Research Article

Ethanol Extracts of Dietary Herb, *Alpinia nantoensis*, Exhibit Anticancer Potential in Human Breast Cancer Cells

Integrative Cancer Therapies
Volume 18: 1–12

DOI: 10.1177/1534735419866924
journals.sagepub.com/home/ict
SAGE

Ching-Ying Kuo, PhD[1], Teng-Song Weng, MS[1,2], K. J. Senthil Kumar, PhD[3], Yen-Hsueh Tseng, PhD[3], Ta-Wei Tung, MS[1], Sheng-Yang Wang, PhD[3,4], and Hui-Chun Wang, PhD[1,5,6]

Abstract

Recent advances in mammography screening, chemotherapy, and adjuvant treatment modalities have improved the survival rate of women with breast cancer. Nevertheless, the breast tumor with metastatic progression is still life-threatening. Indeed, combination therapy with Ras-ERK and PI3K inhibitors is clinically effective in malignant breast cancer treatment. Constituents from genus *Alpinia* plants have been implicated as potent anticancer agents in terms of their efficacy of inhibiting tumor cell metastasis. In this study, we tested the effects of ethanol extracts of *Alpinia nantoensis* (rhizome, stem, and leaf extracts) in cultured human breast cancer cells and particularly focused on the Ras-ERK and PI3K/AKT pathways. We found that the rhizome and leaf extracts from *A nantoensis* inhibited cell migration, invasion, and sphere formation in MCF-7 and MDA-MB-231 cells. The potency was extended with the inhibition of serum-induced PI3K/AKT and Ras-ERK activation and epidermal growth factor (EGF)-mediated EGFR activation in MDA-MB-231 cells. These results indicate that extracts of *A nantoensis* could inhibit signal transduction at least involved in EGFR as well as the PI3K/AKT and Ras-ERK pathways, which are crucial players of tumor cell migration and invasion. Our study strongly supports that the extracts of *A nantoensis* could be a novel botanical drug lead for the development of an antimetastatic agent for the treatment of human malignant breast cancer.

Keywords

Alpinia nantoensis, breast cancer, metastasis, PI3K/AKT, Ras-ERK, EGFR

Submitted January 16, 2019; revised April 26, 2019; accepted June 17, 2019

Introduction

Cancer statistics indicate that breast cancer is the second and fourth leading cause of death in women of the United States and Taiwan, respectively.[1] In breast cancer patients, determination of hormone receptors, such as estrogen receptor (ER) or progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), are considered as biomarkers due to their diagnostic and prognostic implications.[2] Among them, the HER2 subtype tumors are clinically and biologically heterogeneous; therefore, therapeutic options become complicated and varied, but they play a vital role in targeted therapies.[3] Approximately 70% of human breast cancers are hormone receptor–positive, and the hormone therapy following surgery, radiation, or chemotherapy led to a significant improvement in postsurvival and reduction in disease relapse. However, half of the early-stage hormone receptor–positive cells are

[1]Graduate Institute of Natural Products, College of Pharmacy, Kaohsiung Medical University, Kaohsiung 80708, Taiwan
[2]Chi Mei Medical Center, Liouying, Tainan 73657, Taiwan
[3]Department of Forestry, National Chung-Hsing University, Taichung 40227, Taiwan
[4]Agricultural Biotechnology Research Center, Academia Sinica, Taipei 11529, Taiwan
[5]Department of Medical Research Center, Kaohsiung Medical University Hospital, Kaohsiung 80756, Taiwan.
[6]Department of Marine Biotechnology and Resources, National Sun Yat-Sen University, Kaohsiung 80424, Taiwan.

Corresponding Authors:
Hui-Chun Wang, Graduate Institute of Natural Products, College of Pharmacy, Kaohsiung Medical University, Kaohsiung 80708, Taiwan.
Email: wanghc@kmu.edu.tw

Sheng-Yang Wang, Department of Forestry, National Chung-Hsing University, Taichung 40227, Taiwan.
E-mail: taiwanfir@dragon.nchu.edu.tw.

resistant to hormone therapy and highly malignant.[4] More strategies are suggested to overcome resistance and prolong survival. This depends on compensatory signaling pathways; for example, adjuvant systemic therapy with PI3K/AKT inhibitors or receptor tyrosine kinase (RTK) inhibitors.[5] Others, like the 10% to 17% of breast cancer patients without ER, PR, and HER-2 expression called triple-negative breast cancer (TNBC), have limited therapeutic options and are characterized by metastasis with poor prognosis. The molecular profiling of TNBC shown the activation of the PI3K pathway, as well as other genes linked to TNBC.[6] Developing therapeutic strategies for the treatment of recurrent or metastatic tumors is critically important to improving the prognosis of patients with progressive breast cancer. Targeting the PI3K signaling pathway has promising results. However, inhibition of PI3K in case of the tumor with RTKs overexpression leads to compensatory activation of the ERK signaling pathway. Given that both PI3K/AKT and Ras/MAPK are the major effector downstream pathways of RTKs, combined therapy of MAPK or RTKs inhibitors with PI3K inhibitors is suggested.[7]

In the past few decades, a large variety of chemotherapeutic agents have been introduced and most of them have various side effects. To reduce the discomfort and increase the survival rate for patients receiving chemotherapy, current efforts toward anticancer drug development and research are looking forward to finding drugs with high efficacy and low side effects. The bioactive compounds from natural sources are of particular interest.

The plant family Zingiberaceae is one of the largest dietary herb/spice families and have been reported to have various bioactivities, including antitumor, antibacterial, and anti-inflammatory effects.[8-12] The genus *Alpinia* is one of genus in the Zingiberaceae. Previous studies have shown that the extracts or phytocompounds from the genus *Alpinia* showed anticancer activity by directly modulating cellular signaling pathways.[12-14] *Alpinia nantoensis* is a folk plant endemic to Taiwan and used as a food-flavoring and traditional Chinese medicine preparation. Previously, we reported that trans-3-methoxy-5-hydroxystilbene isolated from the rhizome of *A nantoensis* showed antimetastatic effects on human lung carcinoma cells in vitro[15]; however, the anticancer activity of various extracts of *A nantoensis* on other cancer models have not been reported. A recent study has shown that galangin, a flavonol isolated from *Alpinia officinarum*, exhibited anticancer properties against human laryngeal cancer by downregulating PI3K/AKT and Ras/MAPK pathways.[16] In this regard, we examined the antimetastatic potential of leaf, rhizome, and stem extracts of *A nantoensis* against ER-positive (MCF-7) and triple-negative (MDA-MB-231) breast cancer cell lines by determining PI3K/AKT and Ras/MAPK pathways.

Material and Methods

Extracts Preparation

Alpinia nantonensis was collected in February 2015 from Nantou County, Taiwan, and was identified by Prof Yen-Hsueh Tseng, Department of Forestry, National Chung Hsing University. The voucher specimen (TCF Tseng4568) was deposited in the herbarium of the same university. Air-dried rhizome, stem, and leaves of *A nantonensis* were extracted with ethanol at ambient temperature and concentrated under vacuum to yield the different parts of extract, namely, ANR, ANS, and ANL as rhizome extract, stem extract, and leaves extract, respectively. Extracts were dissolved in dimethyl sulfoxide (DMSO) to prepare a final concentrations of 2.5, 5, 10, and 20 mg/mL.

Chemicals and Reagents

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), the PI3K inhibitor wortmannin, DMSO, hydrocortisone, bovine serum albumin, heparin, and methylcellulose were obtained from Sigma-Aldrich (St Louis, CA). The epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) were purchased from PeproTech (Rocky Hill, NJ) and reconstituted by the culture medium. Insulin and B-27 supplement were obtained from Thermo Fisher Scientific (Waltham, MA). Antibodies against AKT, EGFR, phos-AKT^{Ser473}, phos-EGFR^{Tyr1068}, and E-cadherin were obtained from Cell Signaling Technology (Danvers, MA). Antibodies against ERK and phos-ERK1/2$^{Thr202/Thyr204}$ were purchased from Santa-Cruz Biotechnology (Dallas, TX). Anti-vimentin and anti-β-actin antibodies were procured from Gene Tex (Irvine, CA) and Sigma-Aldrich, respectively.

Cell Culture

Human breast cancer cell lines (BCCs), MCF-7 and MDA-MB-231, were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan) and cultured in Dulbecco's modified Eagle medium (DMEM) and Roswell Park Memorial Institute Medium-1640 (RPMI-1640), respectively, and supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Carlsbad, CA). Both cell lines were maintained at 37°C in a humidified 5% CO_2 incubator.

Cytotoxicity Assay

The MTT stock solution was prepared at a concentration of 5 mg/mL in phosphate-buffered saline (PBS). Cells at a density of 1×10^4 cells/well were seeded in 96-well cell culture plates overnight and then treated with different concentrations (25, 50, 100, and 200 μg/mL) of ANR, ANS, and ANL for 48 hours. After treatment, culture media were

replaced with fresh media containing MTT (0.5 mg/mL) and incubated for 4 hours at 37°C in a humidified 5% CO_2 incubator. To determine cell survival, the formazan crystal produced by mitochondrial metabolism was dissolved in DMSO and the intensity measured with a microplate photometer (Multiskan Ascent, MTX Lab Systems, Inc, Bradenton, FL) at 550 nm. Percentage of cell survival was calculated using the following formula: cell survival (% of control) = $OD_{test}/OD_{control} \times 100\%$.

Cell Proliferation Assay

MCF-7 and MDA-MB-231 cells were seeded in a 96-well cell culture plate at a density of 1×10^3 cells/well. After 24 hours incubation, cells were treated with increasing concentrations (2.5, 5, 10, and 20 μg/mL) of ANR, ANS, or ANL for 1 to 4 days. At the end of the indicated time points, the surviving cells were determined by the MTT assay, and the cell growth curve was plotted by the survival of cells in the period of 1 to 4 days.

Western Blot Analysis

The protein extraction and Western blot assay were performed as previously described.[17]

Cell Migration Assay

To determine the capability of cell migration, we used both wound healing and Boyden chamber Transwell assays. For the wound healing assay, a silicon gap insert (Ibidi GmbH, Martinsried, Germany) was placed in a 12-well culture plate to create a cell-free space of 400 μm in width between 2-well chambers. MDA-MB-231 cells (2×10^6 cells/well) were seeded into each chamber of the culture insert. After 24 hours of incubation, the silicon insert was removed and the cells were washed with phosphate-buffered saline to exclude nonadherent cells and debris. Then, cells were treated with 10 μg/mL ANR, ANS, or ANL for indicated times. For the Boyden chamber assay, a Transwell insert with 8.0 μm pore of polyethylene terephthalate polyester membrane (Corning Inc, Corning, NY) can allow cells to migrate through the pore. The MDA-MB-231 cell suspension (5×10^4 cells/well) culture in FBS-free RPMI-1640 medium containing DMSO vehicle or 10 μg/mL ANR, ANS, or ANL were seeded into each top insert. Cells were allowed to migrate to the bottom of the membrane, which was immersed in 10% FBS medium for 16 hours and then fixed with 70% ethanol. Nonmigrated cells were scraped off with a cotton bud, and the migrated cells were stained with 0.1% crystal violet. A digital CCD camera mounted on an inverted microscope obtained all images (Axiovert 40, Zeiss, Thornwood, NY), and the wound distances and cell numbers were calculated, respectively, for the wound healing assay and the Boyden chamber assay using AxioVision Release 4.8 software (Carl Zeiss AG, Oberkochen, Germany).

Cell Invasion Assay

The invasion capability was assessed by the Boyden chamber with an 8 μm pore of polyethylene terephthalate polyester membrane coated with the Matrigel Basement Matrix (BD Biosciences, San Jose, CA). A total of 2.5×10^3 of MDA-MB-231 cells were suspended in FBS-free RPMI-1640 medium containing DMSO vehicle, or 10 μg/mL ANR, ANS, or ANL were seeded into each top insert. Cells were allowed to invade to the bottom of the membrane, which was immersed in 10% FBS medium for 24 hours. The following staining and imaging procedures were the same as the Boyden chamber migration assay.

Sphere Formation Assay

The MCF-7 cells were mechanically separated to the singlet state in the trypsin-EDTA buffer before the experiment. Cells at a density of 5×10^3 cells/well were resuspended in serum-free DMEM with or without various concentrations of ANR, ANS, or ANL, and supplemented with a final concentration of 20 ng/mL EGF, 20 ng/mL of bFGF, 5 μg/mL insulin, 1 μg/mL hydrocortisone, 4 μg/mL heparin, 0.5% bovine serum albumin, 1 × B27 supplement, and 1% methylcellulose in 24-well Ultra-Low Attachment plates (Corning, Corning, NY). Cell spheres appeared after 10 days of incubation; the spheres were imaged and counted by CCD camera.

Gelatin Zymography Assay

A gelatin zymography assay was utilized to determine whether ANR, ANS, or ANL affect matrix metalloprotease-2 and -9 (MMP2 and MMP9) activities in BCCs. MDA-MB-231 cells were seeded in 6-cm dishes overnight after reaching 80% confluency. Cells were incubated with fresh serum-free medium containing various concentrations of ANR, ANS, or ANL for 24 hours. Both cell lysate (containing the cellular form of MMPs) and culture media (containing the secreted form of MMPs) were separated and centrifuged at 7000 rpm at 4°C for 2 minutes. Next, equal amounts of cell lysate or culture media were individually mixed with bromophenol blue dye in 5:1 ratio, and the proteins were prepared on SDS-PAGE containing 0.1% gelatin gel by electrophoresis, which was carried out at 120 V for 90 minutes at ambient temperature. The gel was washed twice with 2.5% Triton X-100 solution for 30 minutes and the enzymatic reaction performed for 24 hours by immersing gelatin gel in the reaction solution (50 mM Tris-HCl [pH 7.5], 2 M NaCl, and 50 mM $CaCl_2$). Next, the gelatin gel was stained with Coomassie brilliant blue G-250 (SimplyBlue Safestain, Thermo Fisher Scientific, Waltham,

MA) for 60 minutes and washed twice with distilled water. Finally, the image was captured by an Image Analyzer System (LAS 4000 mini, Fujifilm, Tokyo, Japan).

Statistical Analysis

The data were plotted and analyzed by GraphPad Prism software version 5.0. All data were expressed as the mean ± standard error of mean. Statistical significance was analyzed by 1-way analysis of variance followed by Dunnett post hoc test for comparison of multiple groups and was represented with a "*" for P values <.05, "**" for P values <.01, and "***" for P values <.001.

Results

Extracts of A nantoensis *Inhibit Growth of BCCs*

The MTT assay was conducted to investigate the cytotoxicity of ANR, ANS, and ANL against MDA-MB-231 and MCF-7 cells. The dose-dependent cytotoxic effect after treatment for 48 hours in cells is presented in Figure 1A and B. The IC_{50} values of ANR, ANS, and ANL were 28.13 ± 2.43, 58.04 ± 3.41, 36.05 ± 3.74 μg/mL in MDA-MB-231 cells and 42.22 ± 3.17, 164.48 ± 5.49, 47.80 ± 3.79 μg/mL in MCF-7 cells, respectively (Figure 1A and B). The cell growth curve was further measured to observe the effect of extracts on cell proliferation. The antiproliferative effect of ANR, ANS, and ANL against MDA-MB-231 and MCF-7 cells was also observed in a dose-dependent manner (Figure 1C and D). This result showed that ANR and ANL are more potent than ANS.

Extracts of A nantoensis *Inhibit Migratory Potential of BCCs*

A previous study reported that compounds isolated from *Alpinia* species inhibit invasion of renal cell carcinoma.[18] Therefore, we hypothesize that extracts of *A nantoensis* have exerted migration and invasion potential of human BCCs. The result of the wound healing assay exhibited that treatment with a nontoxic concentration (10 μg/mL) of ANR and ANL significantly reduced the migratory potential of MDA-MB-231 cells when compared with the DMSO group after treatment for 16 to 24 hours (Figure 2A and B). These results show that the ANR and ANL extracts have a significant anti-migration effect. The epithelial to mesenchymal transition (EMT) plays a crucial role in initiation of tumor cell metastasis through downregulation of epithelial markers, such as E-cadherin and occludin, and upregulation of mesenchymal markers, including N-cadherin and vimentin.[19] Since EGF was reported to reduce E-cadherin expression during the EMT process via MEK/ERK pathway,[20] here we used EGF to induce EMT in BCCs, which was set as a positive control. We examined the effects of ANR, ANS, and ANL on EGF-induced EMT in MDA-MB-231 cells. The results showed that cells exposed to EGF markedly reduced the E-cadherin expression, whereas treatment with ANR, ANS, and ANL significantly increased E-cadherin expression when compared with control and EGF treatment groups (Figure 2C). In addition, compared with the control group, all the extracts significantly inhibited vimentin expression in MDA-MB-231 cells (Figure 2C). The extent of increase in E-cadherin and decrease of vimentin was in parallel to the suppression of cell migration by extracts, supporting the molecular evidence for the antimetastatic effect of *A nantoensis*.

Extracts of A nantoensis *Inhibit Invasive Potential of BCCs*

To inspect the effect of *A nantoensis* crude extracts on cancer metastasis, we performed the Boyden chamber assay to evaluate MDA-MB-231 cell migration and invasion under chamber without or with matrigel coating. The results are consistent with the wound healing assay: the percentage of migrated cells was decreased to 22.9 ± 11.0%, 63.8 ± 17.1%, and 26.8 ± 9.7% when cells were treated with 10 μg/mL ANR, ANS, or ANL for 16 hours, respectively (Figure 3A and B). On the other hand, the percentage of invaded cells was decreased to 29.5 ± 6.1%, 80.6 ± 14.8%, and 42.0 ± 5.5% by 10 μg/mL ANR, ANS, and ANL for 24 hours, respectively (Figure 3C and D). The results of gelatin zymography assay further supported that extracts could inhibit the invasive potential of BCCs. Treatment with 10 and 20 μg/mL ANR, ANS, and ANL for 24 hours significantly decreased the secretive pro-MMP-9, MMP-9, and MMP-2 gelatinase activity (Figure 3E). In addition, the cellular pro-MMP-9, MMP-9, and MMP-2 activities were completely abolished by all the sample extracts (Figure 3F).

Extracts of A nantoensis *Inhibit Mammosphere Formation*

Cancer stem cells (CSCs) have been identified as one of the origins of tumor relapse and drug resistance. To test the effect of *A nantoensis* extracts on CSCs, we used the mammosphere formation assay to enrich the population of cancer stem–like cells.[21] The MCF-7 cells were treated with 5, 10, and 20 μg/mL of ANR, ANS, and ANL for 10 days, and tumorsphere formation was analyzed. As shown in Figure 4A, the size of the spheres was significantly decreased by all the extracts in a dose-dependent manner. Briefly, 100 spheres were picked up for each group to analyze the size, and over 50% of them had a diameter less than 50 μm when treated with ANR or ANL at 10 μg/mL. A similar effect was observed when cells were treated with ANS at a higher concentration (20 μg/mL; Figure 4B). These results indicated that *A nantoensis* extracts could inhibit cancer stem–like cell populations in MCF-7 cells.

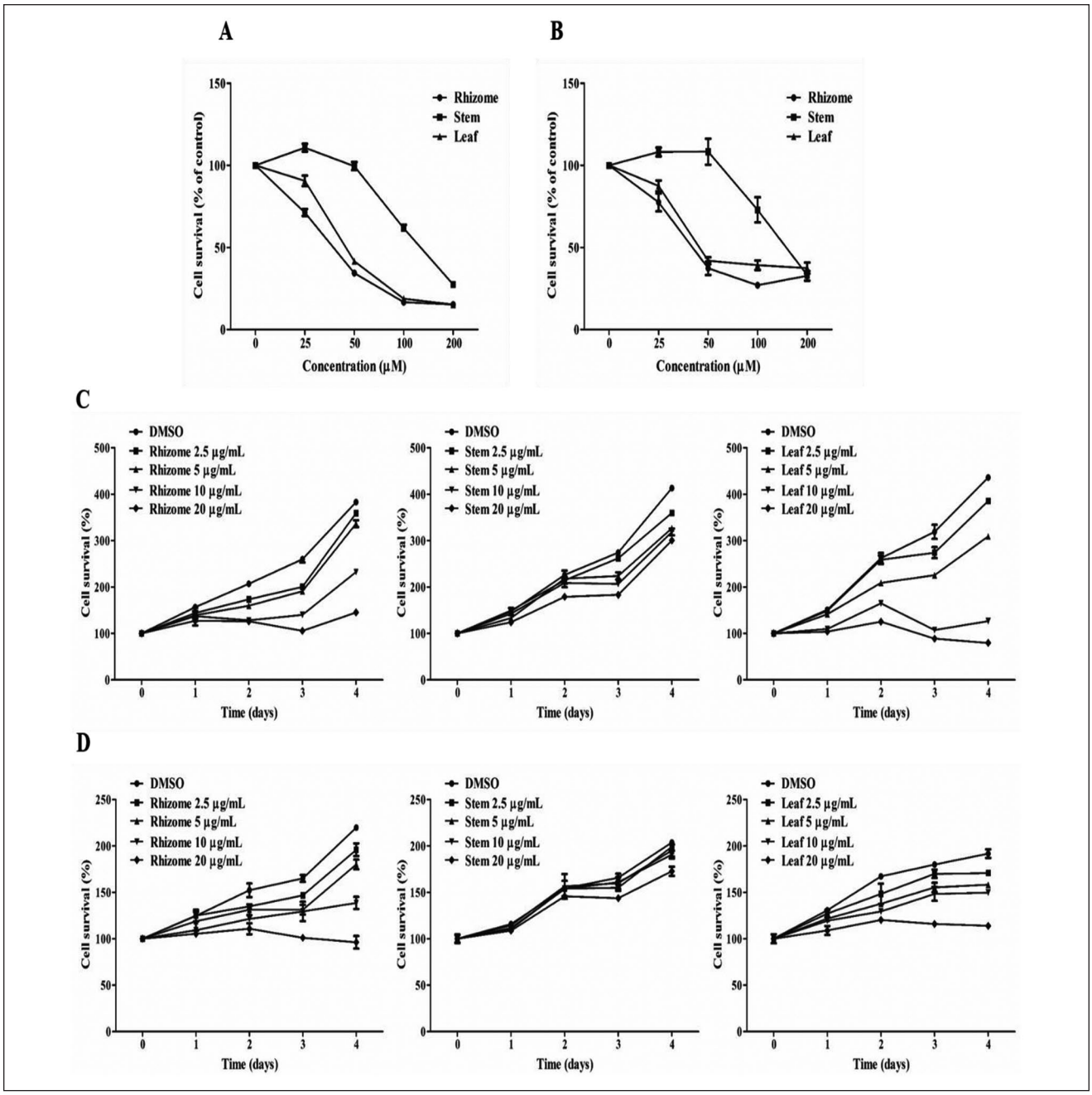

Figure 1. Effect of *Alpinia nantoensis* extracts on cell survival and proliferation in cancer cell lines. Cell viability was measured by MTT assay after 48 hours of treatment with increasing concentrations of ANR, ANS, or ANL in MDA-MB-231 (A) and MCF-7 (B) cells. The survival cells were measured by MTT assay at 0 to 96 hours after exposure to 2.5 to 20 μg/mL *A nantoensis* extracts was used to test the cell doubling in MDA-MB-231 (C) and MCF-7 (D) cells. The insoluble formazan was dissolved by DMSO at the end of the experiment, and the percentage of viable cells to the DMSO group was calculated from the absorbance measured at 550 nm. All results are presented as mean ± standard error from 3 independent experiments, n = 3.

Extracts of A nantoensis *Regulate PI3K/AKT and Ras/ERK Pathways in BCCs*

To evaluate the antiproliferative and antimetastatic mechanisms of *A nantoensis* extracts, we determined the phosphorylation status of AKT and ERK, which are the downstream effectors of PI3K/AKT and the Ras/ERK pathways, respectively. Results of Western blot analysis showed that 10 μg/mL of ANR, ANS, and ANL significantly decreased the phosphorylation of AKT and ERK at Ser347 and Thr202/Tyr204 residues, respectively, in both MDA-MB-231 (Figure 5A) and MCF-7 (Figure 5B) cells.

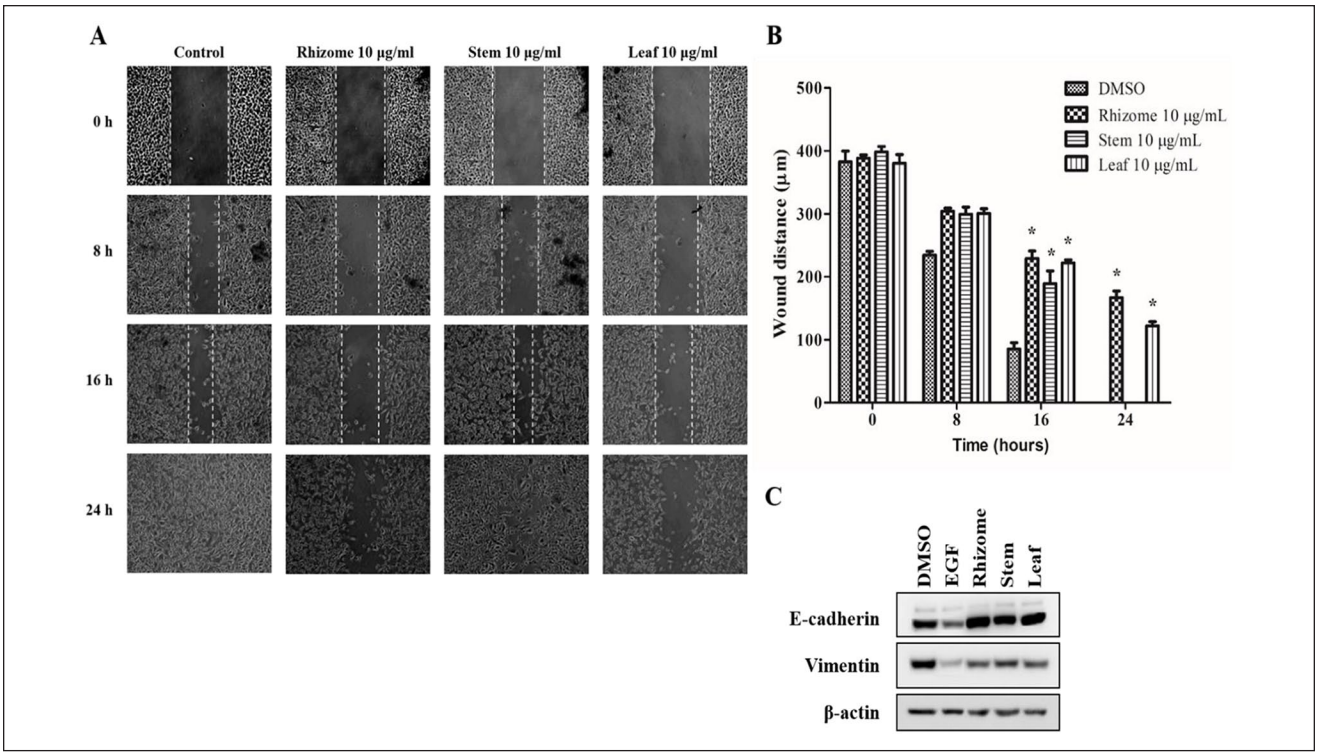

Figure 2. Effect of *Alpinia nantoensis* extracts on cell migration in MDA-MB-231 cells. The ability of cell migration was determined using the wound healing assay and immunoblots for MDA-MB-231 cells. (A) The wounds are 400 µm wide created by a Culture-Insert, and the wound distances were measured on the microscope images at 0, 8, 16, and 24 hours after cells were incubated with or without 10 µg/mL of extracts. These results were shown by representative photomicrographs. (B) Bar graph illustrating the quantitative analysis of wound distances at the different extracts following various exposure times. Data represent the mean ± standard error of 3 independent experiments, $^{*}P < .05$ compared with the DMSO group. (C) Immunoblots results showed the effect of extracts on the E-cadherin phosphorylation following exposure to 10 µg/mL of extracts for 24 hours.

To further clarify whether the phosphorylation of AKT and ERK1/2 are the downstream target of growth factor stimulation, we cultured cells in serum-free medium for 24 hours and induced AKT and ERK phosphorylation by addition of 20% FBS for 30 minutes. Our results showed that pretreatment of 10 µg/mL of ANR and ANL, but not ANS, significantly decreased 20% FBS-induced phosphorylation of AKT and ERK1/2 at Ser347 and Thr202/Tyr204 residues in both MDA-MB-231 (Figure 5C) and MCF-7 (Figure 5D) cells. The inhibitory effects of extracts on AKT and ERK1/2 phosphorylation were highly comparable with the pharmacological inhibitor of PI3K, wortmannin. Next, we examined whether EGF-specific signaling can be affected by extracts. Twenty percent FBS was replaced by 20 ng/mL EGF to induce phosphorylation of AKT and ERK1/2. We found that cells exposed to EGF markedly increased phosphorylation of EGFR at Tyr1068 residue and AKT at Ser347 residue in both cell lines. Indeed, pretreatment with extracts significantly decreased EGF-induced EGFR and AKT phosphorylation in MDA-MB-231 cells (Figure 5E), whereas ANR and ANL, but not ANS, gave a similar effect in MCF-7 cells (Figure 5F).

Discussion

Metastasis is a complex multistep process involving cell adhesion, invasion, and motility. Accumulating evidence has linked the role of CSCs to local migration and invasion.[22-24] Hence, one or more interruptions of these steps may offer a therapeutic approach for antimetastatic therapy. Over a millennium herbal medicine has been used and is still practiced in developing countries as the primary source for the treatment of basic and complicated diseases such as diabetes, heart disease, and cancers. In recent years, many herbal extracts or natural products are identified to have a plethora of effects against human cancers; among them, phytoagents that suppress the metastatic behavior of tumor cells are of special interest.

The genus *Alpinia* is the largest genus in the ginger family with about 230 species. Multiple researchers have identified several *Alpinia* species exhibiting anticancer properties against various cancers, with a major focus on their cytotoxic and tumor growth regression effects, whereas little focus has been put forward to understand the antimetastatic potential of *Alpinia* species.[25-29] Bioactive compounds

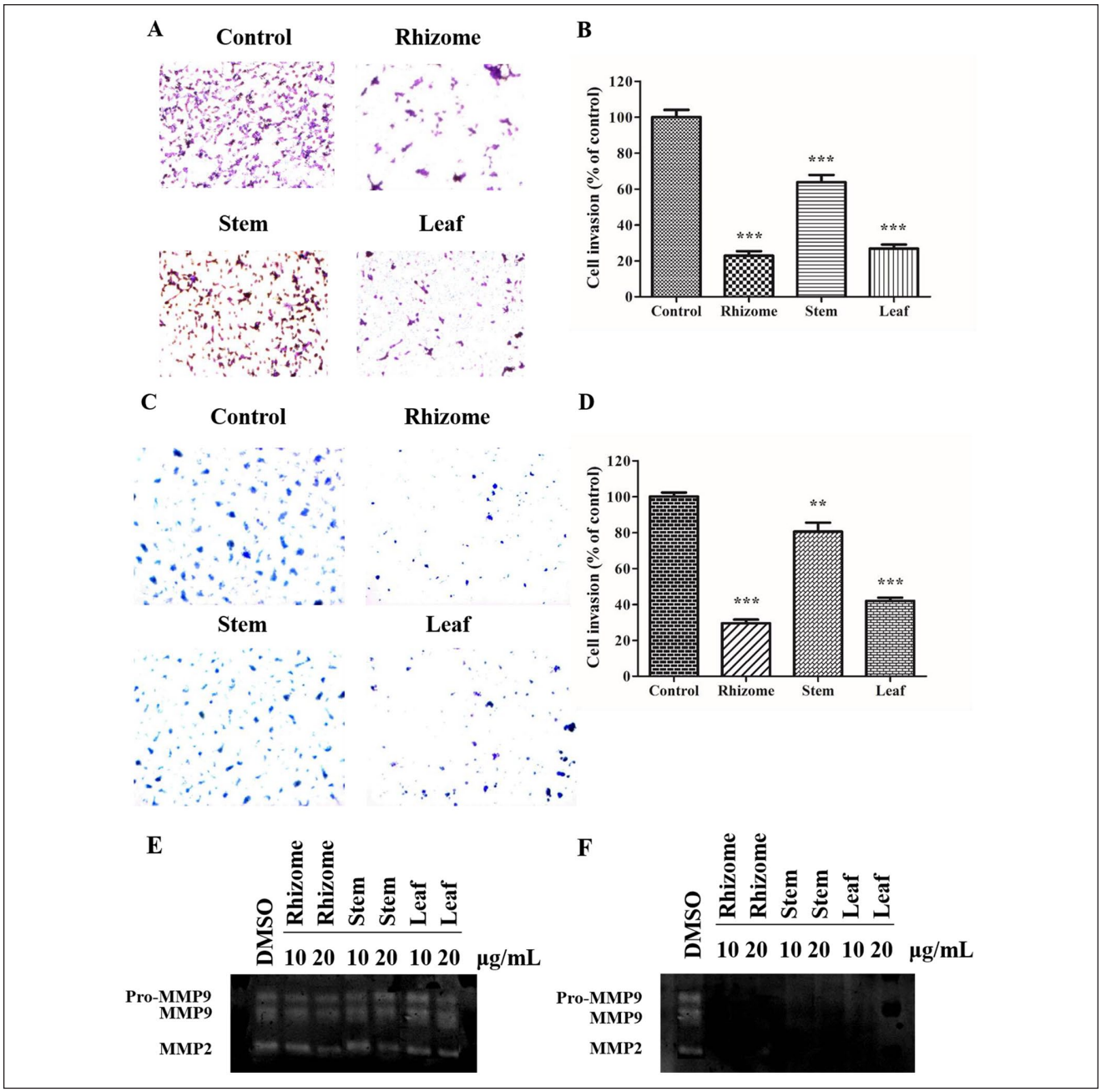

Figure 3. Effect of *Alpinia nantoensis* extracts on cell migration and cell invasion in MDA-MB-231 cells. The ability of cell migration was determined using the Transwell inserts assay, invasion chamber assay, and Griess assay for MDA-MB-231 cells. (A) 5×10^4 cells were plated into noncoding Boyden chambers together with tested extracts for 16 hours. (C) 2.5×10^3 of MDA-MB-231 cells were seeded into the invasion chamber together with extracts for 24 hours. The migratory and invasive cells were stained with 1% w/v crystal violate solution and become visible under an inverted microscope. Representative photomicrographs are shown, and the bar chart for mean ± standard error of mean was calculated from 3 independent experiments (B and D). $^{**}P < .01$ compared with DMSO group. $^{***}P < .001$ compared with the DMSO group. MMP expression and activity assessment of *Alpinia nantoensis* extracts by gelatin zymography in conditioned medium (E) and cell lysates (F).

such as cardamonin, galangin, and flavokawain B isolated from *Alpinia katsumadai, Alpinia officinarum*, and *Alpinia pricei* exhibited antimetastatic properties in Lewis lung carcinoma, fibrosarcoma, melanoma, renal carcinoma, and breast carcinoma cells.[30-33] We previously reported that trans-3-methoxy-5-hydroxystilbene isolated from the rhizome of *A nantoensis* inhibited migration and invasion of small cell lung carcinoma.[15] However, there is no direct

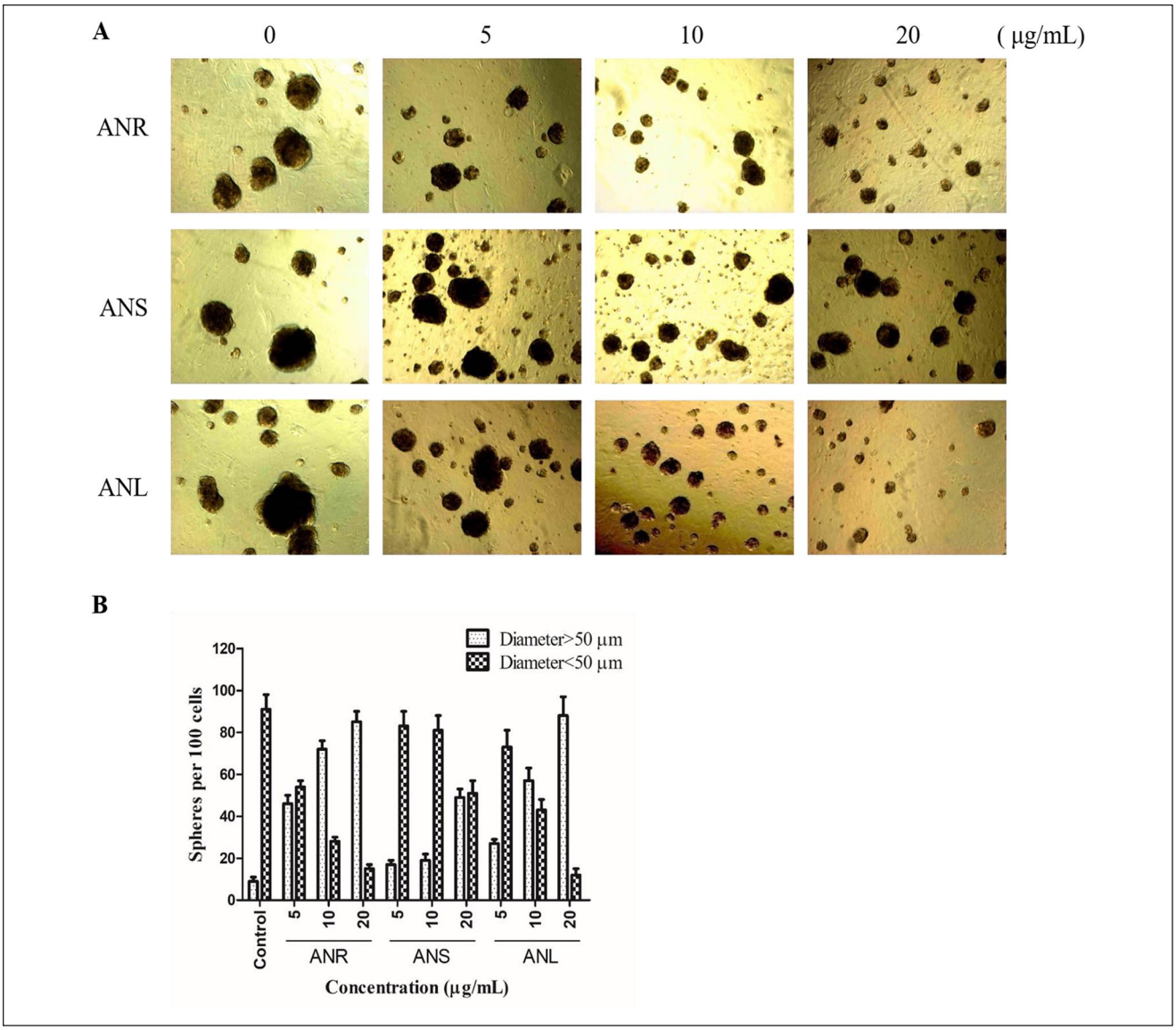

Figure 4. Mammosphere formation of MDA-MB-231 cells treated with *Alpinia nantoensis* extracts at different concentration. (A) Photomicrographs of the mammospheres following treatment with *Alpinia nantoensis* extracts. (B) The number and the size of mammospheres formed per dish were enumerated, and 100 spheres were picked up for each group to analyze. The counts represented are mean ± standard error of mean from 3 independent experiments.

evidence to show that the dietary edible parts (rhizome, leaf, and stem) of *A nantoensis* can inhibit metastasis and stemness of human BCCs. Here, we first report that rhizome and leaf extracts of *A nantoensis* displayed significant inhibition on cell migration, invasion, and cancer stem–like sphere formation in human BCCs, whereas the nonedible part, stem extracts, poorly expressed its antiproliferative and antimetastatic effect. These findings strongly suggest the edible parts of *A nantoensis* are beneficial in preventing cancer progression.

Breast cancer cell lines have been well characterized based on molecular features.[34] In this study, 2 types of BCCs were used to evaluate the anticancer activity of *A nantoensis* extracts. The MCF-7 cells characterized as luminal type and ER-positive, and the MDA-MB-231 cells are basal B–type and triple-negative. The former is endocrine therapy sensitive, epithelial-like, and nonmetastatic, harboring a mutation of *PIK3CA* (E545K) that leads to an increase in PI3K catalytic activity and oncogenic transforming activity dependent on AKT signaling.[35,36] The latter is endocrine therapy insensitive, mesenchymal-like, and metastatic cells that harbor a mutation of *KRAS* (G13D) and high level of RAS activation.[37] Ras activation is required for the metastatic behavior associated with mesenchymal

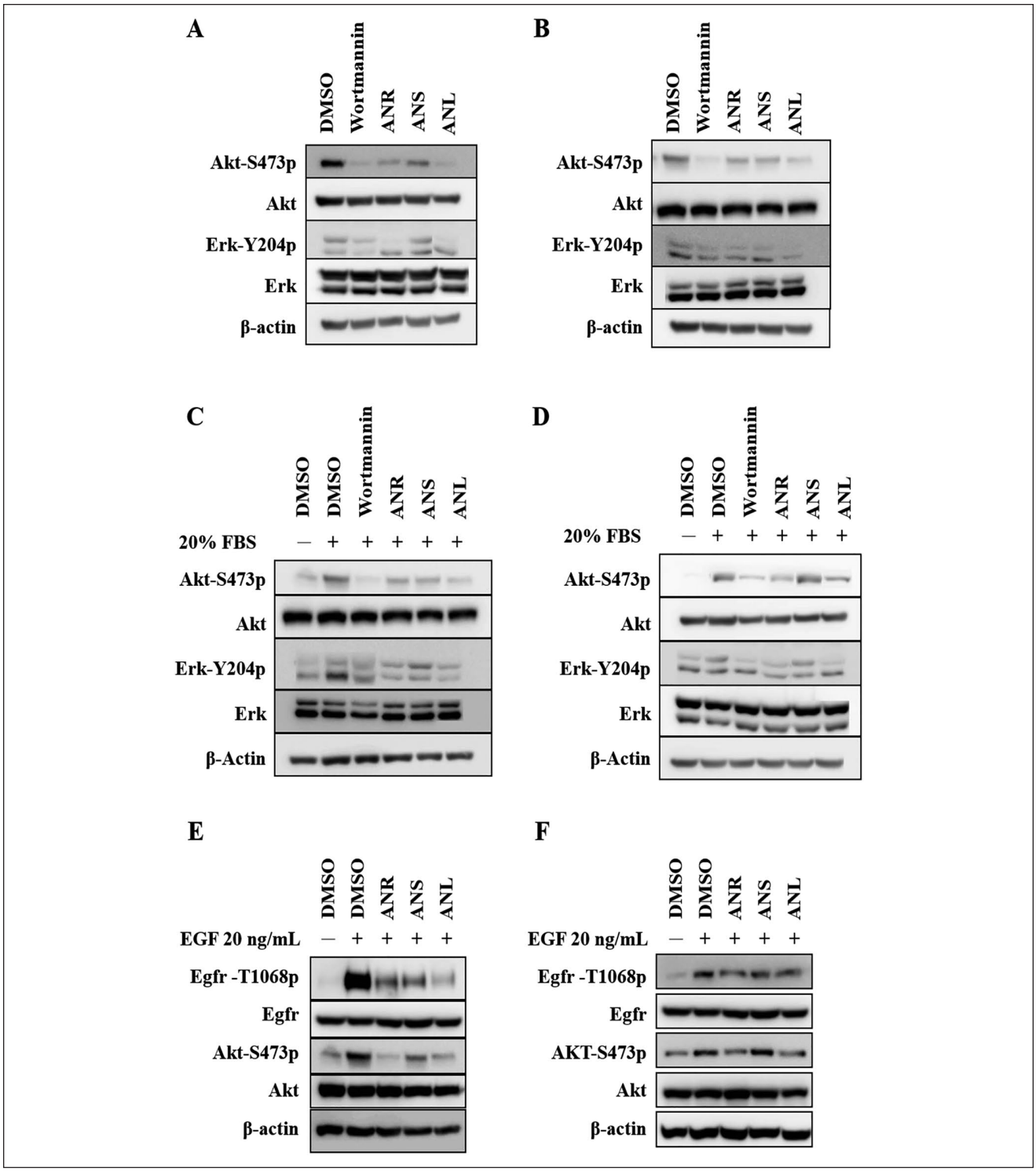

Figure 5. Effect of *Alpinia nantoensis* extracts on the PI3K/AKT and Ras/ERK signaling in MDA-MB-231 cells. Immunoblots results showed the effect of extracts on the AKT and ERK activation by detecting phosphorylation of AKT Ser473 and ERK Tyr204 following exposure to extracts at 10 μg/mL for 24 hours in MDA-MB-231 (A) and MCF-7 (B) cells. Immunoblots results showed the effect of extracts on serum-induced AKT and ERK activations by detecting phosphorylation of AKT Ser473 and ERK Tyr204 following pretreatment to extracts at 10 μg/mL for 30 minutes, then stimulated with 20% FBS (fetal bovine serum) for 30 minutes in MDA-MB-231 (C) and MCF-7 (D) cells. Treatment of a PI3K inhibitor (Wortmannin, 1 μM) was served as a control. Immunoblots results showed the effect of extracts on EGF-induced EGFR and AKT activations by detecting phosphorylation of EGFR Tyr1068 and AKT Ser473 following pretreatment to extracts at 10 μg/mL for 30 minutes before stimulated with EGF for 30 minutes in MDA-MB-231 (E) and MCF-7 (F) cells.

phenotypes of basal-type BCCs.[38] By assaying the CSC-like sphere formation and migration/invasion, respectively, in MCF-7 cells and MDA-MB-231 cells, we provided evidence that *A nantoensis* extracts suppress the malignant phenotypic behavior in both cell lines. At least these extracts were involved in inhibition of AKT and ERK activation, which are, respectively, downstream of PI3K and Ras.

E-cadherin is a calcium-dependent cell adhesion protein associated with tumor progression, and loss of E-cadherin expression could enhance tumor cell migration.[39,40] The constitutive activation of AKT mimics increasing PI3K activity in cancers, which may downregulate E-cadherin expression.[41] In line with previously reported findings in lung carcinoma cells,[15] the present results showed that extracts of *A nantoensis* inhibited AKT activation and increased E-cadherin expression in *PIK3CA*-mutated mesenchymal MDA-MB-231 cells and indicated that reversing the status of the epithelial-mesenchymal transition might underlie the mechanism of postponing cell migration. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that are directly associated with tumor cell invasion, while cancer cell invasion through extracellular matrix degradation needs activation of MMPs.[42] Thus, the elevated MMP activity is critical for transforming tumors to become aggressive; it is also a potential drug target for cancer therapy.[43] The process of MMP maturation is secretion of pro-MMPs secreted to the cell surface and activation by removing the pro-peptide domain through upstream MMPs to expose the "cysteine switch."[44] Based on the use of SDS in the buffers activating latent gelatinase isoforms,[45] gelatin zymography can analyze both the latent (pro) and active forms of enzymatic activity. Our results showed the secreted form of MMP-2 and MMP-9 activities were not changed by extract treatment; if anything, they were only decreased in 20 μM treatment groups. Differently, the gelatinase activity of MMPs isolated from whole cell lysate was abolished by the extracts, indicating that extracts might inhibit MMP gene expression, instead of altering the process of MMP activation, secretion, or catalytic activity itself. We speculated that extracts of *A nantoensis* change the signal pathway leading to MMP gene expression. However, even though the secreted form of MMP-2 and MMP-9 are almost intact, all 3 extracts of *A nantoensis* inhibit MDA-MB-231 cell invasion. This implies that cell invasion requires sustaining of MMP expression, which is compromised by extract treatment.

So far, we could not conclude which specific gene is a definite target for *A nantoensis* crude extracts. These extracts could have multiple targets by complex constituents. To illustrate the impact of *A nantoensis* extracts on cancer growth, we observe the AKT and ERK1/2 activation stimulated by 20% FBS, which is rich in diverse growth factors. We found that 20% FBS can activate AKT and ERK1/2 in both cell lines, and wortmannin inhibited not only AKT but also ERK1/2 activation, indicating the cross-talk between the 2 pathways. In such a situation, extracts of *A nantoensis* inhibited both AKT and ERK1/2 activation in BCCs. The stem extract, with less potency than rhizome and leaf extracts, is exactly reflected in the different pathways in these 2 cell lines. Of these, the stem extract has less inhibition on FBS-induced ERK1/2 activation in MDA-MB-231 cells and AKT phosphorylation in MCF-7 cells. Constitutive EGFR activation was not detected in MCF-7 and MDA-MB-231 cells[37]; we tested EGF as a representative growth factor and found similar potency of the 3 extracts to the situation of FBS stimulation. The most important finding is that *A nantoensis* extracts inhibit EGF-induced EGFR activation in BCCs. This result indicates that the extracts act on the very upstream events under growth factor stimulation.

The sphere formation assay is a putative way to enrich the CSC population,[46] but it is still controversial by modeling in different cell lines and detecting markers.[47] However, we adopted a model by maintaining the MCF-7 cells in sphere growth under EGF and bFGF stimulation. The potency of 3 extracts on inhibiting MCF-7 cell sphere formation is parallel to EGF-induced EGFR and AKT activation, explaining the inhibition of EGF function involved in extract-mediated inhibition of sphere growth in MCF-7 cells. In addition, we found that the TNBC cell line MDA-MB-231 is very sensitive to EGF stimulation. Interestingly, extracts of *A nantoensis* strongly inhibited EGF-induced EGFR activation, indicating that inhibition of EGFR is an effective target for TNBC therapy. This idea is consistent with the findings by Nielsen et al that EGFR is overexpressed in basal-like breast tumors, thus proposing that EGFR is a candidate target for those patients.[48]

Conclusion

Alpinia nantoensis crude extract is a complex mixture, and characterizing the mechanism of action of crude extracts is a difficult task. Here, for the first time, we examined crude extracts from rhizome, stem, and leaf of *A nantoensis* on reversing cancer malignant phenotype and proposed the molecular mechanism involved in inhibiting EGFR, AKT, and ERK activation in human breast cancer cells in vitro. Our study will be a new insight to understand the utility of this edible plant against cancers and also be beneficial to the subsequent identification of pure compounds in further research. To further confirm their antimetastatic effects, in vivo rodent models are highly warranted.

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) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by Grants MOST 103-2320-B-037-007-MY3 from the Ministry of Science and Technology of Taiwan and the NSYSU-KMU Joint Research Project (#NSYSUKMU 108-I006).

ORCID iD

Hui-Chun Wang https://orcid.org/0000-0003-1414-1595

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. *CA Cancer J Clin*. 2016;66:7-30.
2. Kumar RV, Panwar D, Amirtham U, et al. Estrogen receptor, progesterone receptor, and human epidermal growth factor receptor-2 status in breast cancer: a retrospective study of 5436 women from a regional cancer center in South India. *South Asian J Cancer*. 2018;7:7-10.
3. Yersal O, Barutca S. Biological subtypes of breast cancer: prognostic and therapeutic implications. *World J Clin Oncol*. 2014;5:412-424.
4. Lim E, Metzger-Filho O, Winer EP. The natural history of hormone receptor-positive breast cancer. *Oncology (Williston Park)*. 2012;26:688-694, 696.
5. Liu CY, Wu CY, Petrossian K, Huang TT, Tseng LM, Chen S. Treatment for the endocrine resistant breast cancer: current options and future perspectives. *J Steroid Biochem Mol Biol*. 2017;172:166-175.
6. Wang C, Kar S, Lai X, et al. Triple negative breast cancer in Asia: an insider's view. *Cancer Treat Rev*. 2018;62:29-38.
7. Serra V, Scaltriti M, Prudkin L, et al. PI3K inhibition results in enhanced HER signaling and acquired ERK dependency in HER2-overexpressing breast cancer. *Oncogene*. 2011;30:2547-2557.
8. Song W, Yan CY, Zhou QQ, Zhen LL. Galangin potentiates human breast cancer to apoptosis induced by TRAIL through activating AMPK. *Biomed Pharmacother*. 2017;89:845-856.
9. Sim KS, Ibrahim H, Malek SN, Syamsir DR, Awang K. Cytotoxic activity of *Alpinia murdochii* Ridl: a mountain ginger species from Peninsular Malaysia. *Pharmacogn Mag*. 2014;10:70-72.
10. Wang CZ, Yuan HH, Bao XL, Lan MB. *In vitro* antioxidant and cytotoxic properties of ethanol extract of *Alpinia oxyphylla* fruits. *Pharm Biol*. 2013;51:1419-1425.
11. In LL, Azmi MN, Ibrahim H, Awang K, Nagoor NH. 1′S-1′-acetoxyeugenol acetate: a novel phenylpropanoid from *Alpinia conchigera* enhances the apoptotic effects of paclitaxel in MCF-7 cells through NF-κB inactivation. *Anticancer Drug*. 2011;22:424-434.
12. Samarghandian S, Hadjzadeh MA, Afshari JT, Hosseini M. Antiproliferative activity and induction of apoptotic by ethanolic extract of *Alpinia galanga* rhizome in human breast carcinoma cell line. *BMC Complement Altern Med*. 2014;14:192.
13. Reddy AS, Malek SNA, Ibrahim H, Sim KS. Cytotoxic effect of *Alpinia scabra* (Blume) Náves extracts on human breast and ovarian cancer cells. *BMC Complement Altern Med*. 2013;13:314.
14. Basri AM, Taha H, Ahmad N. A review on the pharmacological activities and phytochemicals of *Alpinia officinarum* (Galangal) extracts derived from bioassay-guided fractionation and isolation. *Pharmacogn Rev*. 2017;11:43-56.
15. Kumar KJS, Wang SH, Tseng YH, Tsao NW, Kuo YH, Wang SY. Trans-3-Methoxy-5-hydroxystilbene (MHS) from the rhizome of *Alpinia nantonensis* inhibits metastasis in human lung cancer cells. *Phytomedicine*. 2018;50:223-230.
16. Wang HX, Tang C. Galangin suppresses human laryngeal carcinoma via modulation of caspase-3 and AKT signaling pathways. *Oncol Rep*. 2017;38:703-714.
17. Wang HC, Lee AY, Chou WC, et al. Inhibition of ATR-dependent signaling by protoapigenone and its derivative sensitizes cancer cells to interstrand cross-link-generating agents in vitro and in vivo. *Mol Cancer Ther*. 2012;11:1443-1453.
18. Cao J, Wang H, Chen F, et al. Galangin inhibits cell invasion by suppressing the epithelial-mesenchymal transition and inducing apoptosis in renal cell carcinoma. *Mol Med Rep*. 2016;13:4238-4244.
19. Chao YL, Shepard CR, Wells A. Breast carcinoma cells re-express E-cadherin during mesenchymal to epithelial reverting transition. *Mol Cancer*. 2010;9:179.
20. Tashiro E, Henmi S, Odake H, Ino S, Imoto M. Involvement of the MEK/ERK pathway in EGF-induced E-cadherin down-regulation. *Biochem Biophys Res Commun*. 2016;477:801-806.
21. Ponti D, Costa A, Zaffaroni N, et al. Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer Res*. 2005;65:5506-5511.
22. Li F, Tiede B, Massague J, Kang Y. Beyond tumorigenesis: cancer stem cells in metastasis. *Cell Res*. 2007;17:3-14.
23. Chen D, Bhat-Nakshatri P, Goswami C, Badve S, Nakshatri H. ANTXR1, a stem cell-enriched functional biomarker, connects collagen signaling to cancer stem-like cells and metastasis in breast cancer. *Cancer Res*. 2013;73:5821-5833.
24. Geng SQ, Alexandrou AT, Li JJ. Breast cancer stem cells: multiple capacities in tumor metastasis. *Cancer Lett*. 2014;349:1-7.
25. Pillai MK, Young DJ, Majid HMBHA. Therapeutic potential of *Alpinia officinarum. Mini Rev Med Chem*. 2018;18:1220-1232.
26. Zhang Q, Zheng Y, Hu X, et al. Ethnopharmacological uses, phytochemistry, biological activities, and therapeutic applications of *Alpinia oxyphylla* Miquel: a review. *J Ethnopharmacol*. 2018;224:149-168.
27. Kaushik D, Yadav J, Kaushik P, Sacher D, Rani R. Current pharmacological and phytochemical studies of the plant *Alpinia galanga. Zhong Xi Yi Jie He Xue Bao*. 2011;9:1061-1065.
28. Hseu YC, Chen CS, Wang SY. *Alpinia pricei* rhizome extracts induce cell cycle arrest in human squamous carcinoma KB cells and suppress tumor growth in nude mice. *Evid Based Complement Alternat Med*. 2011;2011:123815.
29. Ma XN, Xie CL, Miao Z, Yang Q, Yang XW. An overview of chemical constituents from *Alpinia* species in the last six decades. *RSC Adv*. 2017;7:14114-14144.
30. Chien ST, Shi MD, Lee YC, Te CC, Shih YW. Galangin, a novel dietary flavonoid, attenuates metastatic feature *via* PKC/ERK signaling pathway in TPA-treated liver cancer HepG2 cells. *Cancer Cell Int*. 2015;15:15.

31. Niu PG, Zhang YX, Shi DH, Liu Y, Chen YY, Deng J. Cardamonin inhibits metastasis of Lewis lung carcinoma cells by decreasing mTOR activity. *PLoS One*. 2015;10:e0127778.
32. Zhang W, Tang B, Huang Q, Hua Z. Galangin inhibits tumor growth and metastasis of B16F10 melanoma. *J Cell Biochem*. 2013;114:152-161.
33. Abu N, Akhtar MN, Yeap SK, et al. Flavokawain B induced cytotoxicity in two breast cancer cell lines, MCF-7 and MDA-MB231 and inhibited the metastatic potential of MDA-MB231 via the regulation of several tyrosine kinases in vitro. *BMC Complement Alternat Med*. 2016;16:86.
34. Dai X, Cheng H, Bai Z, Li J. Breast cancer cell line classification and its relevance with breast tumor subtyping. *J Cancer*. 2017;8:3131-3141.
35. She QB, Chandarlapaty S, Ye Q, et al. Breast tumor cells with PI3K mutation or HER2 amplification are selectively addicted to Akt signaling. *PLoS One*. 2008;3:e3065.
36. Kang S, Bader AG, Vogt PK. Phosphatidylinositol 3-kinase mutations identified in human cancer are oncogenic. *Proc Natl Acad Sci U S A*. 2005;102:802-807.
37. Eckert LB, Repasky GA, Ulkü AS, et al. Involvement of Ras activation in human breast cancer cell signaling, invasion, and anoikis. *Cancer Res*. 2004;64:4585-4592.
38. Kim RK, Suh Y, Yoo KC, et al. Activation of KRAS promotes the mesenchymal features of basal-type breast cancer. *Exp Mol Med*. 2015;47:e137.
39. Rodriguez FJ, Lewis-Tuffin LJ, Anastasiadis PZ. E-cadherin's dark side: possible role in tumor progression. *Biochim Biophys Acta*. 2012;1826:23-31.
40. Techasen A, Loilome W, Namwat N, et al. Loss of E-cadherin promotes migration and invasion of cholangiocarcinoma cells and serves as a potential marker of metastasis. *Tumour Biol*. 2014;35:8645-8652.
41. Grille SJ, Bellacosa A, Upson J, et al. The protein kinase Akt induces epithelial mesenchymal transition and promotes enhanced motility and invasiveness of squamous cell carcinoma lines. *Cancer Res*. 2003;63:2172-2178.
42. Jacob A, Prekeris R. The regulation of MMP targeting to invadopodia during cancer metastasis. *Front Cell Dev Biol*. 2015;3:4.
43. Deryugina EI, Quigley JP. Matrix metalloproteinases and tumor metastasis. *Cancer Metastasis Rev*. 2006;25:9-34.
44. Van Wart HE, Birkedal-Hansen H. The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. *Proc Natl Acad Sci U S A*. 1990;87:5578-5582.
45. Overall CM, Limeback H. Identification and characterization of enamel proteinases isolated from developing enamel. Amelogeninolytic serine proteinases are associated with enamel maturation in pig. *Biochem J*. 1988;256:965-972.
46. Cioce M, Gherardi S, Viglietto G, et al. Mammosphere-forming cells from breast cancer cell lines as a tool for the identification of CSC-like- and early progenitor-targeting drugs. *Cell Cycle*. 2010;9:2878-2887.
47. Calvet CY, André FM, Mir LM. The culture of cancer cell lines as tumorspheres does not systematically result in cancer stem cell enrichment. *PLoS One*. 2014;9:e89644.
48. Nielsen TO, Hsu FD, Jensen K, et al. Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. *Clin Cancer Res*. 2004;10:5367-5374.