoptosis. Dong et al. found that echinacoside could significantly inhibit the activity of human Mut T homolog 1 and increase the oxidation of 8-oxoguanine in tumor cells. Moreover, Ye et al. found that echinacoside can induce the inactivation of the AKT signaling pathway in HepG2 cells, and can reduce the expression of the triggering receptor expressed on myeloid cells 2 (TREM2) protein, thus exerting an antitumor effect. A previous study indicated that echinacoside inhibited breast cancer cells by suppressing the Wnt/β-catenin signaling pathway. In recent years, accumulating studies have shown that natural products such as paclitaxel, curcumin, resveratrol, isoﬂavones, (-)-epigallocatechin-3-gallate, lycopene, 3,3'- diindolylmethane, indole-3-carbinol, or genistein exert their anti-proliferative and/or pro-apoptotic effects through regulating one or more miRNAs, leading to the inhibition of cancer cell growth or induction of apoptosis. It was suggested that some natural products could inhibit cancer progression, increase drug sensitivity, reverse EMT, and prevent metastasis through modulation of miRNAs, which will provide a new therapeutic approach for cancer treatment. However, whether echinacoside affects miRNAs in breast cancer cells has not been investigated to date. As miR-4306 and miR-4508 may be involved in the development of breast cancer, the regulation of echinacoside on them is worthy of exploration.

To identify the role of miR-4306 and miR-4508, and the mechanism of the antitumor effect of echinacoside in breast cancer, the present study first examined the expression of miR-4306 and miR-4508 in breast cancer tissues, and then examined the effect of echinacoside on the expression of miR-4306 and miR-4508, as well as on the viability, apoptosis, cell cycle, and migration and invasion abilities of breast cancer cells. Second, cells and mice bearing breast cancer xenografts were treated with echinacoside and inhibitors of miR-4508 or miR-4306 to confirm their role on the anticancer effect of echinacoside.

### Materials and Methods

#### Treatment With Echinacoside, and Cell Viability, Apoptosis and Cell Cycle Assays

MFC-7 cells were treated with different concentrations of echinacoside (Sigma-Aldrich Corp, St Louis, USA, 98% (HPLC), 5, 10, 20, and 40 μg/ml) at different times (1-6 days). Cell viability was measured with a 3-(4, 5)-dimethylthiahiazol (-z-y1)-3, 5-di- phenytetrazolium-romide (MTT) kit (Beyotime Institute of Biotechnology, China) according to the manufacturer’s instructions. Briefly, MFC-7 cells were seeded in 96-well plates (5000 cells/well) and cultured for 1 to 6 days. After treatments, MTT reagent was added to each well and incubated at 37°C for 6 hours. Finally, the optical density value at a wavelength of 450 nm was determined using a microplate reader (BioTek Instruments, Inc., Shanghai, China).

Cell apoptosis was determined with Annexin V-Fluorescein Isothiocyanate (FITC) Apoptosis Detection Kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer’s instructions. MCF-7 cells were seeded into 12-well plates (3 × 10⁴ cells/well) and cultured for 48 hours at 37°C, followed by digestion with 0.025% trypsin (Beyotime Institute of Biotechnology, Shanghai, China) and staining with FITC-labeled Annexin V and propidium iodide (PI). The cell apoptosis rate was then evaluated using an Attune NxT flow cytometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

For cell cycle measurement, similarly to Cheng et al., after MCF-7 cells were treated with different concentrations of echinacoside (5, 10, 20, and 40 μg/ml) for 24 hours, they were collected, digested, fixed with 70% ethanol and
stained with PI (Beyotime Institute of Biotechnology, Shanghai, China). After 30 minutes of incubation at 37°C in the dark, cells in G0/G1, S, and G2/M phase were detected using a flow cytometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

**Western Blot Analysis**

After treatment of MCF-7 cells with different concentrations of echinacoside (5, 10, 20, and 40 μg/ml) for 48 hours, cells were lysed with cell lysis buffer (Beyotime Institute of Biotechnology) containing 1 μM phenylmethylsulfonyl fluoride, 1.5 μM pepstatin A and 0.2 μM leupeptin. The concentration of protein was measured using a bicinchoninic acid (BCA) kit (Beyotime Institute of Biotechnology, Shanghai, China). About 40 μg of protein was loaded per lane and resolved on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer to a polyvinylidene fluoride (PVDF) membrane. The electrical transfer time was adjusted according to the molecular weight of the target protein. After electrophoresis, the PVDF membrane was placed in 5% non-fat milk and blocked at room temperature for 2 to 3 hours, rinsed with TBST (TBS + 0.1% Tween-20), and incubated with primary antibodies (Sigma-Aldrich Corp, St Louis, USA) against cleaved Caspase-3, Caspase-8, cleaved Caspase-9, and Caspase-9 overnight at 4°C. The next day, the membrane was washed with TBST 3 times for 15 minutes each time under room temperature, incubated with the secondary antibody (Sigma-Aldrich Corp, St Louis, USA) for 1 hours at room temperature, and rinsed with TBST 3 times for 15 minutes each time. Finally, the proteins were visualized using an ECL western blot analysis system (B&D Biosciences, San Diego, CA, USA). The protein levels were semi-quantified using ImageJ software Version v2.1.1 (National Institutes of Health) after normalization to GAPDH.

**Wound Healing and Transwell Assays**

For the wound healing assay, similarly to the method employed by Yue et al., cells in logarithmic growth phase were digested with 0.025% trypsin (Beyotime Institute of Biotechnology, Shanghai, China), and then centrifuged at 300 × g for 5 minutes and counted with a cell counting chamber. The cell concentration was adjusted, and 1 × 10^5 cells/ml were inoculated on a 6-well plate. Cells were treated with different concentrations of echinacoside (5, 10, 20, and 40 μg/ml) for 48 hours. When the cells grew to >80% confluence, the culture medium was removed, a wound was made on the monolayer of cells with a 200-μl sterile pipette tip, and the scratch boundary was marked at the bottom of the plate. The damaged cells were washed with PBS, and the remaining cells were cultured for 24 hours with culture medium without serum. Wound healing and cell proliferation were observed under an inverted microscope (Nikon Coolpix 990; Nikon Corporation, Japan) at 0 and 48 hours. The image processing software of the inverted microscope (ImageJ software Version v2.1.1, National Institutes of Health, USA) was used to measure the closure/gap and to calculate the wound healing rate using the following formula: (0 hour width-48 hours width)/0 hour width × 100%.

The transwell migration assay was conducted similarly to that described by Yue et al. Briefly, the transwell chamber, 24-well culture plate and sterile pipette tip were placed at 20°C for precooling. MCF-7 cells were pretreated in 2% FBS medium for 24 hours, while 60 μl Matrigel gel (B&D Biosciences, San Diego, CA, USA) was added to 300 μl serum-free medium, mixed, and diluted on ice. The bottom of the transwell chamber was coated with Matrigel. MCF-7 cells were treated with different concentrations of echinacoside (5, 10, 20, and 40 μg/ml) for 48 hours, and then digested with 0.025% trypsin (Beyotime Institute of Biotechnology, Shanghai, China) for 4 hours at a37°C cell incubator to prepare a cell suspension. The cell concentration was adjusted to 2 × 10^5 cells/ml and then washed 3 times with serum-free medium. The transwell chamber was placed in a 24-well culture plate. Next, 200 μl MCF-7 cell suspension was added to the chamber and DMEM culture medium (Sigma-Aldrich Corp, St Louis, USA) containing 10% FBS was added outside the chamber. After incubation for 24 hours, the chambers were taken out and cleaned with PBS. The cells in the upper chamber and the Matrigel glue were removed, while the remaining cells were fixed with 10% formalin and stained with hematoxylin and eosin. Under the microscope (magnification, ×100, Nikon Coolpix 990; Nikon Corporation, Japan), the tumor cells penetrating the Matrigel microporous membrane were counted in 6 randomly selected fields to calculate the mean value.

**Patients and Specimen Collection**

Specimens were collected from 30 patients diagnosed with breast cancer (invasive ductal carcinoma: 21 cases; invasive lobular carcinoma: 3 cases; metastatic breast cancer: 6 cases) who were treated at Shandong Provincial Hospital Affiliated to Shandong First Medical University (Jinan, Shandong, China) between January 2017 and January 2020. The control specimens were obtained from the adjacent non-cancer tissues of the patients with breast cancer, which were located at least 5 cm away from the breast cancer tissues. Breast cancer specimens were divided into Ki-67(+) and Ki-67(−) groups according to the expression of these molecules. The mRNA levels of miR-4306 and miR-4508 in the adjacent non-cancer tissues and breast cancer tissues were measured by reverse transcription-quantitative PCR.
(RT-qPCR). Informed consent was obtained from the patients and approved by the Ethics Committee of the Shandong Provincial Hospital Affiliated to Shandong First Medical University. The present study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the Shandong Provincial Hospital Affiliated to Shandong First Medical University (approval no. 2017-8546).

**Cell Culture and miRNA Measurement**

Normal MCF-10A cells and human breast cancer cells (MCF-7, MDA-MB-468, ZR-75-30, MDA-MB-453, and BT-20) were purchased from the Shanghai Institute of Biochemistry and Cellular Biology of the Chinese Academy of Sciences, and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich Corp, St Louis, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂. When the cells were 80% confluent, the cells were harvested by trypsin–EDTA treatment. Cells with a viability of more than 90% were used for the following studies. The total RNAs were extracted from cells with TRIzol Reagent (Invitrogen, Carlsbad CA, USA) and then converted into cDNA with Prime Script Reverse Transcriptase Kit (Takara Biotechnology Co. Ltd, Dalian, China). The expression levels of miR-4306 and miR-4508 in cells were measured by RT-qPCR using an ABI PRISM® 7900HT Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, and then 45 cycles of 95°C for 20 seconds and 55°C for 1 minutes. The primer of U6 was: Forward: 5′-CTCGCTTCGGCAGCACA-3′; Reverse: 5′-AACGCTT

**Breast Cancer Xenograft Mouse Model and Treatments**

BALB/C-nu/nu female nude mice (18-22 g) were purchased from the Animal Experimental Center of Shandong First Medical University and housed in the animal center of Shandong Provincial Hospital under 24°C and 12-/12-hour light/dark cycle, and they were allowed access to water and food ad libitum. The present animal study was approved by the Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong First Medical University (approval no. 2017-8547). After mice were housed for a week, MCF-7 cells in logarithmic growth phase were digested with 0.025% trypsin (Beyotime Institute of Biotechnology, Shanghai, China) for 4 hours at 37°C cell incubator to prepare a cell suspension of 5 × 10⁷ cells/ml. After the nude mice were anesthetized using ketamine hydrochloride (100 mg/kg, intramuscular injection) and xylazine (7.5 mg/kg, intramuscular injection), each nude mouse was inoculated subcutaneously with 0.2 ml suspension of MCF-7 cells (3.0 × 10⁶ cells/site) at the right flank region. When the tumor size was about 100 mm³, mice were randomly assigned into 6 groups: Control, Ech, Ech+ miR-4508 inhibitor, Ech+ miR-4306 inhibitor, Ech+ inhibitor-NC, and inhibitor-NC groups. The miR inhibitors or control miR was cloned into a modified lentiviral vector H1-MCS-CMV-EGFP, respectively (Guangzhou RiboBio Co., Ltd., Guangzhou, China). Mice were injected with lentiviruses containing miR-4508 inhibitor, miR-4306 inhibitor or inhibitor-NC via the tail vein (approximately 2 × 10⁷ transforming units of lentivirus). Mice in the echinacoside group were treated with 10 mg/kg echinacoside dissolved in 2% DMSO/8% olive oil in saline every 3 days, while mice in the control group were treated with 2% DMSO/8% olive oil in saline. The survival rate of mice was monitored for 30 days. The humane endpoints included: The body weight dropping rapidly by at least 15%; the mice unable to feed or drink by themselves; the tumor growing to more than 10% of their original body weight or the average tumor diameter becoming more than 20 mm; and/or sustained infection of body organs. No animal exhibited signs of such endpoints or was found dead during the study. Mice were sacrificed by cervical dislocation. The tumor of each mouse was dissected under aseptic conditions. The long diameter (a) and short diameter (b) of the tumors were measured to calculate the tumor volume (V) using the formula $V = \pi \times \frac{a \times b^2}{6}$. The tumor tissue was weighed to calculate the tumor weight. The tumor tissue was then immediately frozen in liquid nitrogen and stored at −80°C until use for subsequent experiments.

**Treatments of Cells With miR-4306 and miR-4508 Inhibitor**

miR-4306, miR-4508 inhibitor and inhibitor-NC (NC: negative control) were designed and synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China), and transfected into MCF-7 cells with X-fect™ RNA Transfection Reagent (Takara Biotechnology Co. Ltd, Dalian, China). Briefly, MCF-7 cells were seeded in a 6-well plate (1.5 × 10⁶ cells/well). Next, serum-free medium and transfection reagents were added to each well, and incubated for 4 hours before the culture medium was discarded. Next, DMEM culture medium (Sigma, USA) containing 10% FBS was added and incubated for 48 hours. The RNA levels of target miRNAs were measured to verify the success of the transfections.
inhibition rate. The maximum tumor volume observed in the study was 2.0 cm³.

Statistical Analyses
Data are represented as the mean ± SD of 4 independent experiments performed in triplicate. Multiple comparisons were performed by ANOVA followed by Tukey’s post-hoc test using SPSS 17.0 software (SPSS, Inc.). \( P < .05 \) was considered to indicate a statistically significant difference.

Results
Treatment With Echinacoside Affects the Proliferation and Cell Cycle of MCF-7 Cells
Figure 1a shows the molecular structure of echinacoside. Figure 1b shows that echinacoside decreased the viability of MCF-7 cells in a dose and time-dependent manner. Moreover, Figure 1c shows that, after treatment of MCF-7 cells with echinacoside (10, 20, and 40 μg/ml) for 72 hours, their cell cycle distribution was significantly disturbed. Figure 1d presents representative images of cell cycle analysis using a flow cytometer.

Treatment With Echinacoside Promotes the Apoptosis of MCF-7 Cells
The apoptosis rate of MCF-7 cells was measured with a flow cytometer after the cells were treated with echinacoside for 72 hours. As shown in Figure 2a, the apoptosis rate was significantly increased by treatment with echinacoside (20 and 40 μg/ml). Figure 2b to f show representative images of cell apoptosis using a flow cytometer. Figure 2g shows the representative images of western blots for pro-caspase-3, caspase-3, pro-caspase-8, caspase-8, pro-caspase-9, and
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Figure 2. The effect of echinacoside on the apoptosis of MCF-7 cells. (a) The effect of echinacoside on the apoptosis rate of MCF-7 cells. (b-f) The representative images of flow cytometer results of the apoptosis of MCF-7 cells. (g) The representative images of blots form the western blot experiments. (h) The effect of echinacoside on the ratios of cleaved /total Caspase-3, cleaved /total Caspase-8, cleaved /total Caspase-9. Each column represents the mean ± standard error of the mean of each parameter in each group (n = 10 per group).

Caspase-9. Figure 2h shows the results of relative changes in the pro-caspase-3/caspase-3 ratio, pro-caspase-8/caspase-8 ratio, and pro-caspase-9/caspase-9 ratio. The pro-caspase-3/caspase-3 ratio and the pro-caspase-8/caspase-8 ratio were both significantly increased by echinacoside treatment (10, 20, and 40 μg/ml), while the pro-caspase-9/caspase-9 ratio was significantly increased by 20 and 40 μg/ml echinacoside treatment.
Treatment With Echinacoside Inhibits the Migratory and Invasive Abilities of MCF-7 Cells

The migratory ability of MCF-7 cells was determined by wound healing assay after the cells were treated with echinacoside for 48 hours. Figure 3a to e show representative images of the wound healing results. Figure 3f shows the ratios of healing area/wound area. Compared with that of the control, the ratio of healing area/wound area was significantly decreased by 20 and 40 μg/ml echinacoside. The invasive ability of MCF-7 cells was determined by transwell assay after the cells were treated with echinacoside for 48 hours. Figure 4a shows representative images of the results of transwell assay. Figure 4b shows the number of migrated cells. Compared with that of the control group, the number of migrated cells was significantly decreased by 20 and 40 μg/ml echinacoside.

MiR-4306 and miR-4508 Expression is Decreased in Breast Cancer Tissues, and is Associated With Breast Cancer Classification

The expression of miR-4306 and miR-4508 was examined in breast cancer tissues and adjacent tissues. The results showed that miR-4306 and miR-4508 were significantly decreased in breast cancer tissues compared with their expression in adjacent tissues (Figure 5a and b). Next, breast cancer tissues were divided into Ki-67(+), Ki-67(−), PR(+), PR(−), ER-α(+), ER-α(−), HER2(+), and HER2(−) groups according to the positive or negative expression of Ki-67, PR, ER-α, or HER2. The expression levels of miR-4306 and miR-4508 were compared between the positive and negative expression groups. As shown in Figure 5c to j, the expression of miR-4306 and miR-4508 was significantly increased in Ki-67(−) breast cancer tissues.
tissues. There was no significant difference between PR(+) and PR(−) groups, ER-α(+) and ER-α(−) groups, or HER2(+) and HER2(−) groups. The expression of miR-4306 was significantly decreased in PR(−) and ER-α(−) breast cancer tissues compared with that in the PR(+) and ER-α(+) groups (P < .05).

Expression of miR-4306 and miR-4508 is Decreased in MCF-7 Cells

The expression of miR-4306 and miR-4508 was examined in normal MCF-10A cells and human breast cancer cells (MCF-7, MDA-MB-468, ZR-75-30, MDA-MB-453, and BT-20). As shown in Figure 6a and b, the expression of miR-4306 was significantly decreased in MCF-7 and BT-20 cells, while the mRNA expression of miR-4508 was significantly decreased in MCF-7 and MDA-MB-468 cells.

Effects of Echinacoside, miR-4306 and miR-4508 Inhibitors on the Expression of miR-4306 and miR-4508 in MCF-7 Cells

To confirm the role of miR-4306 and miR-4508 in the effects of echinacoside on MCF-7 cells, the present study first examined the effects of different concentrations of echinacoside on the expression of miR-4306 and miR-4508 in MCF-7 cells. As shown in Figure 7a and b, echinacoside upregulated the expression of miR-4306 and miR-4508 in a dose-dependent manner. Next, cells were treated with 20μg/ml echinacoside, and with miR-4306 inhibitor and miR-4508 inhibitor. Next, the expression of miR-4306 and miR-4508 was measured. Figure 7c and d show that miR-4306 inhibitor successfully inhibited the expression of miR-4306, while miR-4508 inhibitor successfully inhibited the expression of miR-4508.

MiR-4306 and miR-4508 Inhibitors Abolish the Effect of Echinacoside on MCF-7 Cells

To further investigate the role of miR-4306 and miR-4508, cells were treated with 20μg/ml echinacoside, and with miR-4306 inhibitor and miR-4508 inhibitor. Next, the cell viability, cell cycle, and migratory/invasive abilities of MCF-7 cells were measured. Figure 8a shows that miR-4306 and miR-4508 inhibitors significantly increased cell viability compared with that of the echinacoside+ inhibitor NC group. Figure 8b shows that miR-4306 and miR-4508 inhibitors significantly changed the cell cycle distribution compared with that of the echinacoside+ inhibitor NC group. Figure 8c and d show that miR-4306 and miR-4508 inhibitors significantly increased the ratio of healing area/wound area and the number of migration cells compared with those of the echinacoside+ inhibitor NC group.

Treatment With miR-4306 and miR-4508 Inhibitors Abolishes the Effects of Echinacoside on Xenograft Tumor

To explore the role of miR-4306 and miR-4508 in the effects of echinacoside in vivo, nude mice were inoculated subcutaneously with MCF-7 cells to establish xenograft tumors, and then treated with echinacoside (10mg/kg) and miR-4306 or miR-4508 inhibitors. Figure 9a shows changes in the survival rate of mice. Treatment with echinacoside (10mg/kg) significantly increased the survival rate of mice compared with that of the control group, while miR-4306 or miR-4508 inhibitor significantly decreased the survival rate of mice compared with that of the echinacoside+ inhibitor NC group. As shown in Figure 9b, echinacoside significantly decreased the tumor size compared with that of the control group, while miR-4306 or miR-4508 inhibitor significantly decreased the tumor size compared with that of the echinacoside+ inhibitor NC group. As shown in Figure 9c, the tumor inhibition rate in the echinacoside group was ~53%, while the tumor inhibition rate in the echinacoside+ miR-4306 inhibitor group and the echinacoside+ miR-4508 inhibitor group was ~12.5% and 18.7%, respectively, which was significantly lower than that of the echinacoside+ inhibitor NC group (46%; P < .05).
Discussion

Previous studies on the molecular mechanism of the occurrence and development of breast cancer suggested that miRNA may become an important factor regulating tumor development,\(^29,30\) which is a potential direction for the treatment of breast cancer. The invasion and metastasis of breast cancer involves changes in a variety of biological behaviors, as well as abnormalities in multiple signal transduction pathways. As a type of non-coding RNA, miRNA plays an important role in the mechanism of invasion and metastasis of multiple types of cancer by regulating the expression of target genes.\(^31-35\) For example, miR-130b inhibits tumor protein p53 inducible nuclear protein 1 to promote the growth and proliferation of CD33-positive liver cancer cells, while miR-10b can promote the invasion and metastasis of breast cancer cells by downregulating the expression of matrix metalloproteinase 2 and inhibiting the degradation of extracellular matrix.\(^36-38\) miRNAs also play an important role in the process of epithelial-mesenchymal transition by regulating the expression of tumor phenotypic proteins.\(^39\) Kim et al.\(^40\) compared the miRNA expression profiles of 10 healthy individuals and 76 patients with breast cancer using gene chip technology, and found that the expression of miR125 and miR145 in breast cancer tissue was decreased, while the expression of miR-21 and miR-155 was increased. Furthermore, miRNA expression was correlated with pathological characteristics of breast cancer, and abnormal expression of certain key miRNAs may lead to breast cancer.\(^41\)

Extraction of active components from natural drugs can provide novel ideas for the treatment of tumors. It has been reported that echinacoside exerted antiproliferative and proapoptotic functions on the HepG2 hepatoblastoma cell line.\(^22\) Another study has shown that echinacoside can regulate the expression of Bax, caspase-3 and Bcl-2 in cells.\(^20\) In addition, echinacoside can induce mitochondrial membrane potential imbalance, thus promoting the mitochondrial internal-dependent apoptosis pathway. Through this pathway, it could promote the apoptosis of tumor cells and inhibit the proliferation of human osteosarcoma, breast cancer and rectal cancer cells.\(^21,42\) These results indicate that echinacoside has a certain inhibitory effect on the proliferation of tumor cells, indicating that echinacoside may become an adjuvant drug for the treatment of tumor cells. The present study confirmed that echinacoside decreased the viability of MCF-7 cells in a dose and time-dependent manner. The cell cycle distribution of MCF-7 cells was significantly disturbed by echinacoside. Furthermore, echinacoside promoted the apoptosis of

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Figure 5. The expression of miR-4306 and miR-4508 in breast cancer tissues and their association with breast cancer classification. The expression of miR-4306 and miR-4508 was examined in breast cancer tissues and adjacent tissues (a and b). The expression of miR-4306 and miR-4508 was examined in Ki-67(+), Ki-67(−), PR(+), PR(−), ER-α(+), ER-α(−), HER2(+), and HER2(−) groups (c-j).
MCF-7 cells, and inhibited the migration and invasion abilities of MCF-7 cells.

For the first time, the present study found that the expression of miR-4306 and miR-4508 was decreased in breast cancer tissues. The expression of miR-4306 and miR-4508 was significantly increased in Ki-67(−) breast cancer tissues, and the expression of miR-4306 was significantly decreased in PR(−), ER-α(−) and HER2(−) breast cancer tissues, suggesting the potential role of miR-4306 and miR-4508 in the development of breast cancer. According to the expression of ER, PR, HER2, and Ki-67, breast cancer can be divided into 4 types: Luminal A, luminal B, HER2, and triple-negative types. Different molecular types of breast cancer have different biological characteristics and deserve different treatment strategies.43 Our results suggested that miR-4508 was not associated with the expression of ER, PR, or HER2, while miR-4508 was positively associated with their expression. ER-α and PR are nuclear receptors whose physiological effects are being promoters of target genes to increase their gene transcription.44 Zhao et al.10 found that ER element and PR element are both miR-4306 promoters; ER-α and PR could transcriptionally promote the expression of miR-4306. HER2, a member of the epidermal growth factor receptor tyrosine kinase family, could form a complex at a specific nucleotide sequence of the gene promoter to stimulate its transcription.45,46 As suggested by Zhao et al.,10 HER2 could directly bind to the promoter of the miR-4306 gene and increase miR-4306 expression, which is consistent with the results of the present study.

Numerous previous studies have investigated the mechanism of the antitumor effect of echinacoside. Dong et al.42 suggested that echinacoside induces apoptosis in human SW480 colorectal cancer cells by induction of oxidative DNA damage. Wang et al.20 reported that echinacoside suppresses pancreatic adenocarcinoma cell growth by inducing apoptosis via the mitogen-activated protein kinase pathway. In another study, echinacoside was found to exert its antitumor activity via decreasing TREM2 expression and PI3K/AKT signaling.22 In breast cancer cells, echinacoside was found to suppress the Wnt/β-catenin signaling pathway.23 However, the association between echinacoside and miRNAs has not been investigated to date. For the first time, the present study examined the effect of echinacoside on the expression of miR-4306 and miR-4508 in MCF-7 cells, and found out that echinacoside upregulated the expression of miR-4306 and miR-4508 in a dose-dependent manner. As our previous investigations (Figure 1) suggested, the role of miR-4306 and miR-4508 in the development of breast cancer, these results indicated that miR-4306 and miR-4508 may play an important role in the anticancer effect of echinacoside. To confirm the role of miR-4306 and miR-4508 in the effects of echinacoside on MCF-7 cells, cells were treated with 20 μg/ml echinacoside, and with miR-4306 inhibitor and miR-4508 inhibitor. Next, the changes in the cell viability, cell cycle and migration/invasion abilities of MCF-7 cells were measured. The present results showed that treatment with miR-4306 and miR-4508 inhibitors abolished the effects of echinacoside on the viability, cell cycle and migration/invasion abilities of MCF-7 cells. Furthermore, in the mouse xenograft tumor model induced by MCF-7 cells, treatment with echinacoside (10 mg/kg) significantly increased the survival rate of mice compared with that of the control group, while miR-4306 or miR-4508 inhibitor significantly decreased the survival rate of mice. miR-4306 and miR-4508 inhibitor also significantly increased the tumor size that was suppressed by echinacoside, and decreased the tumor inhibition rate of echinacoside. These results suggested a novel explanation for the antitumor effect of echinacoside, which involves miR-4306 and miR-4508.
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To confirm the effects of echinacoside on breast cancer in vivo, nude mice were inoculated subcutaneously with MCF-7 cells to establish a xenograft tumor model, followed by treatment with echinacoside (10 mg/kg). Echinacoside inoculation significantly increased the survival rate of mice and decreased the tumor size. The tumor inhibition rate of echinacoside was ~53%, which was significantly higher than seen in that of the control. Similar results were obtained by Tang et al., who found that echinacoside significantly inhibited tumor growth, decreased tumor cell density and reduced proliferation in MDA-MB-231 xenograft tumor tissues, as indicated by Ki-67 staining. To confirm the role of miR-4306 or miR-4508, mice were co-treated with miR-4306 or miR-4508 inhibitor, which significantly reversed the antitumor effect of echinacoside. Co-treatment with miR-4306 or miR-4508 inhibitor decreased the survival rate of mice, increased the tumor size and had a much lower tumor inhibition rate. These results strengthen our hypothesis that echinacoside exerts its antitumor effect by stimulating the expression of miR-4306 or miR-4508. There have been many studies suggesting that some natural products could regulate the expression of several miRNAs involved in cancer development. For instance, curcumin could up-regulate the expression of miRNA-22 and down-regulate miRNA-199a by targeting SP1 transcription factor and estrogen receptor 1, which were important for its anti-tumor efficiency. Resveratrol was found to inhibit breast cancer cell proliferation by activating multiple tumor-suppressive

Figure 7. Effects of echinacoside, miR-4306, and miR-4508 inhibitors on the expression of miR-4306 and miR-4508 in MCF-7 cells. (a) Effects of echinacoside on the expression of miR-4306. (b) Effects of echinacoside on the expression of miR-4508. (c) Effects of miR-4306 and miR-4508 inhibitors on the expression of miR-4306. (d) Effects of miR-4306 and miR-4508 inhibitors on the expression of miR-4508. Each column represents the mean ± standard error of the mean of each parameter in each group (n = 10 per group). Abbreviations: Ech, echinacoside; NC, negative control.
miRNAs (miR-34a, miR-424, and miR-503) expression via the p53 pathway. It is not surprising that echinacoside could stimulate the expression of miR-4306 and miR-4508, but the underlying regulation mechanism needs further clarifying.

In conclusion, the significance of the present results includes that it showed that miR-4306 and miR-4508 were decreased in breast cancer tissues and cells. Echinacoside inhibits cell proliferation, invasion and migration, and promotes the apoptosis of breast cancer cells via downregulating the expression of miR-4306 and miR-4508. This is the first study to show the association between echinacoside and miRNAs in tumor. The present study elucidates an underlying molecular mechanism of the antitumor effect of echinacoside on breast cancer, and thus may contribute to preventive and therapeutic strategies for breast cancer targeting miR-4306 and miR-4508. However, there are several shortcomings that need to be noted. Firstly, the effect of echinacoside on breast cancer was only tested in animal models and cells. As no clinical trial was performed, the effect of echinacoside on human breast cancer still needs to be examined. Secondly, echinacoside may exert antitumor effects through other mechanisms, not only through its regulation on miR-4306 and miR-4508. Thirdly, the exact mechanism of the relationship between miR-4306 and miR-4508 and breast cancer needs to be clarified.

**Figure 8.** Effects of miR-4306 and miR-4508 inhibitors on the effects of echinacoside on the cell viability, cell cycle and migratory/invasive abilities of MCF-7 cells. (a) The changes of cell viability of MCF-7 cells. (b) The changes of cell cycle of MCF-7 cells. (c) The changes of ratio of healing area/wound area in MCF-7 cells. (d) The changes of average migration cell number in MCF-7 cells. Each column represents the mean ± standard error of the mean of each parameter in each group (n = 10 per group). Abbreviations: Ech, echinacoside; NC, negative control.
Author Contributions
PB performed the cell experiments and wrote the paper; CL performed the animal experiments; YD analyzed the clinical data; WH revised the manuscript; SSQ analyzed the data; LL designed and supervised the study.

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Ethics Approval
The present study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the Shandong Provincial Hospital Affiliated to Shandong First Medical University (approval no. 2017-8546). The present animal study was approved by the Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong First Medical University (approval no. 2017-8547).

Consent to Participate
Informed consent was obtained from the patients and approved by the Ethics Committee of the Shandong Provincial Hospital Affiliated to Shandong First Medical University.

Consent for Publication
Informed consent for publication was obtained from the patients and approved by the Ethics Committee of the Shandong Provincial Hospital Affiliated to Shandong First Medical University.

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Availability of Data and Material
The datasets used and/or analyzed for this study are available from the corresponding author upon reasonable request.

References
1. Harbeck N, Gnant M. Breast cancer. Lancet. 2017;389:1134-1150.
2. Hao JJ, Gong T, Zhang Y, et al. Characterization of gene rearrangements resulted from genomic structural aberrations in human esophageal squamous cell carcinoma KYSE150 cells. Gene. 2013;513:196-201.
3. Rhodes LV, Tate CR, Hoang VT, et al. Regulation of triple-negative breast cancer cell metastasis by the tumor-suppressor liver kinase B1. Oncogenesis. 2015;4:e168. doi:10.1038/oncsis.2015.27
4. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. CA Cancer J Clin. 2020;70:7-30.
5. Ahmad A. Pathways to breast cancer recurrence. ISRN Oncol. 2013;2013:209568. doi:10.1155/2013/209568
6. Rojas K, Stuckey A. Breast cancer epidemiology and risk factors. Clin Obstet Gynecol. 2016;59:651-672.
7. Haydu LE, Scolyer RA, Lo S, et al. Conditional survival: an assessment of the prognosis of patients at time points after initial diagnosis and treatment of locoregional melanoma metastasis. J Clin Oncol. 2017;35:1721-1729.
8. Klein U, Lia M, Crespo M, et al. The DLEU2/miR-15a/16-1 cluster controls B cell proliferation and its deletion leads to chronic lymphocytic leukemia. Cancer Cell. 2010;17:28-40.
9. Guil S, Esteller M. DNA methylomes, histone codes and miRNAs: tying it all together. Int J Biochem Cell Biol. 2009;41:87-95.
10. Zhao Z, Li L, Du P, et al. Transcriptional downregulation of miR-4306 serves as a new therapeutic target for triple negative breast cancer. Theranostics. 2019;9:1401-1416.
11. Boo L, Ho WY, Ali NM, et al. MiRNA transcriptome profiling of spheroïd-enriched cells with cancer stem cell properties in human breast MCF-7 cell line. Int J Biol Sci. 2016;12:427-445.
12. Facino RM, Renz I, Brack A. Isolation and constitution of echinacoside, a glycoside from the roots of Echinacea angustifolia DC. *Helv Chim Acta.* 1950;33:1877-1893.

13. Facino RM, Carini M, Aldini G, Saibene L, Pietta P, Mauri P. Echinacoside and caffeoyl conjugates protect collagen from free radical-induced degradation: a potential use of Echinacea extracts in the prevention of skin photodamage. *Planta Med.* 1995;61:510-514.

14. Jia Y, Guan Q, Jiang Y, et al. Amelioration of dextran sulphate sodium-induced colitis in mice by echinacoside-enriched extract of Cistanche tubulosa. *Phytother Res.* 2014;28:110-119.

15. Jiang Y, Tu PF. Analysis of chemical constituents in Cistanche species. *J Chromatogr.* 2009;1216:1970-1979.

16. Zhang D, Li H, Wang JB. Echinacoside inhibits amyloid fibrillization of HEWL and protects against Aβ-induced neurotoxicity. *Int J Biol Macromol.* 2015;72:243-253.

17. Wu Y, Li L, Wen T, Li YQ. Protective effects of echinacoside on carbon tetrachloride-induced hepatotoxicity in rats. *Toxicology.* 2007;232:50-56.

18. Xie H, Zhu H, Cheng C, Liang Y, Wang Z. Echinacoside retards cellular senescence of human fibroblastic cells MRC-5. *Pharmazie.* 2009;64:752-754.

19. Wang S, Zheng G, Tian S, et al. Echinacoside improves hematopoietic function in 5-FU-induced myelosuppression mice. *Life Sci.* 2015;123:86-92.

20. Wang W, Luo J, Liang Y, Li X. Echinacoside suppresses pancreatic adenocarcinoma cell growth by inducing apoptosis via the p73-activated protein kinase pathway. *Mol Med Rep.* 2016;13:2613-2618.

21. Dong L, Wang H, Niu J, et al. Echinacoside induces apoptotic cancer cell death by inhibiting the nucleotide pool sanitizing enzyme MTH1. *Onco Targets Ther.* 2015;8:3649-3664.

22. Ye Y, Song Y, Zhuang J, Wang G, Ni J, Xia W. Anticancer effects of echinacoside in hepatocellular carcinoma mouse model and HepG2 cells. *J Cell Physiol.* 2019;234:1880-1888.

23. Tang C, Gong L, Lvzi XU, Qiu K, Zhang Z, Wan L. Echinacoside inhibits breast cancer cells by suppressing the Wnt/β-catenin signaling pathway. *Biochem Biophys Res Commun.* 2020;526:170-175.

24. Zhang B, Tian L, Xie J, Chen G, Wang F. Targeting miRNAs by natural products: a new way for cancer therapy. *Biomed Pharmacother.* 2020;130:110546. doi:10.1016/j.biopha.2020.110546

25. Sethi S, Li Y, Sarkar FH. Regulating miRNA by natural agents as a new strategy for cancer treatment. *Curr Drug Targets.* 2013;14:1167-1174.

26. Cui YT, Liu B, Xie J, Xu P, Habte-Tsion HM, Zhang YY. The effect of emodin on cytotoxicity, apoptosis and antioxidant capacity in the hepatic cells of grass carp (Ctenopharyngodon idellus). *Fish Shellfish Immunol.* 2014;38:74-79.

27. Cheng C, Jiao JT, Qian Y, et al. Curcumin induces G2/M arrest and triggers apoptosis via FoxO1 signaling in U87 human glioma cells. *Mol Med Rep.* 2016;13:3763-3770.

28. Yue S, Ye X, Zhou T, et al. PGRN(-/-) TAMs-derived exosomes inhibit breast cancer cell invasion and migration and its mechanism exploration. *Life Sci.* 2021;264:118687. doi:10.1016/j.lfs.2020.118687

29. Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. *Nat Rev Drug Discov.* 2017;16:203-222.

30. Li M, Huo X, Davuljigari CB, Dai Q, Xu X. MicroRNAs and their role in environmental chemical carcinogenesis. *Environ Geochem Health.* 2019;41:225-247.

31. Ma S, Tang KH, Chan YP, et al. miR-130b promotes CD133(+) liver tumor-initiating cell growth and self-renewal via tumor protein 53-induced nuclear protein 1. *Cell Stem Cell.* 2010;7:694-707.

32. Yuan K, Xie K, Fox J, et al. Decreased levels of miR-224 and the passenger strand of miR-221 increase MBD2, suppressing maspin and promoting colorectal tumor growth and metastasis in mice. *Gastroenterology.* 2013;145:853-64.e9.

33. Chen DL, Wang DS, Wu WJ, et al. Overexpression of paxillin induced by miR-137 suppression promotes tumor progression and metastasis in colorectal cancer. *Carcinogenesis.* 2013;34:803-811.

34. Kinouchi M, Uchida D, Kuribayashi N, Tamatani T, Nagai H, Miyamoto Y. Involvement of mir-518c-5p to growth and metastasis in oral cancer. *PLoS One.* 2014;9:e115936. doi:10.1371/journal.pone.0115936

35. Kinose Y, Sawada K, Nakamura K, et al. The hypoxia-related microRNA miR-199a-3p displays tumor suppressor functions in ovarian carcinoma. *Oncotarget.* 2015;6:11342-11356.

36. Ma L, Teruya-Feldstein J, Weinberg RA. Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. *Nature.* 2007;449:682-688.

37. Fang JH, Zhou HC, Zeng C, et al. MicroRNA-29b suppresses tumor angiogenesis, invasion, and metastasis by regulating matrix metalloproteinase 2 expression. *Hepatology.* 2011;54:1729-1740.

38. Wang R, Zhao N, Li S, et al. MicroRNA-195 suppresses angiogenesis and metastasis of hepatocellular carcinoma by inhibiting the expression of VEGF, VAV2, and CDC42. *Hepatology.* 2013;58:642-653.

39. Lamouille S, Subramanyam D, Bellerich R, Derynick R. Regulation of epithelial-mesenchymal and mesenchymal-epithelial transitions by microRNAs. *Curr Opin Cell Biol.* 2013;25:200-207.

40. Kim S, Song JH, Kim S, et al. Loss of oncogenic miR-155 expression of clinicopathologic features and survival. *Int J Mol Sci.* 2019;20:11094-11112.

41. Deng CV, Le A, Gao P. MYC-induced cancer cell energy metabolism and therapeutic opportunities. *Clin Cancer Res.* 2009;15:6479-6483.

42. Dong L, Yu D, Wu N, et al. Echinacoside induces apoptosis in human SW480 colorectal cancer cells by induction of oxidative DNA damages. *Int J Mol Sci.* 2015;16:14655-14668.

43. Onitilo AA, Engel JM, Greenlee RT, Mukesh BN. Breast cancer subtypes based on ER/PR and Her2 expression: comparison DNA damages. *Targets.* 2013;25:200-207.

44. Badve SS, Baehner FL, Gray RP, et al. Estrogen- and progesterone-receptor status in ECOG 2197: comparison of immunohistochemistry by local and central laboratories and quantitative reverse transcription polymerase chain reaction by central laboratory. *J Clin Oncol.* 2008;26:2473-2481.
45. Giri DK, Ali-Seyed M, Li LY, et al. Endosomal transport of ErbB-2: mechanism for nuclear entry of the cell surface receptor. *Mol Cell Biol*. 2005;25:11005-11018.

46. Béguelin W, Diaz Flaqué MC, Proietti CJ, et al. Progesterone receptor induces ErbB-2 nuclear translocation to promote breast cancer growth via a novel transcriptional effect: ErbB-2 function as a coactivator of Stat3. *Mol Cell Biol*. 2010;30:5456-5472.

47. Phuah NH, Nagoor NH. Regulation of microRNAs by natural agents: new strategies in cancer therapies. *Biomed Res Int*. 2014;2014:804510. doi:10.1155/2014/804510

48. Li Y, Kong D, Wang Z, Sarkar FH. Regulation of microRNAs by natural agents: an emerging field in chemoprevention and chemotherapy research. *Pharm Res*. 2010;27:1027-1041.

49. Sun M, Estrov Z, Ji Y, Coombes KR, Harris DH, Kurzrock R. Curcumin (diferuloylmethane) alters the expression profiles of microRNAs in human pancreatic cancer cells. *Mol Cancer Ther*. 2008;7:464-473.

50. Otsuka K, Yamamoto Y, Ochiya T. Regulatory role of resveratrol, a microRNA-controlling compound, in HNRNPA1 expression, which is associated with poor prognosis in breast cancer. *Oncotarget*. 2018;9:24718-24730.