Research Article

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

Integrative Cancer Therapies
Volume 20: 1–15

DOI: 10.1177/15347354211062639
journals.sagepub.com/home/ict
SAGE

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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

Submitted July 21, 2021; revised October 24, 2021; accepted November 9, 2021

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.[2,3] 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

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cancer to continue exploring and identifying effective therapeutic targets.

MicroRNA (miRNA or miR) is a class of naturally occurring, non-coding single-stranded RNA. miRNA acts as a key gene expression regulator and participates in almost all biological processes, including tumor invasion and metastasis.[8] 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 mechanism.[9] A previous study demonstrated that low miR-4306 expression is strongly associated with lymph node metastasis and poor survival in triple-negative breast cancer.[10] Another study indicated that miR-4508 showed a potential association with breast cancer chemoresistance and self-renewal capability.[11] 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.[12] Echinacoside can also be extracted from *Cistanche*, which has been widely used in the treatment of the common cold in Europe.[13] *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.[14,15] Some studies have shown that echinacoside has the following functions: neuroprotective, anti-aging, scavenging free radicals, protecting vascular endothelial cells, and promoting hematopoiesis.[16-19] It was also reported that echinacoside induced the apoptosis of pancreatic cancer and other tumor cells.[20] Wang et al[20] 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[21] 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[22] 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.[23] 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.[24,25] It was suggested that some natural products could inhibit cancer progression, increase drug sensitivity, reverse EMT, and prevent metastasis though modulation of miRNAs, which will provide a newer therapeutic approach for cancer treatment.[25] 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)-dimethylthiahiazo (-z-y1)-3, 5-di- phenytetrazoliumromide (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.[26] MCF-7 cells were seeded into 12-well plates (3×10^5 cells per 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,[27] 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 G_0/G_1, S, and G_2/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-3, cleaved Caspase-8, 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,[28] 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[28] 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. MFC-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 in a37°C cell incubator to prepare a cell suspension. The cell concentration was adjusted to 2×10^4 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 MFC-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(+), Ki-67(−), PR(+), PR(−), HER2(+), HER2 (−), ER-α(+) and ER-α(−) 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% CO_2. 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 ofmiR-4306 was: Forward: 5′-ATCGAGCTCACATGATC GTGCGCTCCTGCAAGTG-3′; Reverse: 5′-ACTCTCGA GGCATCTCAGAGTGTTGCTATGGTGA-3′; The primer of miR-4508 was: Forward: 5′ACACTCCAGCTGGGGC GGGGCTGGG-3′; Reverse: 5′-CTCAACTGGTGTC GTGGAGTCGGCAATTCAGTTGAGCGCGCGCC-3′; The amount of miR-4306 and miR-4508 was calculated with $2^{-\Delta\Delta Ct}$ method.U6 small nuclear RNA was used for normalization. The primer of U6 was: Forward: 5′-CTCGCTTCGGCAGCACA-3′; Reverse: 5′-AACGCTT CACGAATTTGCGT-3′.

Treatment 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 (1.5×10^6 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.

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 5×10^7 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 (3.0×10^6 cells/site) at the right flank region. When the tumor size was about 100 mm^3, 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 2×10^7 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 $V = \pi \times a \times b^2/6$. The tumor tissue was weighed to calculate the tumor

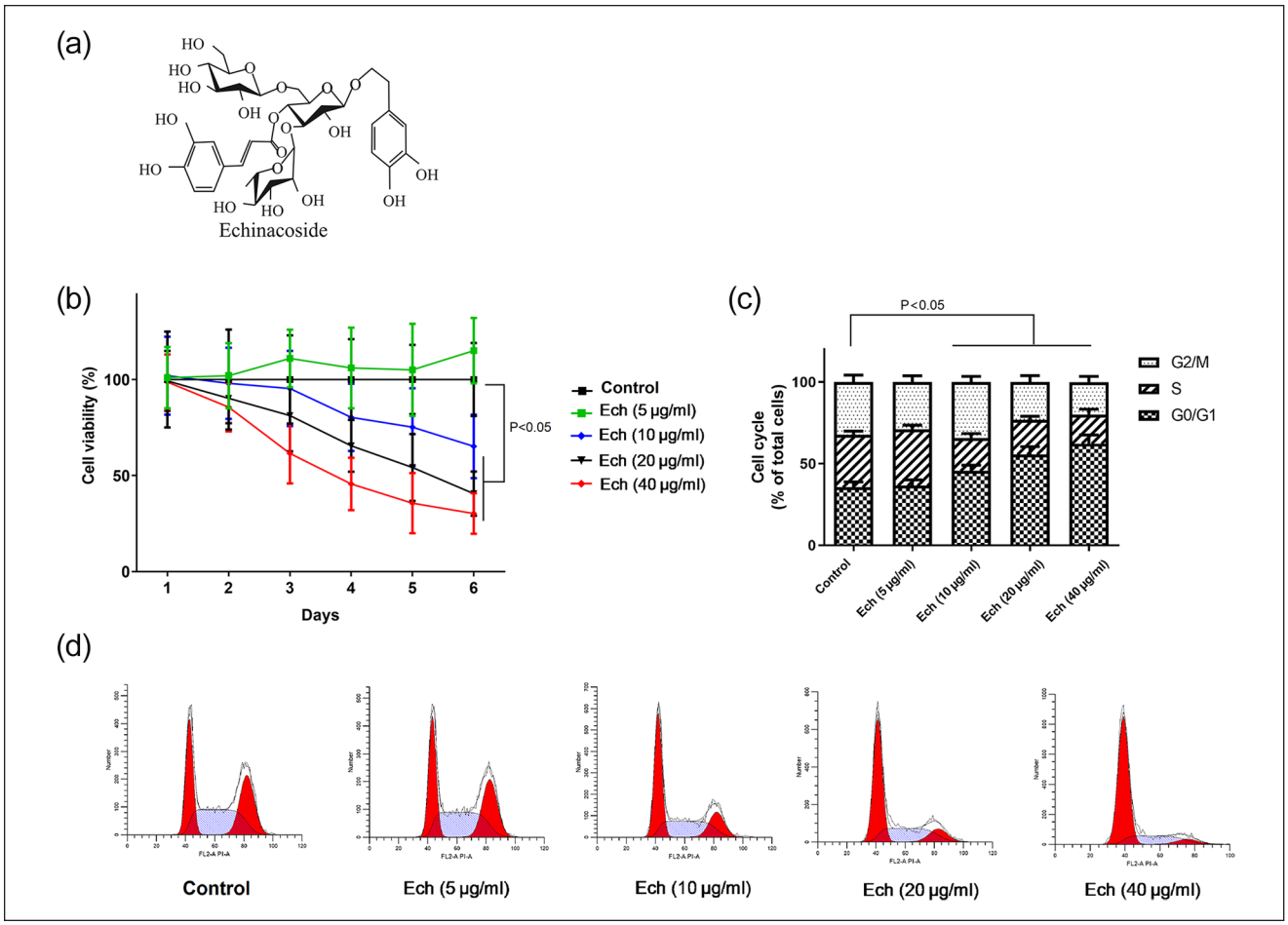

Figure 1. The effect of echinacoside on the proliferation and cell cycle of MCF-7 cells. (a) The molecular structure of echinacoside. (b) The effect of echinacoside on the proliferation of MCF-7 cells. (c) The effect of echinacoside on the cell cycle of MCF-7 cells. (d) The representative images of flow cytometer results of the cell cycle of MCF-7 cells. Each column represents the mean ± standard error of the mean of each parameter in each group (n = 10 per group).

inhibition rate. The maximum tumor volume observed in the study was 2.0 cm^3.

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

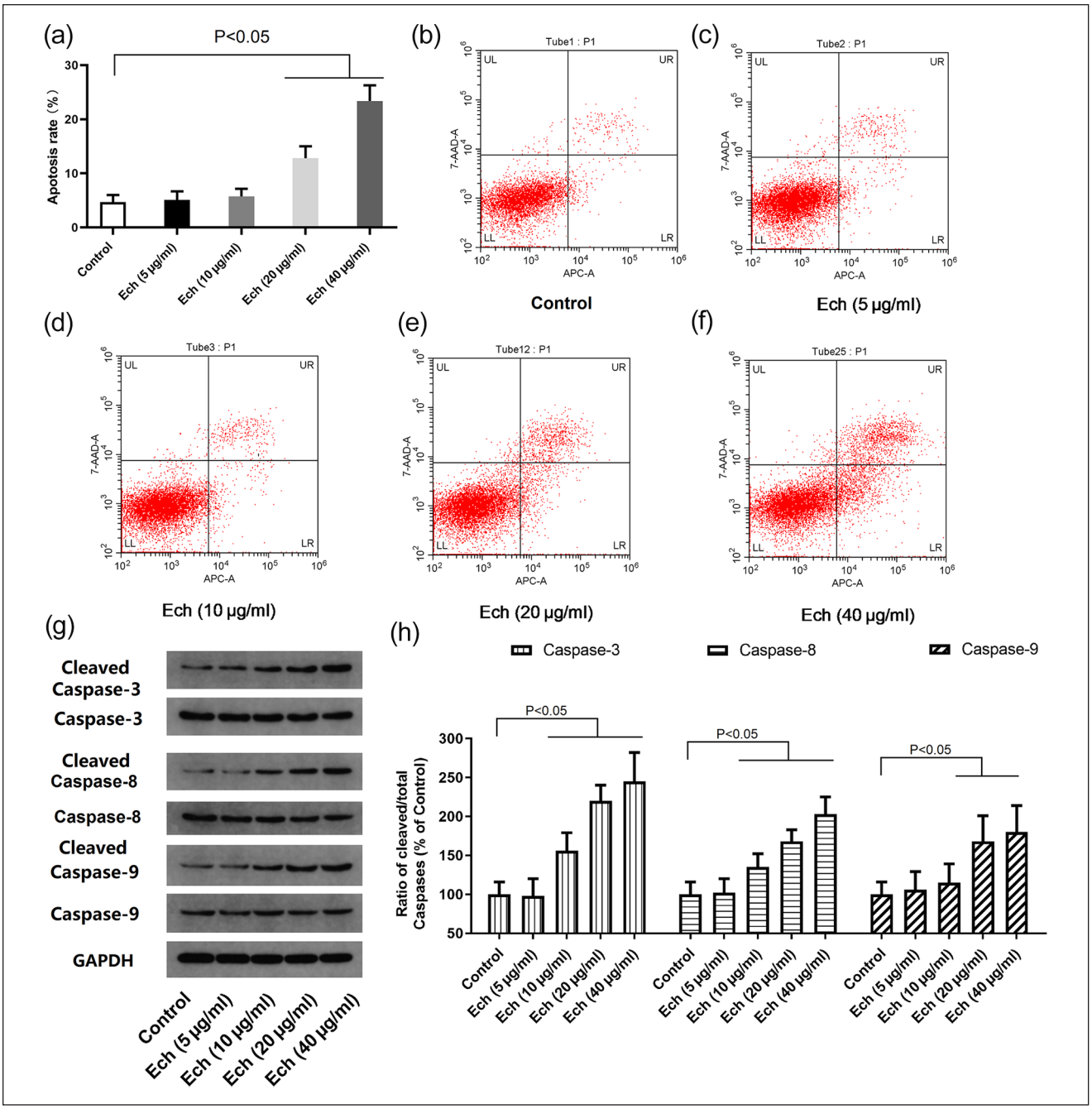

Figure 2. The effect of echinacoside on the apoptosis of MCF-7 cells. (a) The effect of echinacoside on the apoptosis rate of MCF-7 cells. (b-f) The representative images of flow cytometer results of the apoptosis of MCF-7 cells. (g) The representative images of blots form the western blot experiments. (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.

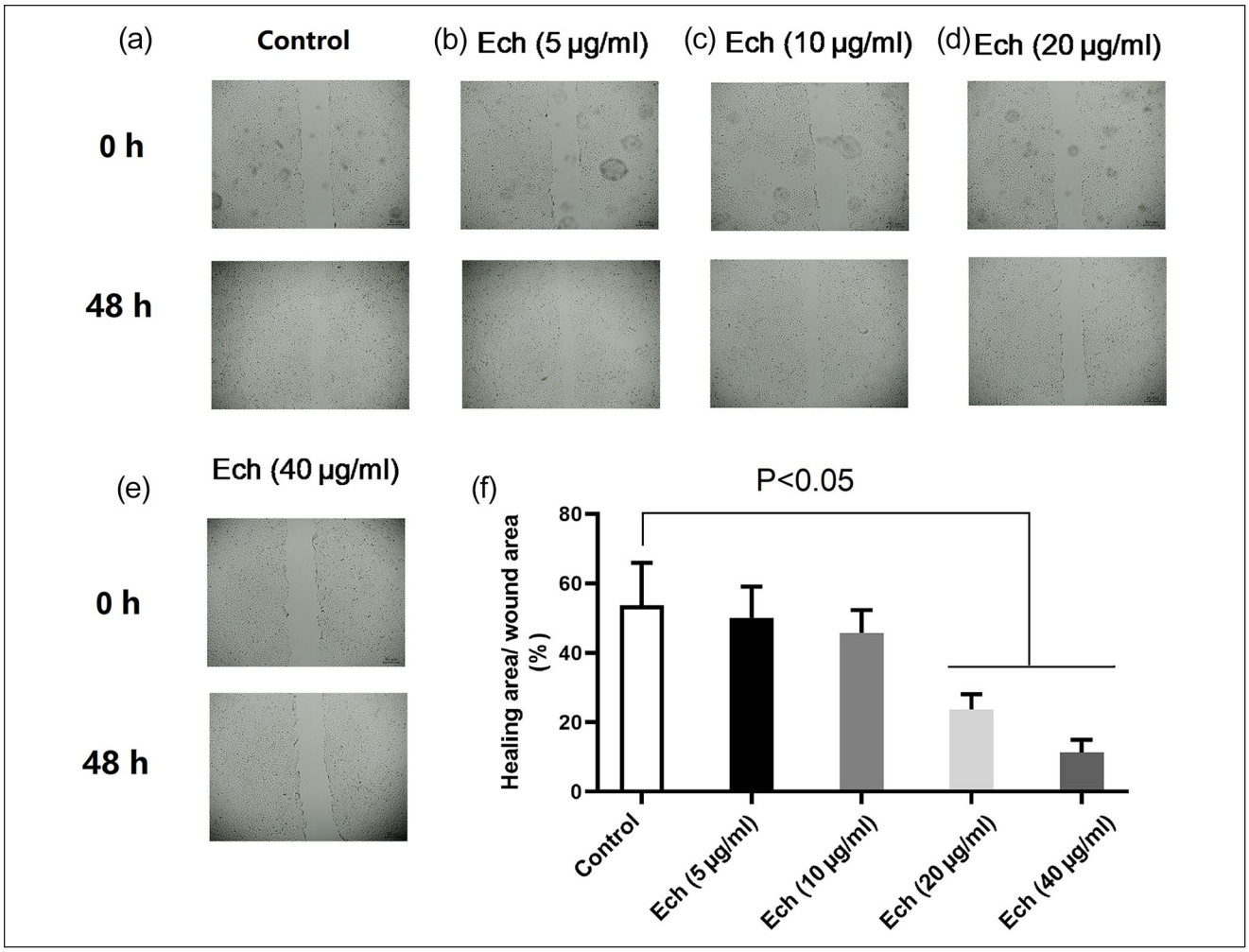

Figure 3. The effect of echinacoside on the migratory ability of MCF-7 cells. (a-e) The representative images of wound healing results of MCF-7 cells; (f) The effect of echinacoside on the ratio of healing area/wound area. Each column represents the mean ± standard error of the mean of each parameter in each group (n = 10 per group).
Abbreviation: Ech, echinacoside.

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

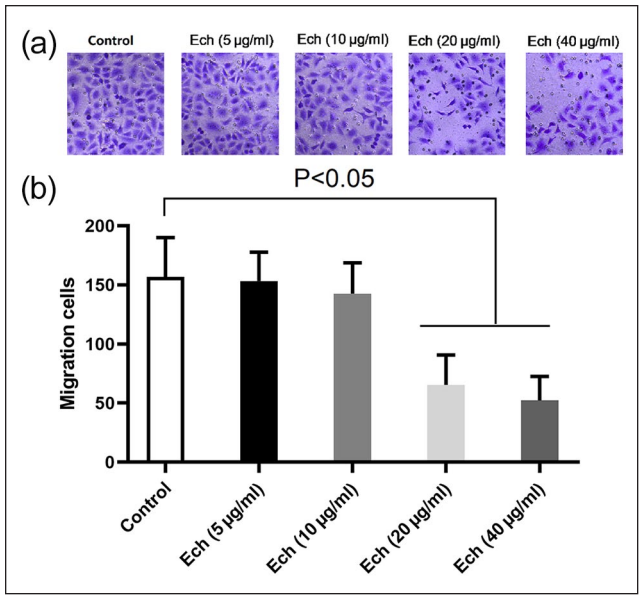

Figure 4. The effect of echinacoside on the invasive ability of MCF-7 cells. (a) The representative images of Transwell results of MCF-7 cells. (b) The effect of echinacoside on the average migration cell number in MCF-7 cells. Each column represents the mean ± standard error of the mean of each parameter in each group (n = 10 per group).
Abbreviation: Ech, echinacoside.

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 20 μg/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 20 μg/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 (10 mg/kg) and miR-4306 or miR-4508 inhibitors. Figure 9a shows changes in the survival rate of mice. 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 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 increased it 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%; $P < .05$).

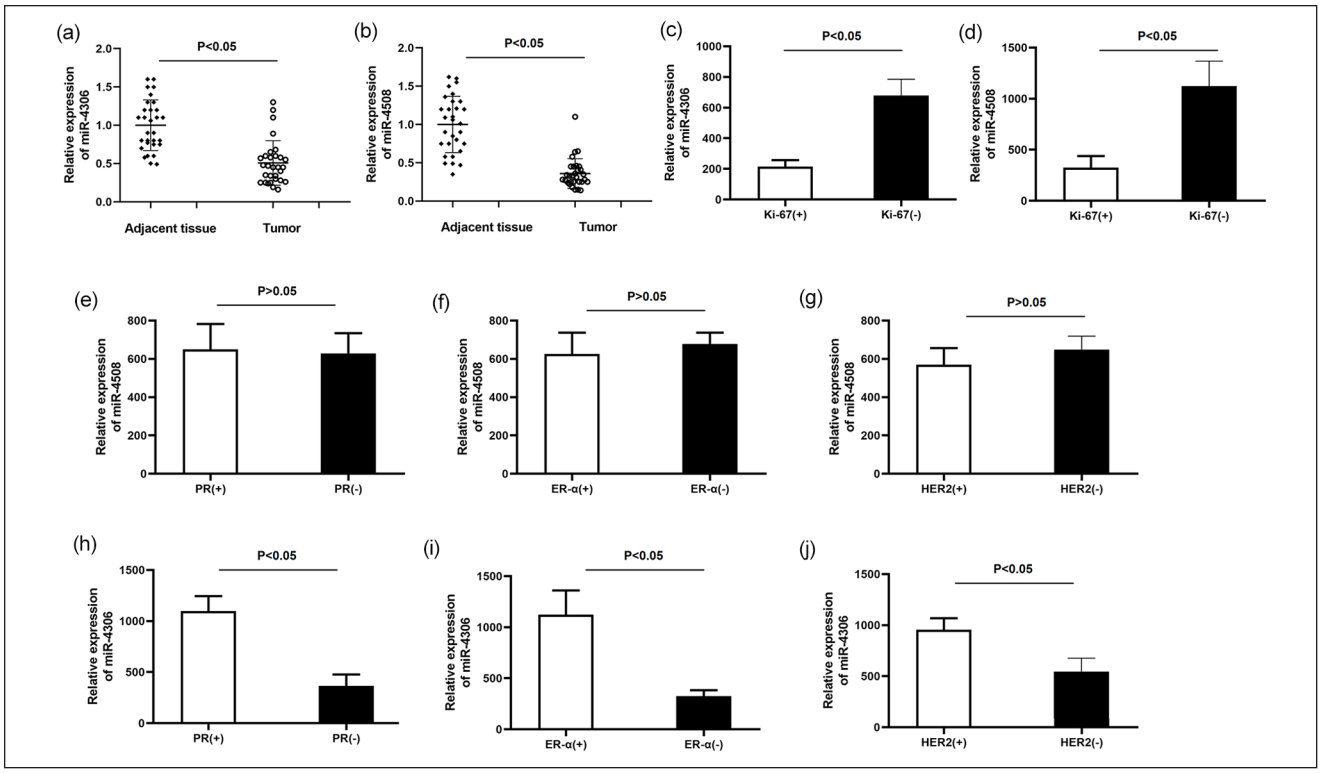

Figure 5. The expression of miR-4306 and miR-4508 in breast cancer tissues and their association with breast cancer classification. The expression of miR-4306 and miR-4508 was examined in breast cancer tissues and adjacent tissues (a and b). The expression of miR-4306 and miR-4508 was examined in Ki-67(+),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

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

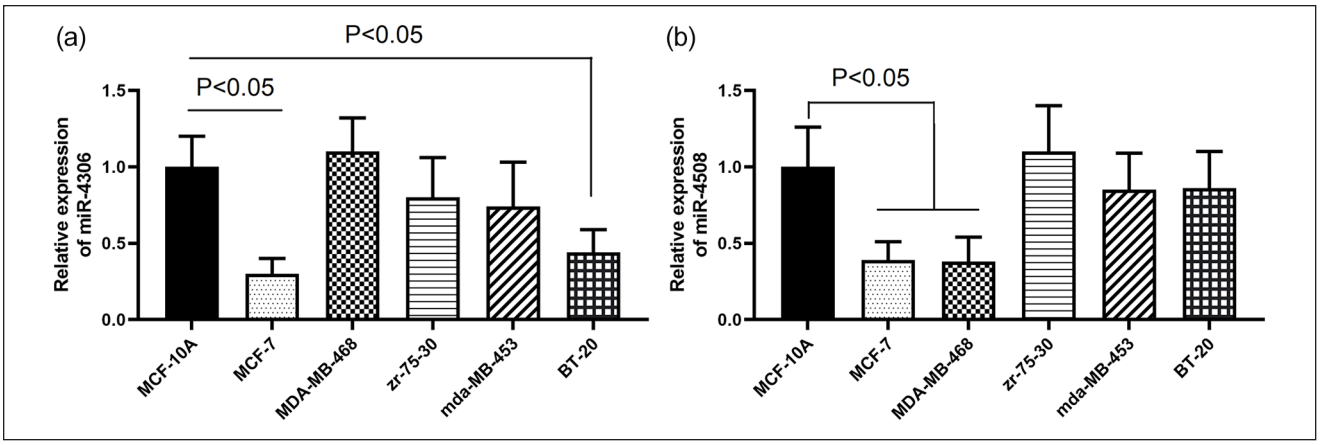

Figure 6. The expression of miR-4306 and miR-4508 in normal breast cell and breast cancer cells. (a) The expression of miR-4306 in normal breast cells and breast cancer cells; (b) The expression of miR-4508 in normal breast cells and breast cancer cells. Each column represents the mean ± standard error of the mean of each parameter in each group (n = 10 per group).

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-4306 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.

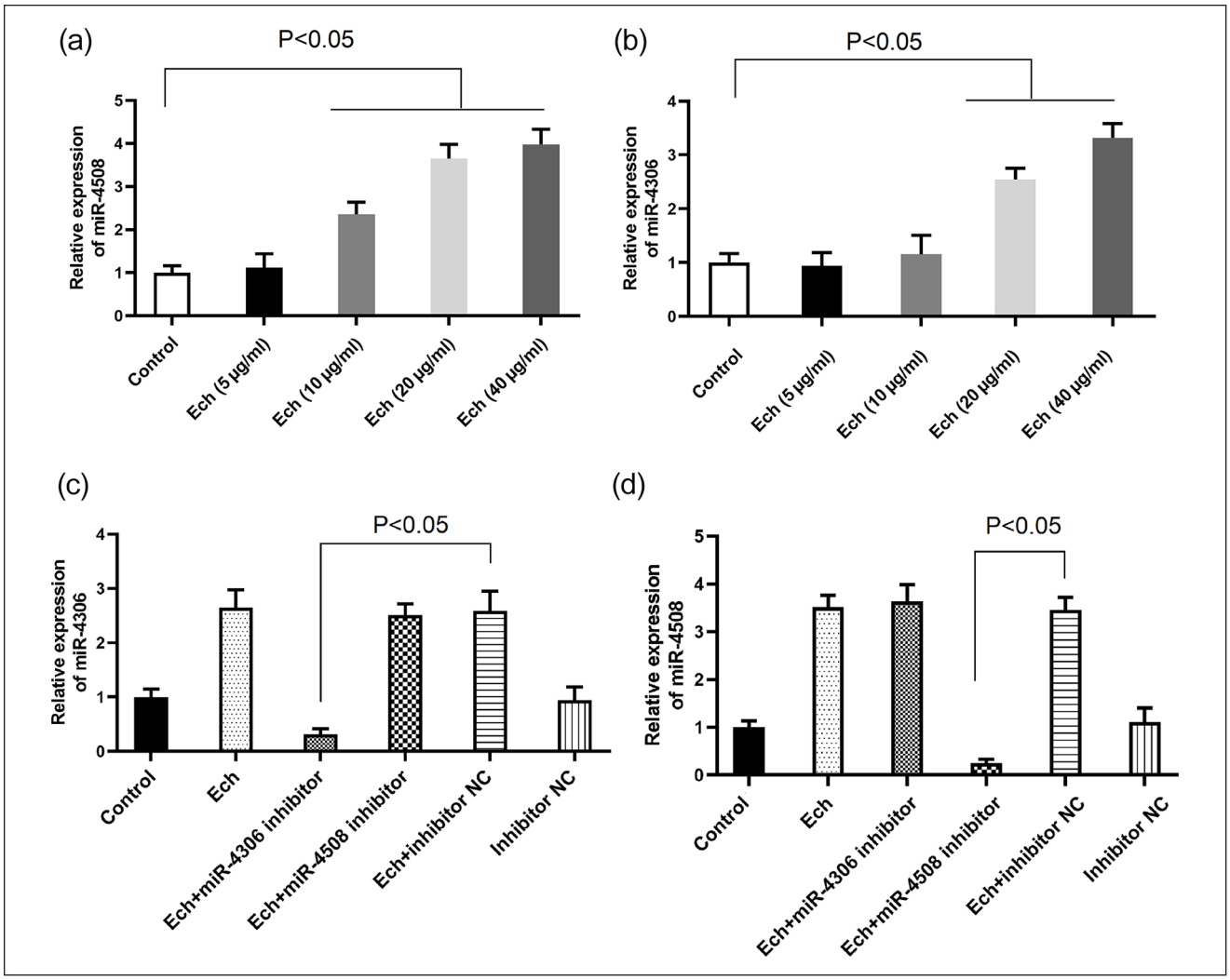

Figure 7. Effects of echinacoside, miR-4306, and miR-4508 inhibitors on the expression of miR-4306 and miR-4508 in MCF-7 cells. (a) Effects of echinacoside on the expression of miR-4306. (b) Effects of echinacoside on the expression of miR-4508. (c) Effects of miR-4306 and miR-4508 inhibitors on the 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.

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,[23] 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.[47,48] 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.[49] Resveratrol was found to inhibit breast cancer cell proliferation by activating multiple tumor-suppressive

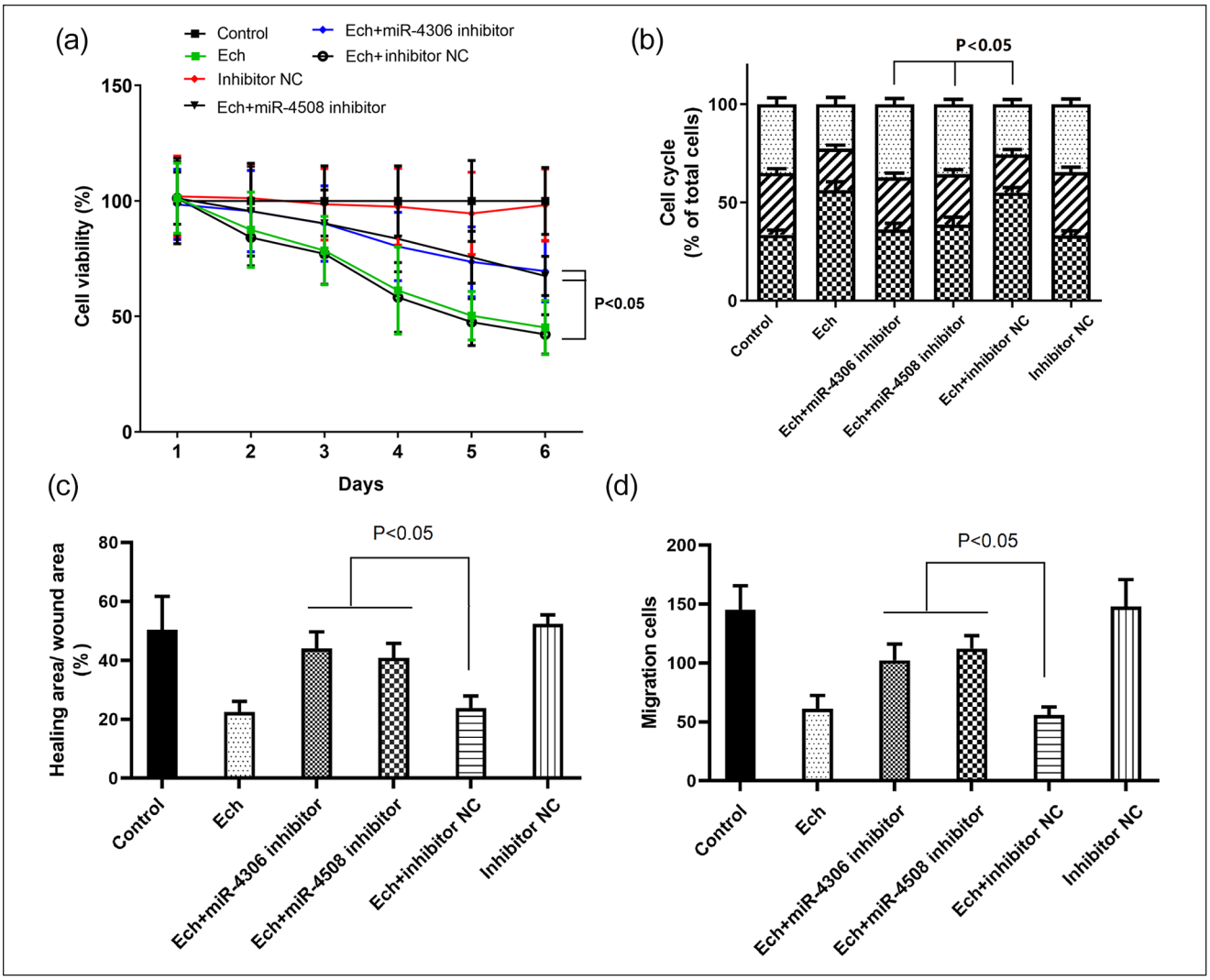

Figure 8. Effects of miR-4306 and miR-4508 inhibitors on the effects of echinacoside on the cell viability, cell cycle and migratory/invasive abilities of MCF-7 cells. (a) The changes of cell viability of MCF-7 cells. (b) The changes of cell cycle of MCF-7 cells. (c) The changes of ratio of healing area/wound 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.

miRNAs (miR-34a, miR-424, and miR-503) expression via the p53 pathway.[50] 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.

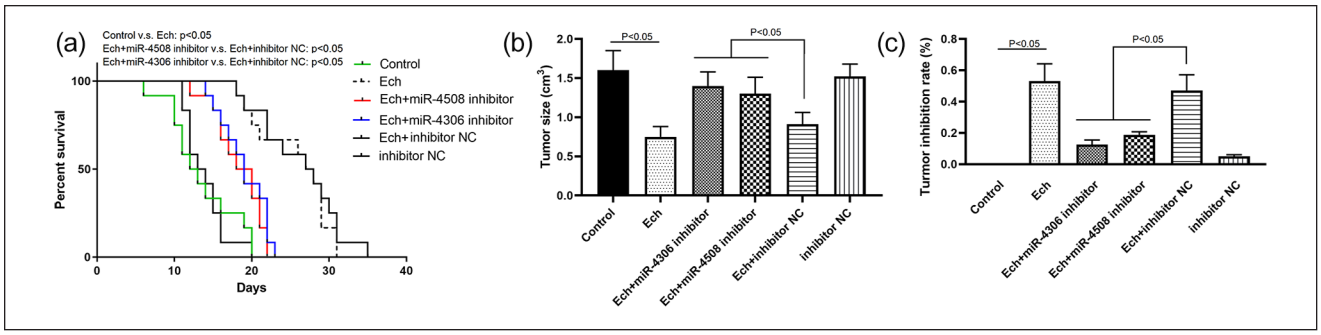

Figure 9. Effects of miR-4306 and miR-4508 inhibitors on the effects of echinacoside on xenograft tumor. Nude mice were inoculated subcutaneously with MCF-7 cells to establish xenograft tumors, and then treated with echinacoside and miR-4306 or miR-4508 inhibitors. The survival rates (a), tumor size (b) and tumor inhibition rate (c) were measured. 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 analzed the data; LL designed and supervised the study.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

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.

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