

Aloperine inhibits proliferation, migration and invasion and induces apoptosis by blocking the Ras signaling pathway in human breast cancer cells

DELONG TIAN^{1*}, YANHAI LI^{2*}, XINXIN LI¹ and ZHENZHEN TIAN¹

Departments of ¹Rehabilitation and ²General Surgery, Binzhou Central Hospital, Binzhou, Shandong 210557, P.R. China

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Abstract. Aloperine (Alo), as a quinolizidine alkaloid extracted from *S. alopecuroide*, has the positive activities of anti-inflammatory, anti-allergenic, antitumor and anti-viral. However, the role and mechanism of Alo in breast cancer have not been studied yet. In the present study, Alo markedly inhibited the proliferation and suppressed the colony formation ability of the breast cancer cell lines MCF-7 and MDA-MB-231 in a dose-dependent manner by Cell Counting kit-8 and colony formation assays, respectively. In addition, the results of confocal microscopy analysis and flow cytometry detection revealed that Alo induced the apoptosis of MCF-7 and MDA-MB-231 cells, and western blotting indicated that Alo upregulated the protein levels of Bax, caspase-3 and caspase-9, and downregulated the expression of Bcl-2. Furthermore, the results of wound healing, Transwell migration and invasion assays demonstrated that Alo inhibited the migration and invasion of MCF-7 and MDA-MB-231 cells, and reduced the protein levels of matrix metalloproteinase (MMP)-2 and MMP-9. Alo also downregulated the protein expressions of Ras, phosphorylated (p)-Raf proto-oncogene, serine/threonine kinase 1 and p-extracellular signal-regulated kinase 1/2. Furthermore, ISIS 2503, a Ras inhibitor, inhibited colony formation, induced apoptosis, and suppressed the migration and invasion of MCF-7 and MDA-MB-231 cells. These effects were more marked in the presence of ISIS 2503 and Alo, when compared with those of either agent alone. In conclusion, the present study reported a novel use of Alo in inhibiting the proliferation, migration and invasion, and inducing the apoptosis of human breast cancer cells by blocking the Ras signaling pathway.

Introduction

Breast cancer is the most common malignant tumor and the leading cause of cancer mortality in women, resulting in 14% of cancer-related deaths (1,2). Besides, Breast cancer is a major malignant tumor threatening women's health in China (3). According to statistics, the new cases and dead cases of breast cancer account for 12.2 and 9.6% annually, respectively (4). Although the incidence of breast cancer in China is low, it is noteworthy that the incidence of breast cancer in China grows rapidly at 3% per year in recent years, which has become one of the leading causes of death in Chinese cities (5). However, the cause of breast cancer is not clear yet, and even about 50% of the causes with breast cancer can not be explained at all, which seriously threatens the life and health of women (6).

Along with the increasing study of molecular mechanisms of breast cancer, the level of comprehensive treatment for breast cancer has been greatly improved. In recent years, multiple treatments have been developed for breast cancer, such as local surgical treatment, radiotherapy and chemotherapy, endocrine therapy, molecular targeted treatment, auxiliary treatment of traditional Chinese medicine and so on, which significantly improves the quality of life and prolongs the survival period of patients (7-11). A large number of clinical data and experimental studies have shown that the adjuvant treatment of breast cancer patients with traditional Chinese medicine can improve the body's metabolism, reduce the toxic and side effects of radiotherapy and chemotherapy, improve the surgical resection rate and endocrine therapy efficacy, and so on (12,13). The development of traditional Chinese medicine plays an important role in reducing the recurrence and metastasis of tumor, prolonging the survival time, and improving the quality of patients' life (14). Moreover, traditional Chinese medicine still has many positive advantages of rich resources, relatively less cytotoxicity, easy access and low cost. Therefore, the study of the targets and mechanisms of traditional Chinese medicine in the comprehensive treatment for breast cancer has been an important subject in the study of breast cancer prevention and treatment.

Aloperine (Alo) (Fig. 1) is an alkaloid extracted from the traditional Chinese medicine *Sophora alopecuroides* (*S. alopecuroides*) (15,16). It has been reported that Alo induced apoptosis and inhibited invasion in MG-63 and U2OS human osteosarcoma cells, and induced G2/M phase cell cycle arrest

Correspondence to: Professor Delong Tian, Department of Rehabilitation, Binzhou Central Hospital, 6 Tangfang Road, Binzhou, Shandong 210557, P.R. China
E-mail: tiandelong031@163.com

*Contributed equally

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and apoptosis in HCT116 human colon cancer cells (17,18). However, the impact of Alo on breast cancer has not been reported yet. Therefore, the article was designed to investigate the antitumor potential and the underlying molecular mechanisms of Alo on breast cancer.

Materials and methods

Cell culture. Human breast cancer cells MCF-7 and MDA-MB-231 were purchased from the American Type Culture Collection (Manassas, VA, USA). MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Tianhang, Hangzhou, China), 100 mg/ml streptomycin and 100 U/ml penicillin in 5% CO₂ atmosphere at 37°C. MDA-MB-231 cells were cultured in L-15 medium (both Gibco; Thermo Fisher Scientific, Inc.) medium supplied with 10% FBS, 100 mg/ml streptomycin and 100 U/ml penicillin in normal air atmosphere at 37°C.

Cell counting kit-8 (CCK-8) assay. The viabilities of MCF-7 and MDA-MB-231 cells were measured by CCK-8 assay. Briefly, MCF-7 and MDA-MB-231 cells at a density of 1x10⁴ cells/well in 100- μ l of complete culture medium were seeded in 96-well plates. After culturing for 24 h, the medium was replaced with serum-free media or serum-free media containing various concentrations of Alo (0, 0.1, 0.2 and 0.4 mM) according to previous studies (17,18), and incubated in a humidified incubator at 37°C for 12, 24 and 48 h, respectively. After incubation, 10 μ l of CCK-8 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added to each well for another 2 h at 37°C. The optical density (OD) was recorded at 450 nm using a microplate reader (Dojindo Molecular Technology, Rockville, MD, USA).

Colony formation test. Colony formation assay was conducted to evaluate the role of Alo in the proliferative potential of MCF-7 and MDA-MB-231 cells. MCF-7 and MDA-MB-231 cells (1x10³ cells/well) were plated in 6-well plates and cultured at 37°C with 5% CO₂. The medium was replaced with fresh culture media or fresh culture media containing Alo (0, 0.1, 0.2 and 0.4 mM) every 2-3 days. After two weeks, the plates were fixed with 4% paraformaldehyde for 20 min and stained using 10% crystal violet for 30 min. Then the number of stained colonies was manually counted.

Hoechst 33342 staining. MCF-7 and MDA-MB-231 cells at a density of 2x10⁴ cells/well were seeded in 24-well plates, and after incubation for 24 h, fresh culture media or fresh culture media containing Alo (0, 0.1, 0.2 and 0.4 mM) were added, and incubated in a humidified incubator at 37°C for 24 h. Then the cells were stained with Hoechst 33342 (10 mg/ml) in culture medium at room temperature in the dark for 20 min. Subsequently, the cells were washed twice with PBS and immediately evaluated by a microscope.

Apoptosis analysis. Cell apoptosis was performed using Annexin V Apoptosis Detection kit I (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, MCF-7 and MDA-MB-231

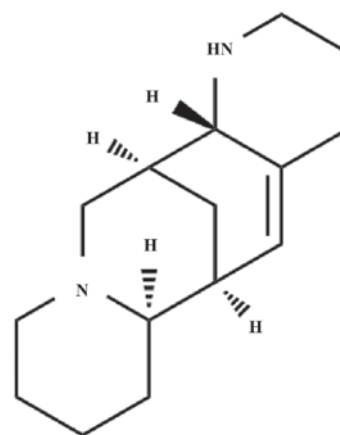


Figure 1. Structure of Aloperine.

at a density of 2x10⁴ cells were treated with indicated doses of Alo (0, 0.1, 0.2 and 0.4 mM) for 24 h. Then the treated cells were digested with trypsin and washed in cold 1X PBS (4°C) twice, followed by resuspending the cell pellet with 300 μ l of 1X Binding Buffer. Next, 5 μ l of Annexin V-PE were added to the cell suspension for 15 min in the dark at room temperature, according to the manufacturer's instructions. 7-AAD solution (5 μ l) was added in the cell suspension 5 min before flow cytometry analysis and then 200 μ l of 1X Binding Buffer was added for flow cytometry analysis. The percentage of apoptotic cells was evaluated by FACS Calibur (BD Biosciences).

Wound healing assay. MCF-7 and MDA-MB-231 cells were cultured in fresh culture media to full confluence. After that, we created a wound using a plastic scraper. After being washed with PBS, the medium was replaced with fresh culture media or fresh culture media containing Alo (0, 0.1, 0.2 and 0.4 mM) and incubated at 37°C for 48 h. Then, the cells were washed twice with PBS and the wound was observed under a microscope (Nikon, Tokyo, Japan).

Transwell migration and invasion assay. Cell migration and invasion assays were performed using Trans-well chambers (8 μ m pore-size, Corning Co., Corning, NY, USA). In migration assay, 5x10⁴ cells in fresh culture media were added into the upper chamber. In invasion assay, Matrigel was purchased from BD Biosciences and stored at -20°C. After thawing at 4°C overnight, the Matrigel was diluted in serum-free medium, and 30 μ l of the diluted Matrigel were evenly inoculated into the upper chamber to form a gel at 37°C. Cells (1x10⁵) suspended in 300 μ l of fresh culture media were seeded into the upper compartments. For trans-well migration and invasion assay, the lower compartments were filled with 600 μ l of medium with 20% FBS. After incubation for 24 h, the non-migrate or non-invasive cells were removed from the upper surface of the membrane by scrubbing. The cells that migrated or invaded to the lower surface of the membrane were fixed with 4% paraformaldehyde, stained in 10% crystal violet, and cells were counted under a microscope (Olympus, Tokyo, Japan).

Western blot analysis. The total protein of cells was extracted according to the manufacturer's recommended protocol (Vazyme, Piscataway, NJ, USA). The protein concentrations

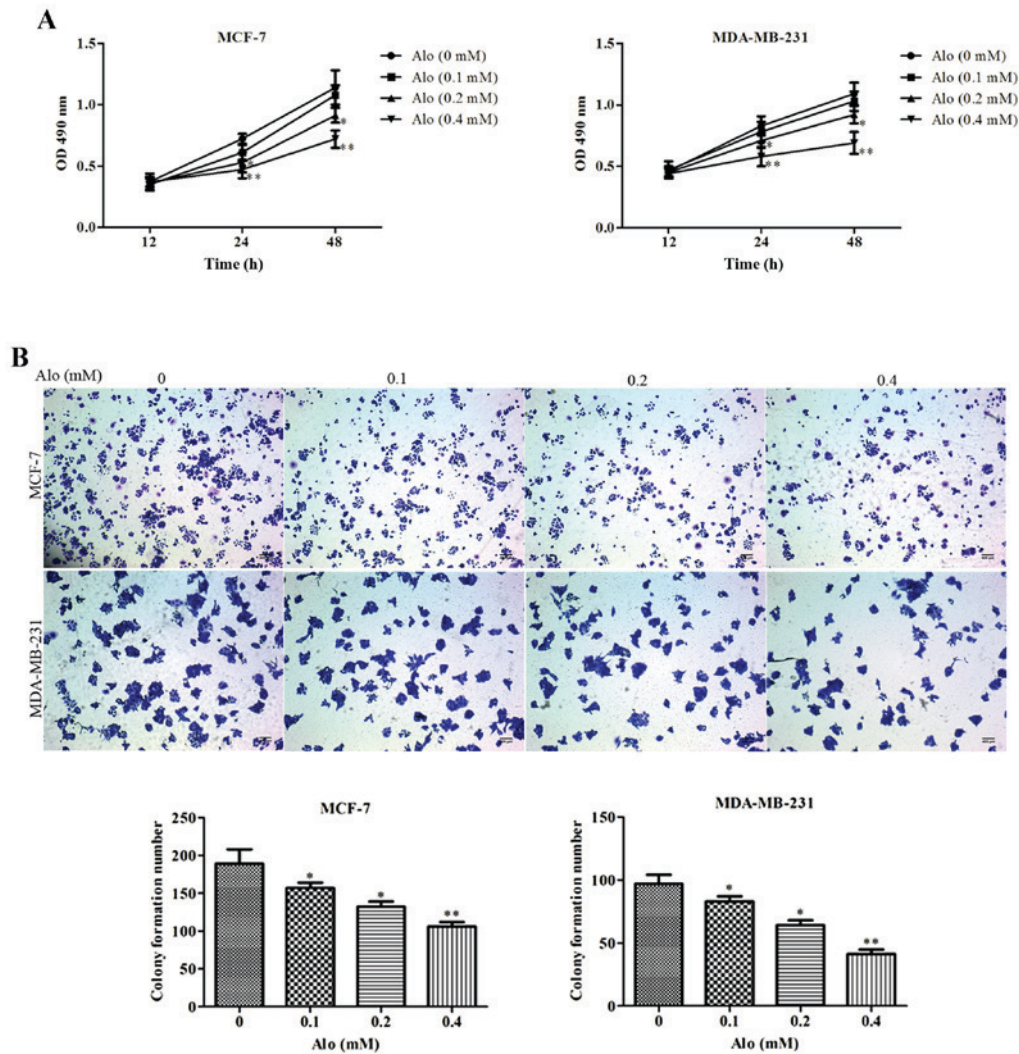


Figure 2. Alo inhibits breast cancer cell proliferation and colony formation. (A) MCF-7 and MDA-MB-231 cell proliferation was evaluated at the indicated time points following treatment with Alo (0.1, 0.2 and 0.4 mM). (B) MCF-7 and MDA-MB-231 cell colony formation was detected following cultured with Alo (0.1, 0.2 and 0.4 mM) for 14 days (scale bars, 400 μ m). The results were expressed as the mean \pm standard deviation of three independent experiments and each was performed in triplicate. *P<0.05 and **P<0.01 vs. non-Alo treated group (0 mM Alo). Alo, Aloperine; OD, optical density.

were determined using the BCA Protein Assay kit (Vazyme, Piscataway, NJ, USA). Samples with equal amounts of protein (50 μ g) were fractionated on 10% SDS polyacrylamide gels, transferred to polyvinylidene difluoride membranes (PVDF), and blocked in 5% skim milk in TBST for 1.5 h at 25 \pm 1 $^{\circ}$ C. The membranes were then incubated at 4 $^{\circ}$ C overnight with 1:1,000 dilutions (v/v) of the primary antibodies. After washing the membranes with TBST, incubations with 1:1,000 dilutions (v/v) of the secondary antibodies were conducted for 2 h at 25 \pm 1 $^{\circ}$ C. Protein expression was detected using an Enhanced Chemiluminescence Detection System. GAPDH was used as a loading control. Antibodies in western blot were purchased Cell Signaling Technology (Beverly, MA, USA), including matrix metalloproteinase (MMP)-2 (cat. no. 4022), MMP-9 (cat. no. 3852), caspase-3 (cat. no. 9662), caspase-9 (cat. no. 9508), Bax (cat. no. 2774), Bcl-2 (cat. no. 2872), Ras (cat. no. 3965), p-Raf1 (cat. no. 9421), Raf1 (cat. no. 4432), p-Erk1/2 (cat. no. 4370), Erk1/2 (cat. no. 9102), GAPDH (cat. no. 8884).

Statistical analyses. GraphPad Prism 5.0 statistical software (GraphPad Software, Inc., La Jolla, CA, USA) was utilized

to analyze the above experimental data. Measurement data were represented as the mean \pm standard deviation. Statistical differences between means among multiple groups were analyzed by one-way analysis of variance followed by a Bonferroni post hoc analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Alo inhibited human breast cancer cells proliferation and colony formation. To investigate the impact of Alo on human breast cancer MCF-7 and MDA-MB-231 cells proliferation, MCF-7 and MDA-MB-231 cells were treated with Alo (0.1, 0.2 and 0.4 mM) for 12, 24 and 48 h, respectively. The results of CCK-8 assay showed that Alo suppressed cell proliferation in a dose and time dependent manner (Fig. 2A). Subsequently, to evaluate whether Alo regulated the colony growth of human breast cancer cells, MCF-7 and MDA-MB-231 cells were treated with Alo (0.1, 0.2 and 0.4 mM), and incubated for two weeks. The results revealed that Alo reduced colony formation of MCF-7 and MDA-MB-231 cells compared with the untreated group (Fig. 2B).

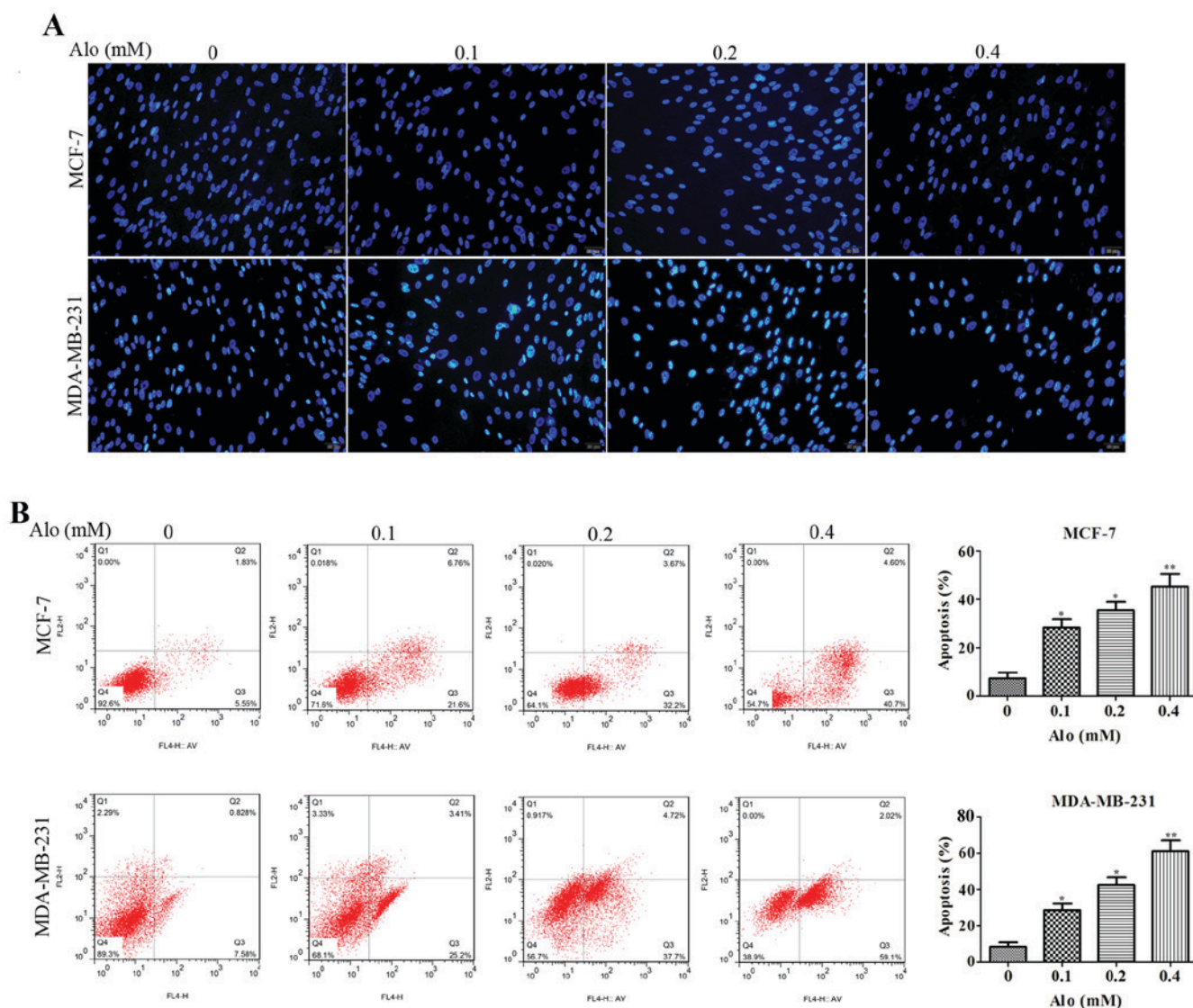


Figure 3. Alo induces breast cancer cell apoptosis. (A) Images of apoptotic MCF-7 and MDA-MB-231 cells incubated with vehicle or Alo (0.1, 0.2 and 0.4 mM) for 24 h, as determined by Hoechst 33342 staining (scale bars, 50 μ m). (B) MCF-7 and MDA-MB-231 cells exposed to Alo (0.1, 0.2 and 0.4 mM) for 24 h were harvested and analyzed by flow cytometry. The results were expressed as the mean \pm standard deviation of three independent experiments and each was performed in triplicate. *P<0.05 and **P<0.01 vs. non-Alo treated group (0 mM Alo). Alo, Aloperine.

Alo induced human breast cancer cells apoptosis. In order to determine whether Alo induced human breast cancer MCF-7 and MDA-MB-231 cells apoptosis, first Hoechst 33342 staining was adapted to evaluate cell apoptosis by morphological examination. The results demonstrated the nuclei of MCF-7 and MDA-MB-231 cells were uniformly stained in the untreated group, indicating these cells had intact cell membrane morphology. However, both MCF-7 and MDA-MB-231 cells treated with Alo (0.1, 0.2 and 0.4 mM) for 24 h clearly exhibited significant morphological changes compared with the untreated group, showing that MCF-7 and MDA-MB-231 cells apoptosis remarkably increased (Fig. 3A). Additionally, the effect of Alo on MCF-7 and MDA-MB-231 cells apoptosis was evaluated by flow cytometry with Annexin V-FITC/PI staining. After treatment of MCF-7 and MDA-MB-231 cells with Alo (0.1, 0.2 and 0.4 mM), we found degree of apoptosis in the Alo group was higher than that in the untreated group (Fig. 3B). These findings suggested that Alo dramatically abated MCF-7 and MDA-MB-231 cells

proliferation rate in a dose dependent manner, which was possibly associated with increasing apoptosis.

Furthermore, the expressions of apoptosis-related proteins, including caspase-3, caspase-9, Bax and Bcl-2, were detected by western blotting analysis. The results showed that Alo could downregulate the expression of Bcl-2 and upregulate the expressions of caspase-3, caspase-9 and Bax compared with the untreated group in MCF-7 and MDA-MB-231 cells (Fig. 4).

Alo suppressed human breast cancer cells migration and invasion. To further confirm the effects of Alo on cell motility, wound healing, trans-well migration and invasion assays were conducted. The results of wound healing and Trans-well migration assays revealed that Alo could significantly suppress MCF-7 cells migration and also remarkably inhibit MDA-MB-231 cells migration in a dose dependent manner compared with the untreated group (Fig. 5A and B). Besides, a similar results in cell invasion was observed. Alo treatment in MCF-7 and MDA-MB-231 cells led to a decreased percent

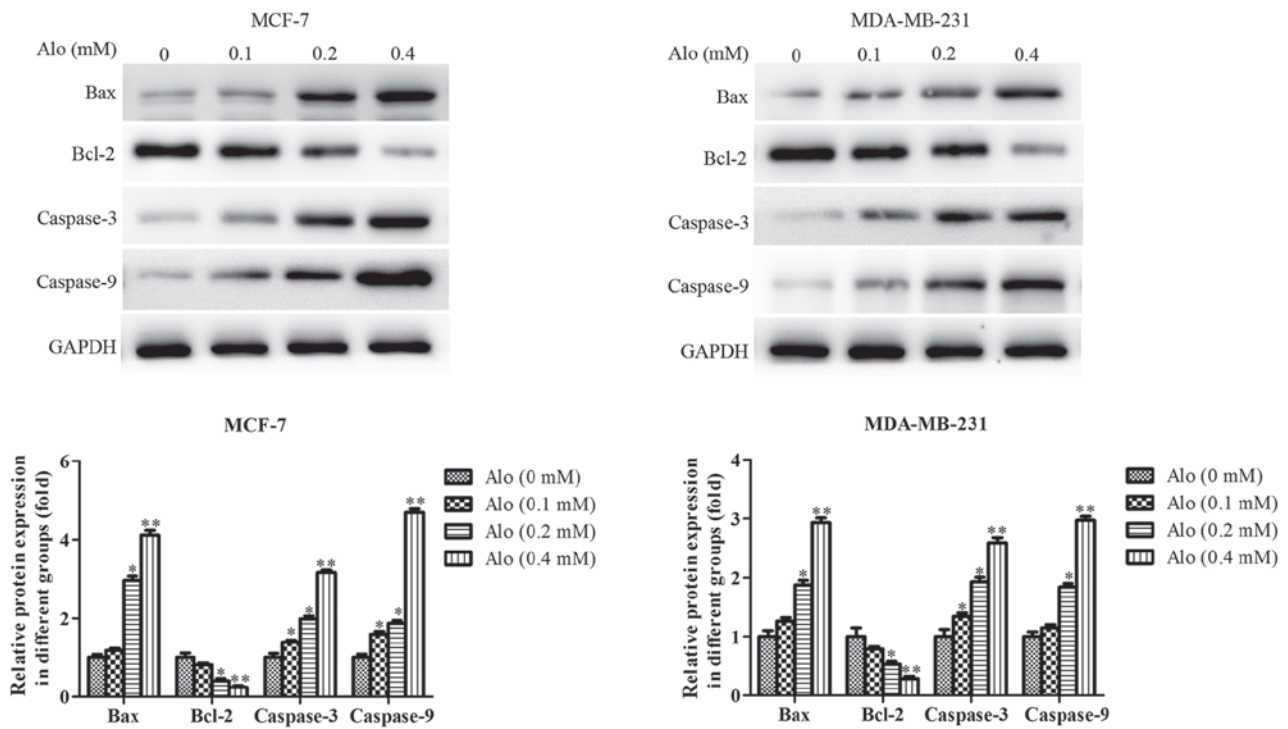


Figure 4. Alo induces breast cancer cell apoptosis by regulating apoptosis-associated protein expression. Western blotting was performed to analyze the expressions of Bax, Bcl-2, caspase-3 and caspase-9 following Alo (0.1, 0.2 and 0.4 mM) treatment for 24 h. Then the band intensity was quantified by ImageJ software. The results were expressed as the mean \pm standard deviation of three independent experiments. * P <0.05 and ** P <0.01 vs. non-Alo treated group (0 mM Alo). Alo, Aloperine; Bcl, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

of invasion cells compared with the untreated group (Fig. 5C). These data indicated Alo dramatically depressed cell migration and invasion in MCF-7 and MDA-MB-231 cells.

MMPs are zinc-dependent proteolytic enzymes of the extracellular matrix (ECM), widely involved in cell migration and invasion (19,20). And as the members of MMPs, MMP-2 and MMP-9 are closely related to the migration and invasion of various types of cancer cells (21,22). Thus, the protein expressions of MMP-2 and MMP-9 were detected to evaluate the effect of Alo on the motility of MCF-7 and MDA-MB-231 cells. It showed that Alo significantly suppressed the protein expressions of MMP-2 and MMP-9 (Fig. 5D).

Alo blocked Ras in human breast cancer cells. Ras family is usually recognized as an oncogene, including H-Ras, Ha-Ras, K-Ras, Ki-Ras and N-Ras, and Ras/Raf1/ERK1/2 pathway is considered to be the downstream of epidermal growth factor receptor (EGFR), which is closely associated to the incidence and prognosis of various cancers (23). Activated ERK1/2 regulates gene expression and ultimately mediates cell growth, differentiation, migration, invasion and other processes through phosphorylation on transcription factors in the cell cytoplasm and nucleus (24,25). Thus, in this study, we observed that, after treatment of human breast cancer cells MCF-7 and MDA-MB-231 with Alo (0.1, 0.2 and 0.4 mM), the level of Ras was downregulated, and the levels of phosphorylation of Raf1 and Erk1/2 were decreased as well (Fig. 6), which indicated that Alo could block the Ras/Raf1/Erk1/2 signaling pathway.

Alo inhibited proliferation, migration and invasion and induced apoptosis via Ras pathway in human breast cancer

cells. After demonstrating decreased level of Ras following incubation of MCF-7 and MDA-MB-231 cells with Alo (0.1, 0.2 and 0.4 mM), further, we evaluated whether activation of these kinases contributed to the progress of MCF-7 and MDA-MB-231 cells. To further verify the involvement of Ras signaling in inhibiting proliferation, migration and invasion and inducing apoptosis of human breast cancer cells, ISIS 2503, a novel Ras inhibitor with selective activity against H-Ras, was employed to inhibit the Ras expression (26,27). First, we found both Alo (0.4 mM) and ISIS 2503 inhibited the expression of Ras, and co-treatment of Alo and ISIS 2503 inhibited the level of Ras more than either one of them in MCF-7 and MDA-MB-231 cells (Fig. 7A). Then the results demonstrated that Alo (0.4 mM) and ISIS 2503 reduced colony formation (Fig. 7B), and could apparently downregulate the expression of Bcl-2 and upregulate the expressions of caspase-3, caspase-9 and Bax (Fig. 7C). However, co-treatment of Alo (0.4 mM) and ISIS 2503 had better inhibitory effect on the cell colony formation and apoptosis compared with that of either agent alone in MCF-7 and MDA-MB-231 cells. Moreover, we adapted the wound healing (Fig. 8A), trans-well migration (Fig. 8B) and invasion (Fig. 8C) assays to evaluate the impact of ISIS 2503 on cell motility, and the results demonstrated co-treatment of Alo (0.4 mM) and ISIS 2503 remarkably inhibited migration and invasion more than either one of them in MCF-7 and MDA-MB-231 cells. In addition, co-treatment of Alo (0.4 mM) and ISIS 2503 showed more positive activity of inhibition of MMP-2 and MMP-9 levels (Fig. 8D). Furthermore, we found co-treatment of Alo (0.4 mM) and ISIS 2503 had better effects on the decrease of the phosphorylation of Raf1 and Erk1/2 (Fig. 9). These findings suggested that Alo

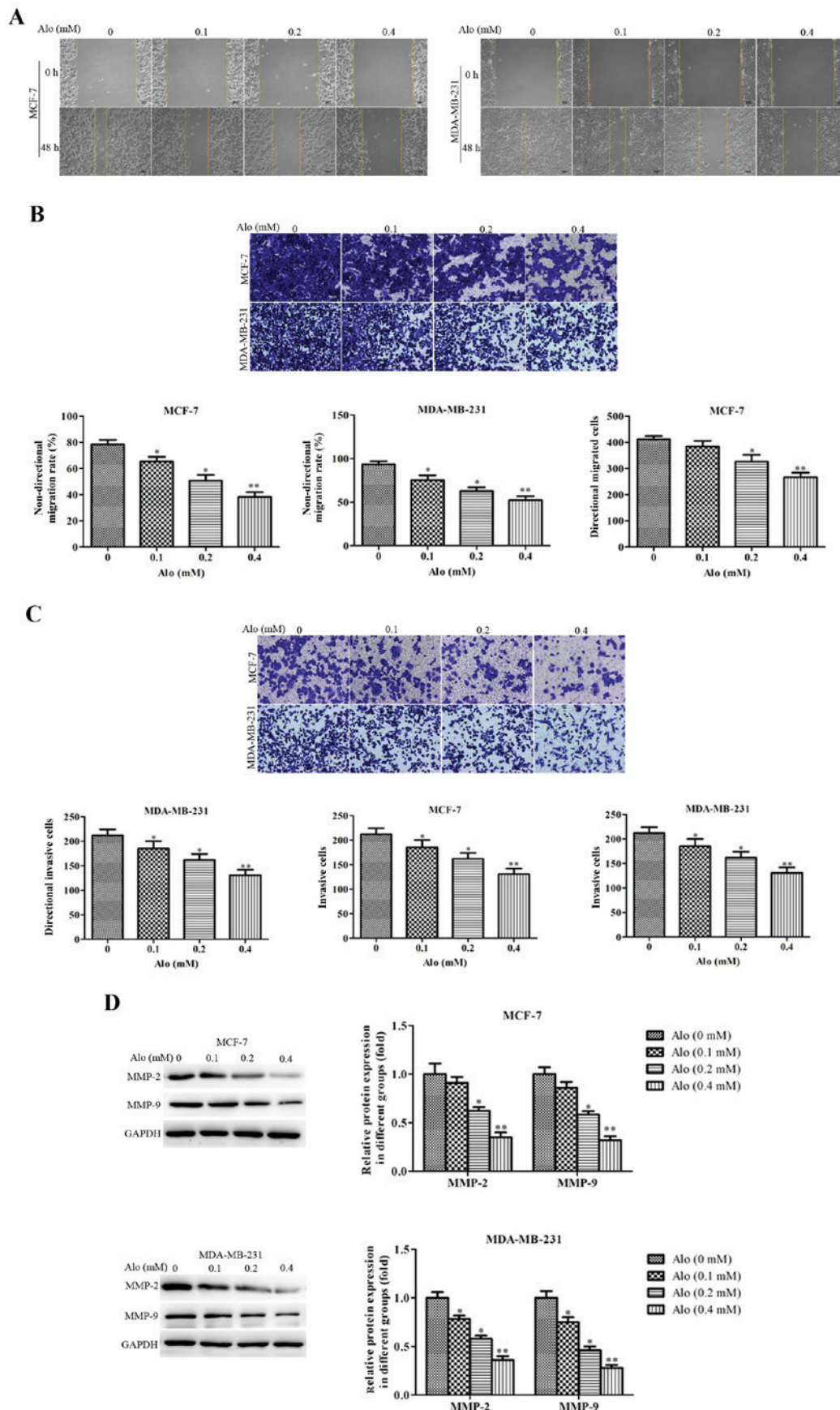


Figure 5. Alo suppresses human breast cancer cell migration and invasion. (A) MCF-7 and MDA-MB-231 cell non-directional migration was detected by wound healing assay (scale bars, 200 μ m; magnification, x100). (B) MCF-7 and MDA-MB-231 cell directional migration was evaluated by Transwell assay (scale bars, 200 μ m; magnification, x200). (C) MCF-7 and MDA-MB-231 cell invasion was examined by Transwell invasion assay (scale bars, 200 μ m; magnification, x200). (D) Western blotting analyzed the expression of MMP-2 and MMP-9 following Alo (0.1, 0.2 and 0.4 mM) treatment for 24 h. Then the band intensity was quantified by ImageJ software. The results were expressed as the mean \pm standard deviation of three independent experiments and each was performed in triplicate. * P <0.05 and ** P <0.01 vs. non-Alo treated group (0 mM Alo). Alo, Aloperine; MMP, matrix metalloproteinase.

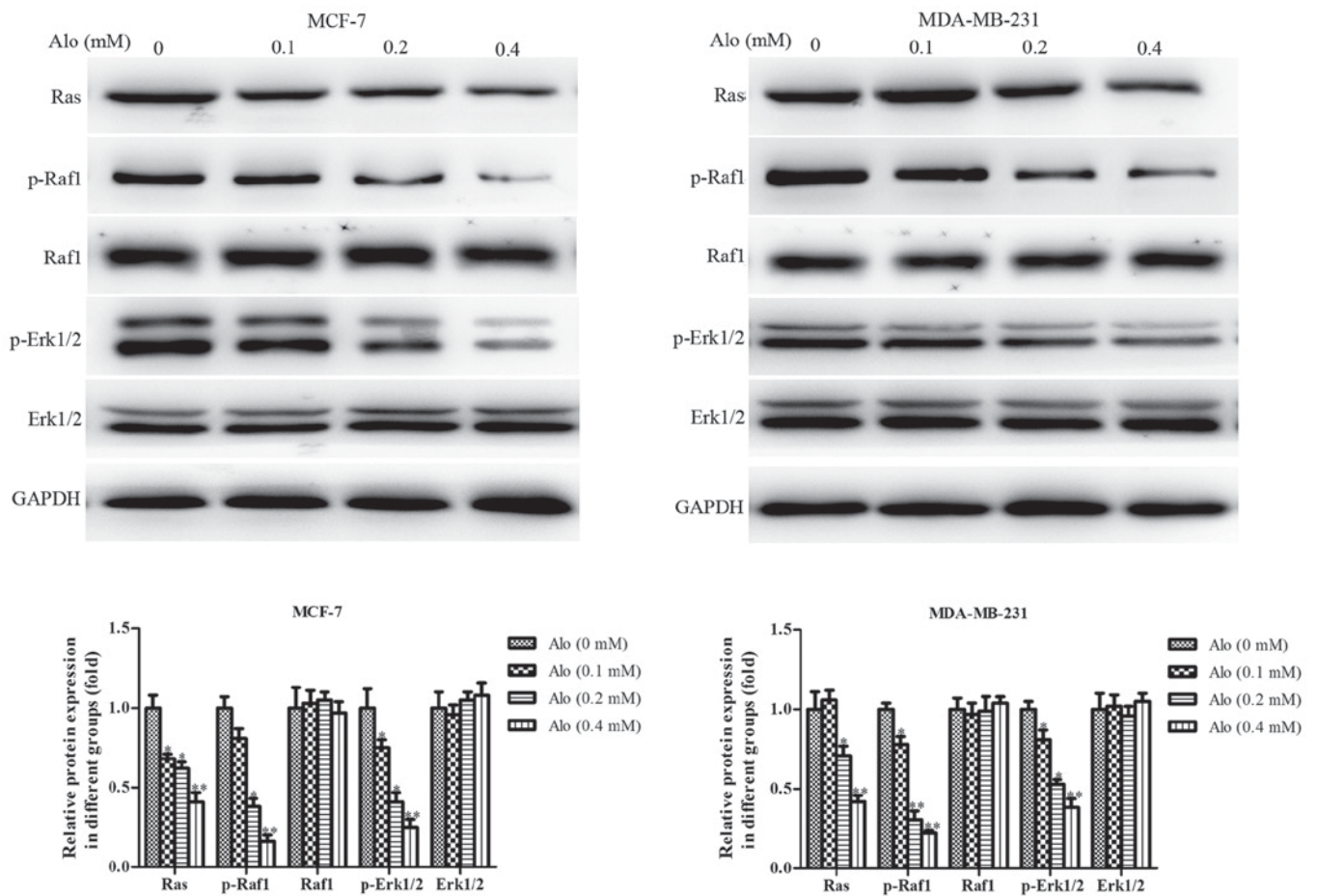


Figure 6. Alo blocks the Ras/Raf1/Erk1/2 signaling pathway in breast cancer cells. Western blotting was performed to analyze the expression of Ras, p-Raf1, Raf1, p-Erk1/2 and Erk1/2 following Alo (0.1, 0.2 and 0.4 mM) treatment for 24 h. Then the band intensity was quantified by ImageJ software. The results were expressed as the mean \pm standard deviation of three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs. non-Alo treated group (0 mM Alo). Alo, Aloperine; Raf, Raf proto-oncogene, serine/threonine kinase; Erk1/2, extracellular signal-regulated kinase 1/2; p-, phosphorylated.

inhibited proliferation, migration and invasion and induced apoptosis maybe through Ras pathway in human breast cancer cells.

Discussion

Breast cancer is one of the most common malignant tumors that leads to the death of women, and hematogenous spread occurs easily in the early stage, which threatens the health of women seriously. At present, the treatments of breast cancer include surgery, chemotherapy, radiotherapy, targeted biotherapy and so on, which is certain to have to cancer cell kill and wound action. However, there are still 25-30% of early breast cancer patients with distant metastasis after 10-15 years of follow-up (28). At the same time, the drug resistance of tumor cells and the side effects of chemotherapeutic drugs are also another difficult problem in the process of cancer treatment. Therefore, the natural compounds from animal and plant have become the main research direction of anticancer drugs (29). Currently, traditional Chinese medicine is still the mainstream of the prevention and treatment of tumor, and the characteristics of multi-component, multi-link and multi-target play a significant role in polygene regulation, complicated pathogenesis, and prevention and treatment of various tumors (30). Moreover, traditional Chinese medicine

has been paid more attention because of its unique antitumor effect, sensitivity of radiotherapy and chemotherapy, little toxic and side effects and prolonged the survival time of patients (31). Therefore, this article was designed to explore the effects and mechanisms of Chinese medicine monomer Alo on proliferation, apoptosis, migration and invasion of breast cancer cells. In this study, we found Alo inhibited MCF-7 and MDA-MB-231 cells proliferation and colony formation in a dose dependent manner, changed cell membrane morphology and promoted the apoptosis in MCF-7 and MDA-MB-231 cells. Bcl-2 family includes pro-apoptotic proteins and anti-apoptotic proteins. A pro-apoptotic bcl-2 family protein, Bax, promotes cell apoptosis by activation of caspase and the release of cytochrome c from mitochondria, while Bcl-2, an anti-apoptotic bcl-2 family protein, restrains cell apoptosis via blocking the release of cytochrome c (32,33). Our data showed that Alo could dramatically downregulate the expression of Bcl-2 and upregulate the expression of caspase-3, caspase-9 and Bax in MCF-7 and MDA-MB-231 cells. These results were similar to previous studies (17,18), which further confirmed that Alo had positive inhibitory activity on the progression and development of many types of cancer.

The main cause of death in patients with breast cancer is the invasion and metastasis of the tumor (34). Wound healing, trans-well migration and invasion assays were conducted to

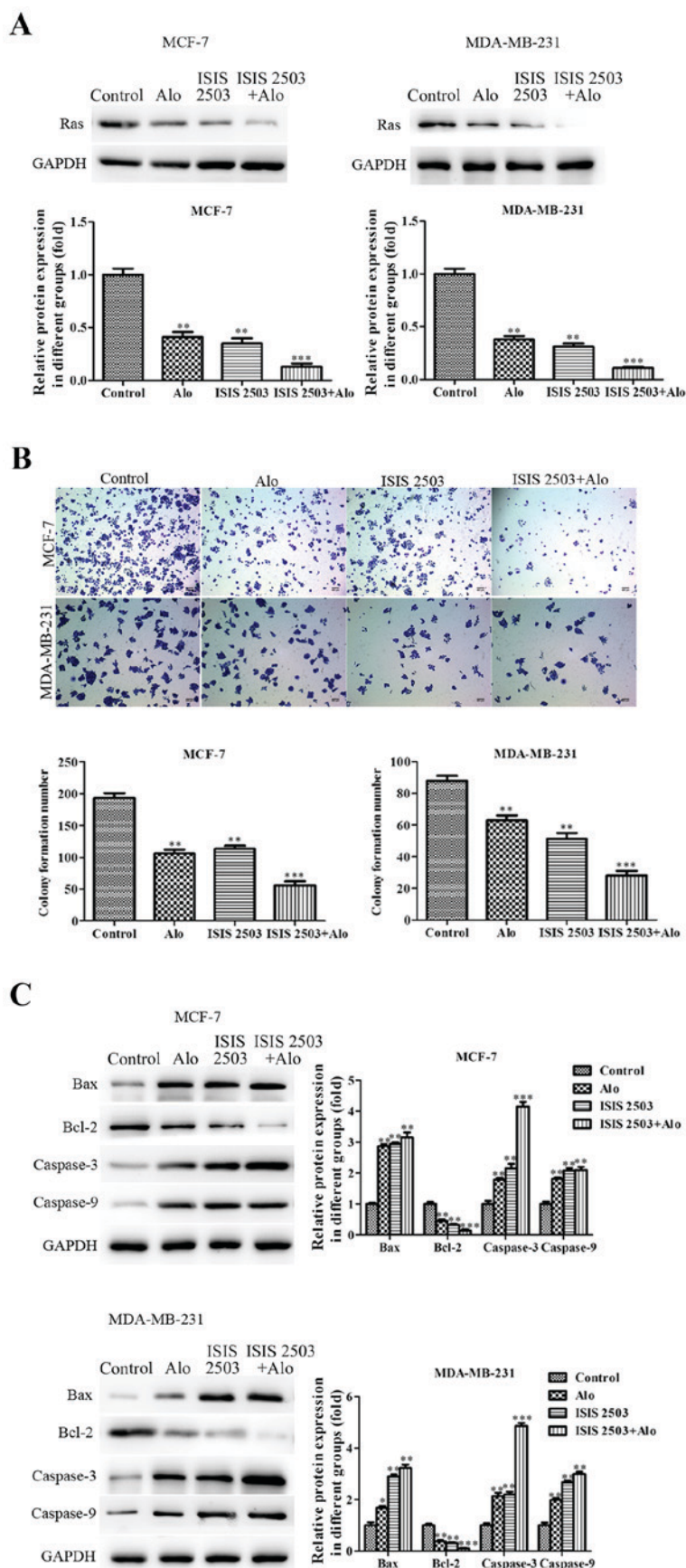


Figure 7. Alo inhibits proliferation and induces apoptosis via the Ras signaling pathway in breast cancer cells. (A) Alo and the Ras inhibitor ISIS 2503 inhibited the expression of Ras, as determined by western blotting analysis. Then the band intensity was quantified by ImageJ software. (B) Images of colony formation of MCF-7 and MDA-MB-231 cells cultured with Alo (0.4 mM) or Ras inhibitor (ISIS 2503) for 14 days (scale bars, 400 μ m). (C) Western blotting analyzed the expressions of Bax, Bcl-2, caspase-3 and caspase-9 following Alo (0.4 mM) or Ras inhibitor (ISIS 2503) treatment for 24 h. Then the band intensity was quantified by ImageJ software. The results were expressed as the mean \pm standard deviation of three independent experiments and each was performed in triplicate. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. control group. Alo, Aloperine; Bcl, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

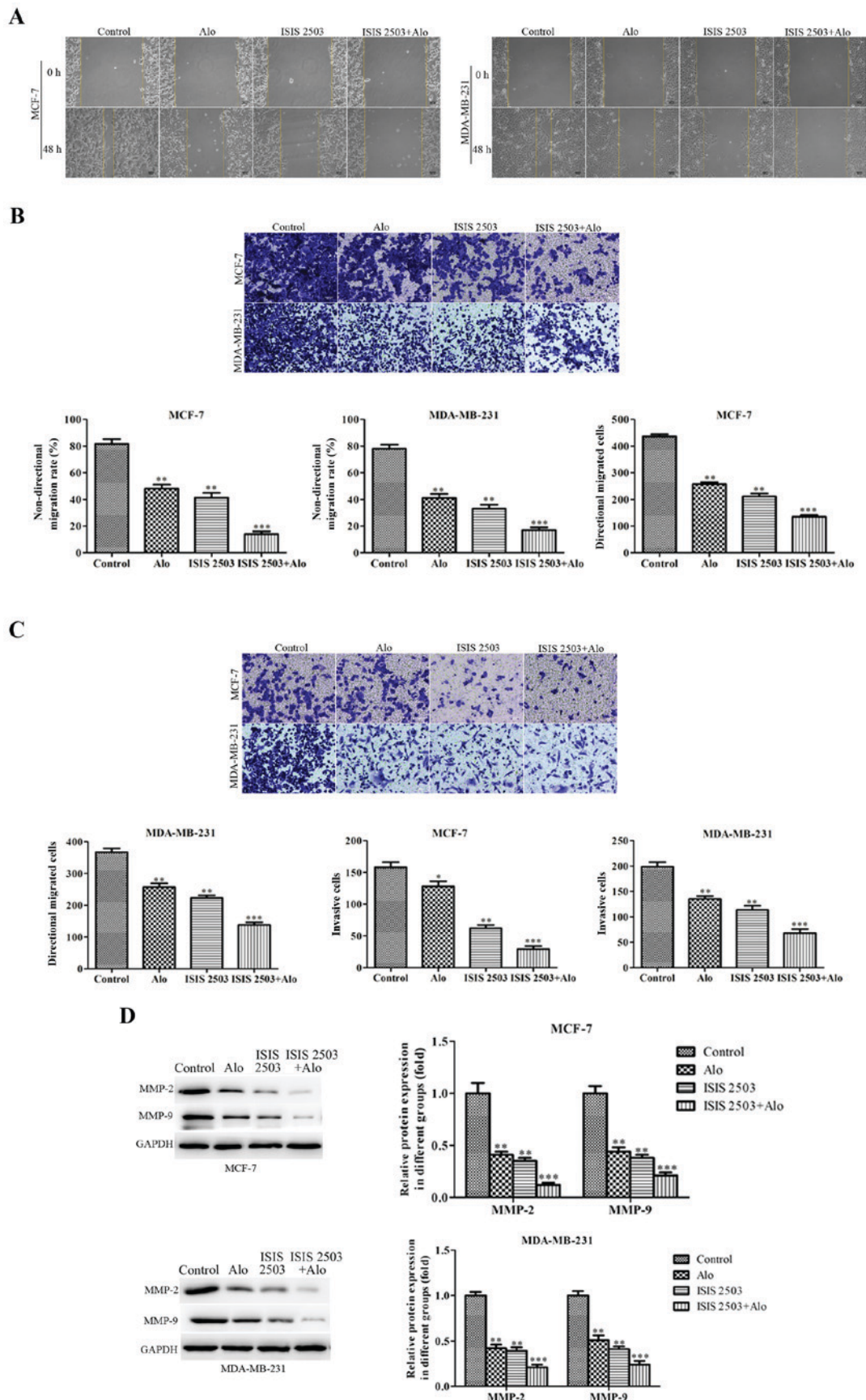


Figure 8. Alo inhibits migration and invasion via the Ras signaling pathway in breast cancer cells. (A) MCF-7 and MDA-MB-231 cell non-directional migration was detected by wound healing assay (scale bars, 200 μ m; magnification, x100). (B) MCF-7 and MDA-MB-231 cell directional migration was evaluated by Transwell assay (scale bars, 200 μ m; magnification, x200). (C) MCF-7 and MDA-MB-231 cell invasion was examined by Transwell invasion assay (scale bars, 200 μ m; magnification, x200). (D) Western blotting analyzed the expressions of MMP-2 and MMP-9 following Alo (0.4 mM) or Ras inhibitor (ISIS 2503) treatment for 24 h. Then the band intensity was quantified by ImageJ software. The results were expressed as the mean \pm standard deviation of three independent experiments and each was performed in triplicate. * P <0.05, ** P <0.01 and *** P <0.001 vs. control group. Alo, Aloperine; MMP, matrix metalloproteinase.

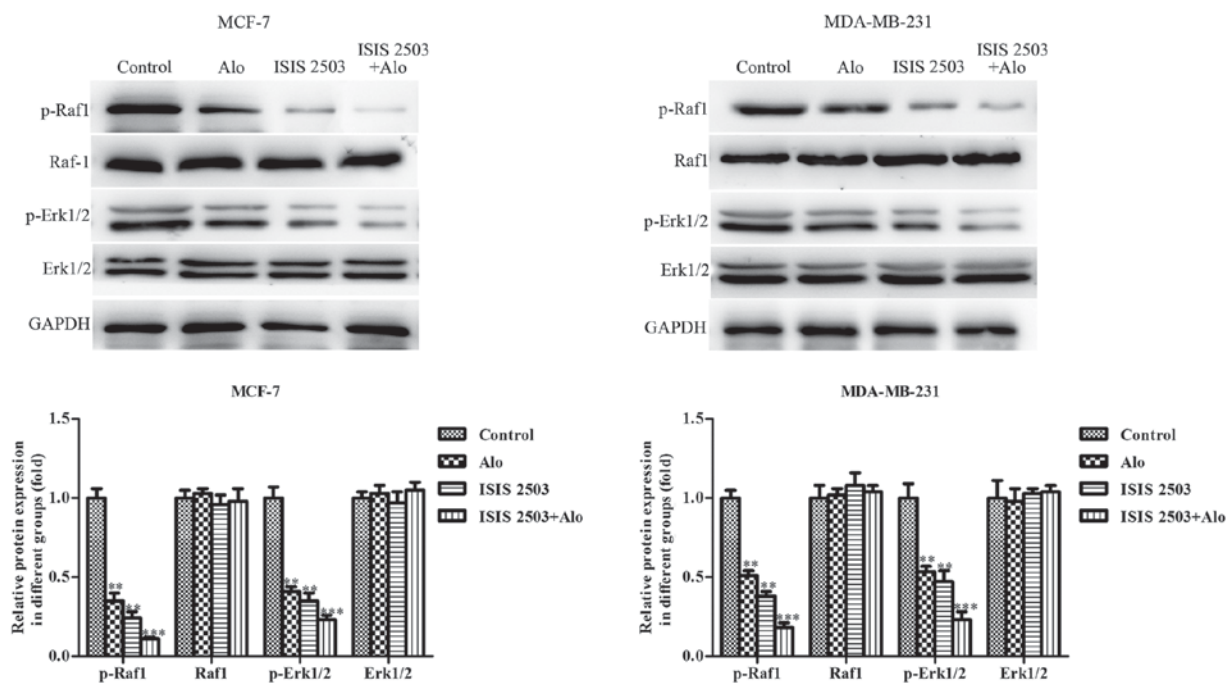


Figure 9. Synergistic effect of Alop and Ras inhibitor (ISIS 2503) on the blockage of the Ras/Raf1/Erk1/2 signaling pathway in breast cancer cells. Western blotting analyzed the expressions of p-Raf1, Raf1, p-Erk1/2 and Erk1/2 following Alop (0.4 mM) or Ras inhibitor (ISIS 2503) treatment for 24 h. Then the band intensity was quantified by ImageJ software. The results were expressed as the mean \pm standard deviation of three independent experiments. ** $P < 0.01$ and *** $P < 0.001$ vs. control group. Alop, Aloperine; Raf, Raf proto-oncogene, serine/threonine kinase; Erk1/2, extracellular signal-regulated kinase 1/2; p-, phosphorylated.

further confirm the effects of Alop on cell motility, and the results revealed that Alop could significantly suppress MCF-7 and MDA-MB-231 cells migration and invasion. In the process of tumor invasion and metastasis, the degradation of ECM plays an important role. Moreover, the degradation of ECM mainly depends on proteolytic enzymes, and the most important is MMPs, which are highly conserved zinc dependent endonuclease protease family with the positive activities on degrading most proteins of basement membrane and ECM (35). Furthermore, MMP-2 and MMP-9 have been strongly correlated with the invasiveness of many types of cancer cell (36). Thus, the protein expressions of MMP-2 and MMP-9 were detected to evaluate the effect of Alop on the motility of MCF-7 and MDA-MB-231 cells and we found that Alop significantly suppressed the protein expressions of MMP-2 and MMP-9.

At present, it is believed that the occurrence of human tumor is the result of multiple gene mutations that control the proliferation, differentiation and apoptosis of normal cells, and these mutations include the activation of the oncogenes and the inactivation of the anti-oncogenes. It has been found that mutation or activation of Ras gene and abnormal over-expression of Ras protein exist in about 30% human tumors (37,38). Therefore, research on the regulation of Ras signal transduction pathway plays an important role in the design of antitumor drugs targeting the cellular signal transduction pathway (39). Ras protein is considered as an important element to regulate the signal pathway of cell growth and proliferation. If Ras protein is activated continuously, it can bind downstream effective proteins and transmit signals to downstream signaling elements, which may cause abnormal proliferation of cells and lead to the occurrence of tumors (40). Therefore,

we evaluated the role of Alop in Ras and its downstream signaling (41). From the results, we found Alop could significantly downregulate the level of Ras and suppress the levels of phosphorylation of Raf1 and Erk1/2, indicating that Alop could block the Ras/Raf1/Erk1/2 signaling pathway. To further verify role of Ras signaling in occurrence and development of human breast cancer cells (42), we chose a Ras inhibitor, ISIS 2503, to inhibit the Ras signaling pathway, and we found that ISIS 2503 could remarkably inhibit the expression of Ras. Moreover, we also found that co-treatment of Alop and ISIS 2503 had better inhibitory effects on proliferation, migration, invasion and apoptosis of MCF-7 and MDA-MB-231 cells than either one of them. These results indicated that Alop had better inhibitory effects on the occurrence and development of breast cancer via blockage of Ras pathway.

The current research is a preliminary study on the antitumor effect of Alop, the other effects of Alop and the mechanisms of Alop in breast cancer are not very clear. Based on the current findings, we will further explore the role of Alop in development of cancer both *in vivo* and *in vitro*, and explore the anticancer mechanism of Alop by genomics, proteomics and metabolomics in future. Taken together, the present data suggest that Alop possesses anticancer effects in human breast cancer cells via inhibiting the potential of cell proliferation, migration and invasion, and inducing apoptosis. Furthermore, Alop might act its role in suppressing human breast cancer cells via Ras inactivation. Based on our findings, Alop could be considered as a potential candidate to treat breast cancer.

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Availability of data and materials

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

Authors' contributions

DT and YL designed the experiments. DT, YL, XL and ZT performed the experiments. DT and YL wrote the manuscript. All authors reviewed the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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