ORIGINAL RESEARCH

Naringin Inhibits the Proliferation, Migration, Invasion and Epithelial-to-Mesenchymal Transition of Gastric Cancer Cells via the PI3K/AKT Signaling Pathway

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ABSTRACT

Background • Gastric cancer is a common malignant tumor of the human digestive system. Currently, the treatment of gastric cancer is still dominated by radiotherapy, chemotherapy and surgery. Although the treatment is very effective, we are also trying to find new treatment methods. Traditional Chinese Medicine (TCM) may play an important role in the treatment of gastric cancer.

Study Objective • The aim of this study is to explore the effects of naringin on the proliferation, migration, invasion and apoptosis of gastric cancer and its potential mechanisms.

Methods • MGC803 and MKN45 viability were detected by MTT assay. The effects of naringin on cell cloning, migration and invasion were determined by colony formation assay, cell scratch test and transwell assay (ThermoFisher Scientific™, Waltham, Massachusetts USA), respectively. Cell cycle and apoptosis were assayed by flow cytometry. Associated proteins were measured using Western blot and immunohistochemistry (IHC). The experimental results were further verified in nude mice.

Setting • This study was carried out in Department of Experimental Animal Center of Xi'an Jiaotong University and the Translation Medicine Center of the First Affiliated Hospital of Xi'an Jiaotong University in China.

Results • Cells remained mainly in G0/G1 phase and apoptosis was increased. The nude mouse model showed that naringin treatment could inhibit the growth of tumors in nude mice. Cell scratch tests and transwell assay showed that the invasion and migration abilities of the gastric cancer cell line were significantly reduced after naringin treatment. Western blot showed that the expression of Vimentin, Zeb1 and P-AKT was downregulated and that E-cadherin was upregulated after naringin treatment.

Conclusion • Naringin can block the cell-cycle, induce cancer cell apoptosis, and inhibit the epithelial mesenchymal transition (EMT) process by inhibiting the PI3K-AKT/Zeb1 pathway in gastric cancer cells. Therefore, naringin can inhibit the development of gastric cancer. (*Altern Ther Health Med.* 2023;29(1):191-197).

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INTRODUCTION

Gastric cancer (GC) is a common malignant tumor of the human digestive system, which could be related to *Helicobacter pylori*, diet, region, age, etc. GC is the fifth most common cancer and the third most common cause of cancer death globally.[1] Especially in Asia, GC is the second most common cause of death from cancer.[2] In 2020, China had 478 508 newly diagnosed GC and 373 789 cancer-related deaths.[3] At present, gastric cancer is still surgery-based comprehensive treatment, and other treatment methods include chemotherapy, radiotherapy, immunotherapy and targeted drug therapy. Traditional Chinese Medicine (TCM) may play an important role in the treatment of gastric cancer. However, the ingredients of most TCM are complex and their mechanisms for treating cancer are not clear. Therefore, further studies of natural compounds in TCM are urgently needed.

The chemical formula for naringin is $C_{27}H_{32}O_{14}$ and it is either a light yellow or quasi-white powder. Naringin exists mainly in the peel and pulp of citrus grandis, citrus paradise and orange, which is the main ingredient in *Zhike, Chenpi,* and other TCM. Recent studies have shown that naringin has characteristics that include antioxidation, anti-inflammation, anti-ulcer, anti-osteoporosis and anticarcinogenic. First, naringin has a certain therapeutic effect on many diseases of the central nervous system, including Parkinson disease, epilepsy and Alzheimer's disease.[4-7] Second, naringin reduces metabolic syndrome by upregulating AMPK and downregulating the expression of key glycosylases.[4, 8-11] Third, naringin can promote the proliferation and differentiation of osteocytes.[12,13] Fourth, naringin can scavenge reactive oxygen species (ROS) and free radicals and has the function of anti-oxidation.[14] Fifth, in the verification model, it was proven to be able to reduce the expression of signal molecules related to inflammation, such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α).[15] Sixth, naringin has been shown to promote tumor cell apoptosis and to inhibit the proliferation of tumor cells, including triple negative breast cancer (TNBC) cells,[16] human cervical cancer (SiHa) cells[17] and bladder cancer cells.[18] Many basic studies have shown that naringin induces apoptosis of most tumor cells, inhibits cell proliferation and plays a role in reducing cell invasion and migration.

Although naringin has a certain inhibitory effect in a variety of tumors, the role of naringin in gastric cancer is not completely clear. A study by Raha, et al[19] showed that naringin can induce gastric cancer cells autophagy, which in turn inhibits tumor cells. However, the researchers did not clarify the effect of naringin on gastric cancer in general. Therefore, further exploration of the mechanism of naringin in inhibiting gastric cancer cells is needed.

MATERIALS AND METHODS

Cell Culture and Naringin Treatment

A total of 2 human gastric cancer cell lines, MGC803 and MKN45, were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences, and the detection report was provided. The cells were grown in DMEM medium (Gibco BRL, Carlsbad, California, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL) at 5% CO_2 at 37° C. Naringin was purchased from Solarbio, Beijing, China, cat. No. 8370; purity >98%. Naringin was totally dissolved in dimethylsulfoxide (DMSO). Gastric cancer cells were exposed to different concentrations of naringin for various intervals.

MTT Tetrazolium Reduction Assay

A total of 5000 cells were seeded in 96-well plates for MTT assay, treated with an increasing concentration (0, 25, 50, 100 μmol/L) of naringin for 24, 48 and 72 hours, and the half-maximal inhibitory concentration (IC50) of naringin was determined. Cell viability was tested via a microplate reader (Thermo Fisher, Waltham, USA). A total of 6 parallel wells were set for each experiment and this process was repeated 3 times. Data were analyzed with IBM® SPSS 22.0 software (Amonk, New York, USA) and were presented as mean ± standard deviation (SD).

Colony Formation Assay

Single cells after suspension were seeded in 6-cm culture dishes (2×10^3 cells/well). After overnight incubation, cells were treated with naringin for 24 hours. The cells were then incubated in fresh medium for 2 weeks and culture medium was replaced every 3 days. Finally, the cell colonies were first fixed with 4% paraformaldehyde and then stained with 0.5% crystal violet. The number of visible colonies was determined and the experiment was performed at least 3 times.

Cell Cycle Analysis

Gastric cancer cells cultured with naringin at the IC50 dose for 24 hours were harvested, fixed with 70% ethanol and pretreated with 250 μg/ml RNAse (cat.70856; Sigma-Aldrich, Burlington, Massachusetts USA). Cells were stained with propidium iodide (PI) (cat. P4864, 50 μg/mL, Sigma), and the cell cycle profile was determined via flow cytometer (Beckman Coulter, Brea, California USA).

Cell Apoptosis

The effect of naringin on cell apoptosis was evaluated via flow cytometry. Gastric cancer cells were seeded in 6-well plates and administered with the IC50 dose of the 2 cells for 24 hours. Gastric cancer cells were washed with cold phosphate buffer saline (PBS) 3 times. Cell apoptosis was detected using the Annexin V-APC/7-ADD apoptosis kit (cat. AP105-60, Multi Sciences, Nanjing, China) according to the manufacturers' instructions.

Mitochondrial Transmembrane Potential Detection

The mitochondrial transmembrane potential (MTP) was detected by mitochondrial transmembrane potential kit JC-1 (cat. M8650; Solarbio, Beijing, China). Briefly, cells treated with naringin were harvested, then incubated with JC-1 for 30 min at 37° C. Then, cells were washed twice with incubation buffer and resuspended in incubation buffer and analyzed via flow cytometry.

Wound Healing Assay

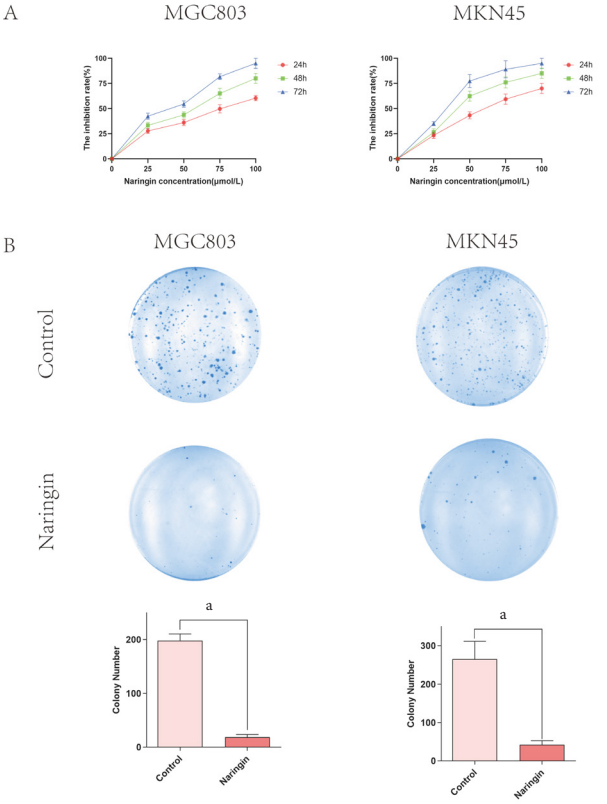
Gastric cancer cells were seeded in 6-well plates with complete medium. When the cells formed a confluent monolayer, a scratch wound was made in the middle of each well with a 10 μl pipette tip. The medium and cell debris were aspirated away and replaced with medium either with or without IC50 naringin. The scratch gap was recorded at 0, 24 and 48 hours by inverted phase microscope. ImageJ software (National Institutes of Health, USA) was used to analyze the wound area.

Transwell Assay

The invasion ability of gastric cancer cells treated with naringin was evaluated via transwell assays (ThermoFisher

Scientific) using Millicell Hanging Cell Culture Inserts (Millipore Sigma, Cleveland, Ohio USA). First, the chambers were coated with 100μl of 5 mg/ml Matrigel at 37° C for 1 hour. MGC803 and MKN45 were treated with naringin at the concentration of 39.54μM and 34.37 μM respectively for 24 hours without fetal bovine serum (FBS). Gastric cancer cells suspension (1×10^5/mL, 200 μl) was added to the inserts and a total of 500μl of 10% FBS was added to the lower chamber. After 72 hours of incubation, the inserts were removed from the 24-well plates, fixed with 4% paraformaldehyde for 5 minutes, and stained with crystal violet for 30 minutes. Non migrated cells on the upper layer were gently wiped out with cotton balls. The number of invaded cells per file was quantified under an inverted phase microscope (Nikon, Tokyo, Japan).

Figure 1. Inhibitory effect of naringin on the growth of gastric cancer cell lines. **(1A)** Gastric cancer cells MGC803 and MKN45 were treated with various concentrations (0, 25, 50, 75,100 μmol/L) of naringin for 24, 48 and 72 hours. **(1B)** MGC803 and MKN45 were treated with naringin at the dose of IC50 (MGC803 cells:76.21 μmol/L, MKN45 cells: 64.42 μmol/L) for 24 hours.



[a]indicates $P < .01$. Three independent experiments were carried out.

Abbreviations: IC50, half maximal inhibitory concentration.

Western Blot

Cell protein extraction was performed using the RIPA Protein Extraction Kit (cat. E121-01; GenStar, China) according to the operating manual. Western blot was performed as described in the literature. The extracted proteins were subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% milk for 1 hour, the membrane was incubated with the primary antibody at 4° C overnight. Information about the antibodies used in Western blot was shown as follows: P21 (cat.109199,1:1000l; Abcam, Cambridge UK), Cyclin D1 (cat.16663,1:1000; Abcam) Caspase3 (cat. 9662,1:1000; Cell Signaling Technology, Danvers, Massachusetts USA), Cleaved-Caspase3 (cat. 9664,1:1000; CST), Bcl2 (cat. YM3041,1:1000; Immunoway, Plano, Texas USA), Bax (cat. YM3619,1:1000; Immunoway), AKT (cat. 4691,1:1000; CST), P-AKT (cat. 4060,1:2000; CST), E-Cadherin (cat. 3195,1:1000; CST), Zeb1 (cat. 21544-1-AP,1:1000; Proteintech, Wuhan, China), Vimentin (cat. 3295,1:1000; CST) and GAPDH (cat.60004-1-Ig,1:1000, Proteintech). After incubation, the PVDF membrane was rinsed and then incubated with the secondary antibody for 1 hour at room temperature. Finally, protein expression was detected using a chemiluminescence detection system (Millipore, USA).

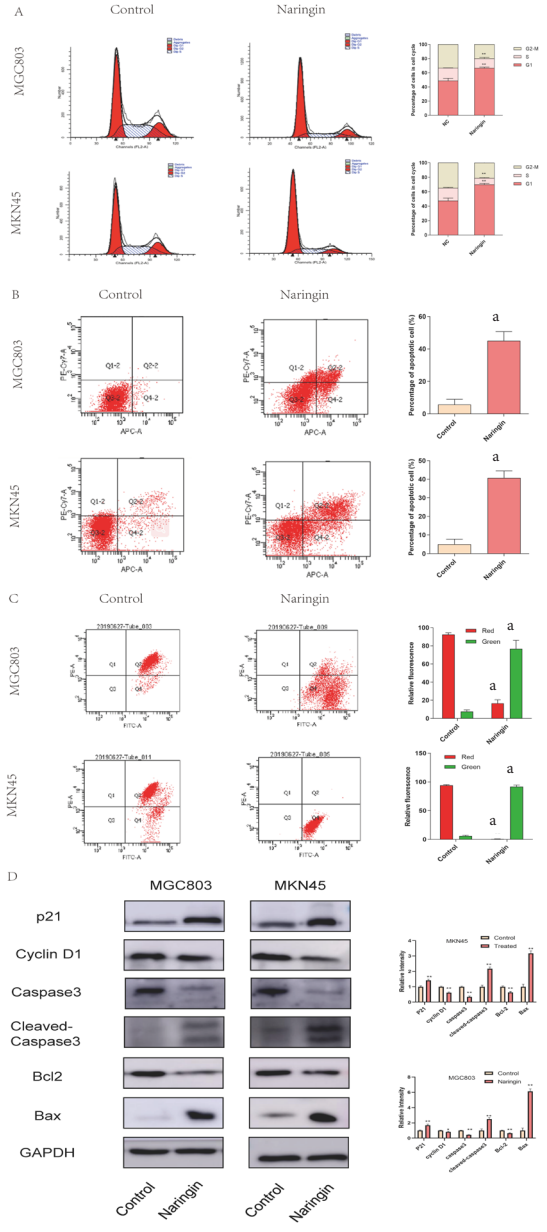
Antitumor Effects in Nude Mice

Adult BALB/C nude mice (female, age 35 to 40 days, weight 18g to 22g) were purchased and raised at the Experimental Animal Center of Xi'an Jiaotong University. All mice were housed with a 12-h light/12-h dark cycle at 25° C, 60%±10% humidity with free access to food and water. Each nude mouse was injected with 0.1 ml of cell suspension (cell concentration 1×10^6/ml). When their tumor reached 100 mm^3, it was treated with naringin (0.2 ml 150 mg/kg) for 10 days by intraperitoneal injection according to Li, et al[20] while a 0.9% sodium chloride solution of the same volume was injected into mice in the control group. The change in tumor volume and weight of the mice were recorded. Tumor volume was calculated using the following formula, $V= (x \times y^2) / 2$ (tumor long diameter length (x); short diameter (y); volume (V). Mice were sacrificed after 21 days, and tumors were dissected and weighed.

Immunohistochemistry

The tissue obtained from the mice was first soaked in formalin for 24 hours and then made into continuous 4μm pathology sections with paraffin embedding. The procedure was performed per the manufacturer's instructions. The concentration of the primary antibodies was: Ki67 (cat. ab1667; 1:100; Abcam and Zeb1 (cat. 21544-1-AP; 1:100; Proteintech). For each section, we selected at least 5 high-magnification fields to access the results. In addition, the immunohistochemistry (IHC) score was evaluated by the area and intensity of positively stained cells, and the immunoreactivity score (IRS) was the product of the area and the intensity. IRS <3 was attributed to the negative group and the others were defined as the positive group. A total of

Figure 2. Naringin induces G0/G1 phase cell cycle arrest and apoptosis in gastric cancer cells. **(2A)** The cell cycle of gastric cancer cell lines MGC803 and MKN45 treated with and without naringin at the dose of IC50. **(2B)** Apoptosis of the gastric cancer cell lines MGC803 and MKN45 treated with and without naringin. Naringin was administered at the dose of IC50 in the 2 cells for 24 hours, respectively. **(2C)** The mitochondrial membrane potential of the gastric cancer cell line MGC803 treated with and without naringin. Naringin was administrated at the dose of IC50 in the 2 cells for 24 hours, respectively. **(2D)** Key proteins related to apoptosis and the cell cycle.



[a]$P < .01$. Three independent experiments were carried out.

Abbreviations: IC50, half maximal inhibitory concentration.

2 independent pathologists who were blinded to the experimental design performed this process.

Statistical Analysis

IBM® SPSS 22.0 and GraphPad software were used to analyze the data. *t* test, chi-square test, and one-way ANOVA test were used. All experiments were repeated 3 times. The results were expressed as mean ± standard deviation (SD). $P < .05$ was considered significant.

RESULTS

Naringin Inhibits Proliferation of Gastric Cancer Cells

MTT proliferation assay showed that naringin inhibited the proliferation of MGC803 and MKN45 gastric cancer cells after treatment with different concentrations of naringin for 24, 48 and 72 hours. The IC50 naringin dose in gastric cancer cells MGC803 and MKN45 was 76.21 μM (44.2 μg/ml) and 64.42 μM (37.4μg/ml), respectively (see Figure 1A).

To further explain the effect of naringin on the proliferation of gastric cancer cells, we carried out a plate clone formation experiment. The results (see Figure 1B) showed that gastric cancer cells MGC803 and MKN45 treated with naringin for 24 hours can significantly inhibit cancer cell proliferation ability.

Naringin Induces G0/G1 Phase Cell Cycle Arrest and Apoptosis in Gastric Cancer Cells

To find out how naringin suppressed gastric cancer cell proliferation, we performed a flow cytometry assay to detect changes in cell cycle after treatment with naringin. IC50 naringin concentration was used for the assay. As expected, naringin treatment led to more cells having a G0/G1 phase arrest, while the cell ratio in phase S and phase G2/M decreased significantly (see Figure 2A).

Since naringin caused G0/G1 cell cycle arrest, we next investigated whether naringin could induce gastric cancer cell apoptosis. The results showed that gastric cancer cell lines MGC803 (control group: 5.8% ± 0.7%; naringin group: 1%±5.8%; $P = .005 < .05$) and MKN45 (control group: 5.1% ± 0.6%; naringin group: 40.8% ± 5.2%; $P = .002 < .05$) treated with naringin could significantly increase the occurrence of apoptosis (see Figure 2B). MTP (ΔΨm) is a marker for mitochondrial dysfunction in the early stage of cell apoptosis. We then used a JC-1 fluorogenic probe to detect the decrease in MTP in gastric cancer cells after naringin treatment. As shown in Figure 2C, compared with the control group, naringin induced a significant depolarization of the mitochondrial membrane after 24 hours of treatment. It is suggested that naringin causes apoptosis mainly through the mitochondria apoptosis pathway.

Western blot revealed that naringin could reduce the expression of Cyclin D1, Bcl2, and Caspase-3, and increase the expression of P21, Bax and Cleaved Caspase-3, which contributed to the arrest of the cell cycle and apoptosis (see Figure 2D).

Naringin Inhibits the Migration and Invasion of Gastric Cancer Cells

To clarify the effect of naringin on the migration and invasion of gastric cancer cells, cell scratches and a transwell assay were performed. Cell scratch test showed that the migration ability of gastric cancer cell lines MGC803 and MKN45 decreased significantly after treatment with naringin (see Figure 3A). A further transwell assay showed that naringin could significantly reduce the invasion of gastric cancer cell lines, as shown in Figure 3B.

Naringin Inhibits the EMT Process Through the PI3K/AKT/Zeb1 Axis

To assess the mechanism by which naringin may affect gastric cancer cells, EMT-related proteins were evaluated using Western blot. As the bands revealed, the protein expression level of Vimentin was significantly reduced, whereas that of E-cadherin was enhanced in both cell lines, indicating that naringin inhibited EMT in gastric cancer cells. In addition, we then evaluated the expression of Zeb1, and the result shows that Zeb1 decreased in the naringin group. Since the PI3K/Akt signaling pathway plays a crucial role in cancer progression, we investigated whether the PI3K/Akt signaling pathway was involved in the anti-tumor mechanism of naringin. The results of Western blot showed that naringin could significantly reduce the expression of P-AKT (see Figure 3C).

Naringin Inhibits Tumor Cells Growth *in Vivo*

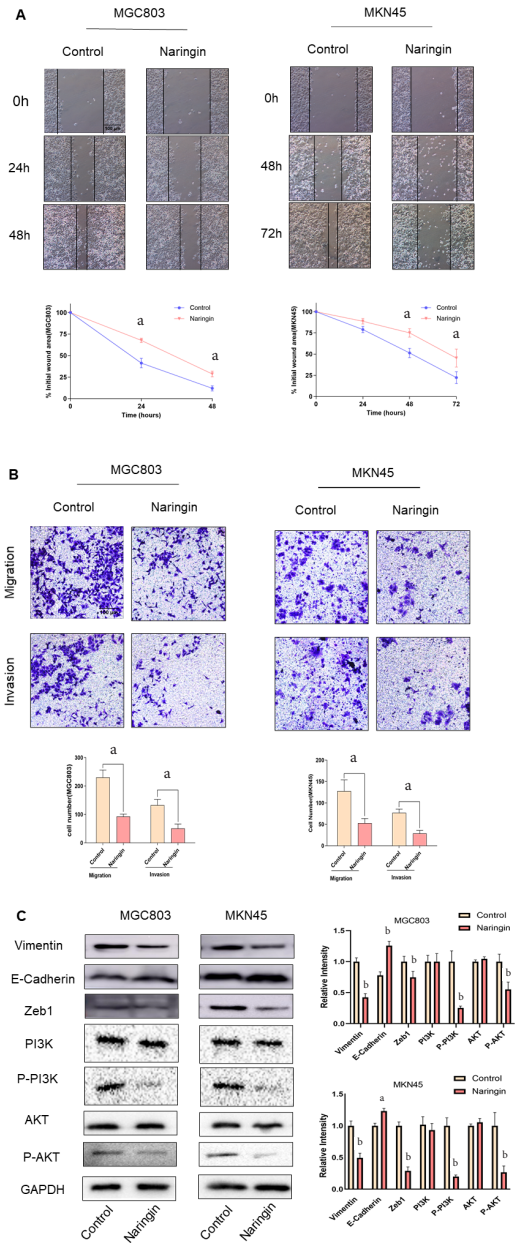
To further examine the antitumor effect of naringin, MGC803 cancer cell-based xenograft model was established in the dorsal flanks of nude mice. Tumor burden was monitored during and at the end of treatment. The results showed that naringin (150 mg/kg; 0.2 ml) could obviously inhibit tumor growth in nude mice (see Figure 4A), the growth rate of tumor volume was significantly decreased and tumor weight was reduced (see Figure 4B). Subsequently, we performed IHC staining of the tumor and detected Zeb1 and Ki67. The results showed that the expression of Zeb1 and Ki67 was significantly decreased in the naringin-treated group (Figure 4C).

DISCUSSION

Recently, natural compounds and extracts have become attractive in the treatment of gastric cancer. In this study, we confirmed the anticancer effect of naringin in gastric cancer using *in vitro* and *in vivo* models. As a natural flavonoid glycoside, naringin could not only effectively inhibit the proliferation, migration and invasion of gastric cancer cells *in vitro*, but also possessed good antitumor activity in the xenograft mouse model. Our study can provide a theoretical basis for the application of naringin in gastric cancer.

Naringin could induce G0/G1 cell arrest by promoting p21 and downregulating the expression of cyclin D1. Studies by Li, et al[16] shown that naringin treatment could lead to G0/G1 phase arrest of breast cancer cells mediated by P21 via the

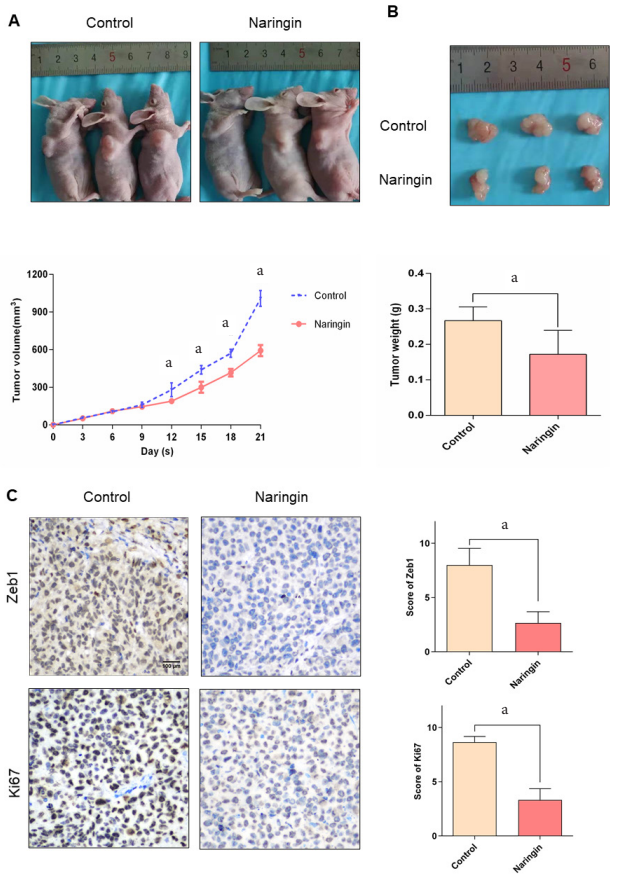
Figure 3. Naringin inhibits gastric cancer cell migration and invasion and inhibits the EMT process via the PI3K/AKT/Zeb1 axis. (**3A**) Scratches of gastric cancer cell lines MGC803 and MKN45 at different time points after naringin IC50 treatment (100×). **(3B)** Representative pictures of gastric cancer cell lines MGC803 and MKN45 in transwell assay after naringin treatment for 24 hours (100×) at the concentration of 39.54 μM and 34.37 μM, respectively. (**3C**) Naringin inhibits invasion and migration of gastric cancer cell lines by inhibiting Zeb1 expression by downregulating the PI3K-AKT pathway.



[a]$P < .05$;
[b]$P < .01$. Three independent experiments were carried out.

Abbreviations: EMT, epithelial mesenchymal transition; IC50, half maximal inhibitory concentration.

Figure 4. Naringin inhibits the growth of tumor cells *in vivo*. **(4A)** Photograph of BALB/C nude mice in the control and naringin groups, and the tumor growth curve in the 2 groups. **(4B)** Photograph of tumor dissected from BALB/C nude mice treated with and without naringin, and comparison of tumor weight in the 2 groups. **(4C)** Expression of Zeb1 and Ki67 in tumor tissue and the immunohistochemical score in the 2 groups (200×); 2 independent pathologists blinded to the experimental design performed this process.



[a]$P < .05$. Three independent experiments were carried out.

β-catenin pathway. Studies by Suat Erdogan, et al[21] also shown that naringin treatment could lead to upregulation of p53 in prostate cancer. Our study showed that naringin inhibits gastric cancer cells by inducing arrest of G0/G1 cells.

This study also demonstrated that naringin can induce gastric cancer cell apoptosis, which we confirmed via flow cytometry. To better understand the possible mechanism, we evaluated MTP and apoptosis-relative proteins, such as Caspase3, Caspase9, Bax, and Bcl2. The results showed that MTP was lost after treatment with naringin and the expression level of apoptosis-related protein cleaved-Caspase3 and Bax increased and Caspase3 and Bcl2 decreased.

As a highly malignant digestive tract tumor, the high invasion and migration ability is one of the main factors affecting prognosis in gastric cancer.[22] In this study, we verified that naringin treatment could significantly reduce the invasion and migration capacity of gastric cancer cells MGC803 and MKN45 and reduce the EMT process by cell scratch and transwell assay.

In the process of tumor progression, tumor cells will lose epithelial polarity and adhesion, lose some epithelial phenotypes and obtain some characteristics of mesothelial cells, which will improve the ability of tumor cells to invade and metastasize via the epithelial-mesenchymal transformation (EMT).[23,24] During EMT, a series of molecular changes occurs, including decreased expression of epithelial phenotype-related molecule E-cadherin and increased expression of mesenchymal phenotype-related molecule Vimentin. Some molecules can directly regulate the occurrence of EMT, such as Zeb1, etc. In the EMT process, the PI3K/AKT signal pathway also plays a direct or indirect role.[25] After naringin treatment in gastric cancer, E-cadherin increased and Zeb1, Vimentin, P-PI3K and P-AKT were decreased.

In this study, we first found that naringin can inhibit scratch healing and invasion and migration of gastric cancer cells MGC803 and MKN45, suggesting that naringin could affect the metastatic ability of gastric cancer cells, as seen in previous studies.[26-28] Further research showed that naringin could negatively regulate Zeb1expression and inhibit the P-AKT level, suggesting that naringin could further reduce the expression of Zeb1 by inhibiting the PI3K/AKT pathway to inhibit EMT, which was consistent with the research of Raha, et al.[19]

CONCLUSIONS

Our study explored the effects of naringin, an active ingredient of TCM, on the proliferation, apoptosis, invasion and migration of gastric cancer cells and illustrated its possible mechanism. This provided a theoretical basis for the possibility that naringin might be selected as one of the natural compounds for tumor treatment. It is believed that with a deepening of people's understanding of the molecular mechanism of tumors and the improvement in drug preparation methods, the role of naringin in the anti-tumor effect in TCM can be observed.

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CONFLICT OF INTEREST

None.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The experimental protocol was established according to the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University, China.

AUTHOR CONTRIBUTIONS

Funding acquisition: Xingjie Wang and Xuejun Sun; Investigation: Wei Zhao and Lin Zhu; Methodology: Jing Shi; Project administration: Xingjie Wang, Xuejun Sun and Wei Zhao; Resources: All authors; Software: Mingchao Mu and Chenye Zhao; Supervision: Xuejun Sun and Wei Zhao; Validation: All authors; Visualization: Zilu chen, Chao Qu, and Changchun Ye; Roles/Writing – original draft: Lin Zhu; Writing – review & editing: All authors.

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