Magnolol inhibits growth and induces apoptosis in esophageal cancer KYSE-150 cell lines via the MAP kinase pathway

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**Background:** Magnolol has shown anti-cancer activity against a variety of cancers, such as liver, breast, lung and colon cancer. However, the role of magnolol in esophageal cancer cells is unknown.

**Methods:** In this study, esophageal cancer cell lines including TE-1, Eca-109 and KYSE-150 were used to evaluate the cytotoxic effect of magnolol on cell proliferation, apoptosis and migration.

**Results:** We found that magnolol inhibits cellular proliferation of all three cell lines in a time- and dose-dependent manner, 20 μM magnolol markedly inhibited the migration ability of KYSE-150 cell which was accompanied with a decreased expression of MMP-2. Treatment with 100 μM magnolol significantly increased KYSE-150 cell apoptosis. We found that cleaved caspase-3, cleaved capsese-9 and Bax protein expression was increased and Bcl-2 protein expression was decreased after magnolol treatment. In addition, Magnolol had no effect on JNK but induced the phosphorylation of p38 and ERK1/2 in a concentration-dependent manner, suggesting the involvement of these kinases in the initiation of the apoptosis process. Finally, magnolol treatment significantly suppressed KYSE-150 tumor cell growth in nude mouse xenograft models.

**Conclusions:** The results of this study provide a basis for the understanding and development of magnolol as a potential novel drug for esophageal cancer.

**Keywords:** Esophageus cancer; natural compound; magnolol; apoptosis

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**Introduction**

Esophageal cancer is an extremely aggressive malignant tumor with a high clinical incidence. It ranks ninth in terms of incidence and sixth for mortality worldwide (1-3). It affects more than 400,000 people worldwide every year and the incidence rates are rapidly increasing, especially in China (4). Pickled vegetables, tobacco and alcohol are the most common carcinogenic environmental factors that have contributed to the increased incidence of esophageal cancers (5). The morbidity of patients with esophageal cancer in China accounts for over 40% of patients worldwide. It has become a severe threat to people’s health and a heavy financial burden to society in general (6). The therapeutic strategy for patients with early-stage esophageal cancer is surgery as the first choice. It can play a role in improving the patient’s prognosis. However, the 5-year survival rate for patients with esophageal cancer is only around 20% (7). Most patients are at the advanced stage of the disease when they are diagnosed. Different therapeutic interventions are available, such as chemo- and
radiation therapy. However, these treatment strategies are often inadequate or associated with severe side effects. The alternate treatment options that have gained prominence are phytochemicals, i.e., natural compounds that have fewer side effects, higher specificity and cost-effectiveness.

Medicinal herbs have been used to treat a variety of diseases and have a long history of demonstrated therapeutic benefit. In recent years, there has been a worldwide trend in research of natural compounds present in herbs, vegetables and fruits for their anti-oxidant and anti-cancer properties (8-12). It has been reported that natural compounds have a unique advantage for the treatment of esophageal cancer. They have been shown to enhance immunity, inhibit the growth of cancer cells and reduce cancer relapses and metastases (13,14). Furthermore, natural compounds have been shown to improve the therapeutic efficacy of chemo- and radiotherapy, and reduce their side effects (15). Luteolin, a natural plant flavonoid, obtained from Chrysanthemum morifolium, have been shown to exert an anti-proliferative effect on esophageal cancer cells and induce apoptosis though the mitochondrial pathway (16,17). Berberine has been shown to have potent anti-cancer effects on a variety of cancer cell lines. Berberine has been shown to inhibit the migration and metastasis of esophageal cancer cells (18) and exert anti-proliferative effects by interfering with the mTOR pathway (19). The natural compound, curcumin, has been demonstrated to enhance cancer cell chemosensitivity (20,21), increase cleaved caspase-3 activity, and activate the notch signaling pathway (22).

Magnolol (5,5′-diallyl-2,2′-dihydroxybiphenyl), extracted from a well-known traditional Chinese medicine, Magnolia officinalis, possesses a variety of pharmacological properties, especially anti-cancer properties. Magnolol inhibits the proliferation of human lung squamous carcinoma CH27 cells at low concentrations and induces apoptosis at high concentrations (23). Magnolol inhibits the proliferation, migration, and invasion of hepatocellular carcinoma HepG2 cells in vitro in a dose-dependent manner. In addition, magnolol has been shown to reduce HCC tumor volume and weight in mouse xenograft tumor models (24), and significantly inhibit angiogenesis in vitro and in vivo evidenced by the attenuation of hypoxia and vascular endothelial growth factor (VEGF)-induced tube formation in human bladder cancer cells (25). These findings have led us to investigate the mechanism by which magnolol exerts its anti-cancer activity in esophageal cancer cells.

Methods

Reagents and cell culture

Magnolol (>98% of purity) was purchased from Sigma-Aldrich Co., Ltd. Stock solutions of magnolol were prepared at 100 mM in dimethyl sulfoxide (DMSO) and stored at -80°C. Antibodies for cleaved caspase-3, cleaved -caspase-9, Bcl-2, Bax, JNK, and p-JNK were purchased from Abcam Technology, Inc. Antibodies for GAPDH, cleaved caspase-8, mmg-2, p38, and p-p38 were purchased from Cell Signaling Technology. Anti-ERK and P-ERK were purchased from Santa Cruz Biotechnology. FITC-Annexin V/propidium iodide (PI) apoptosis detection kits and Matrigel were purchased from BD Biosciences. The Caspase-Glo® 3/7 and Caspase-Glo® 9 Assay System were purchased from Promega Corporation. Human esophagus cancer cell lines (TE-1, Eca-109 and KYSE-150) were purchased from the Institute of Biochemistry and Cell Biology (Shanghai Institutes for Biological Sciences, CAS). Cells were maintained in RPMI-1640 supplemented with 10% FBS in a humidified incubator with 5% CO2 at 37 °C.

Cell viability assays

Cell viability treated with different Magnolol concentrations were measured using the CCK-8 kit. Cells were cultured in 96-well plates (1×10⁴/well), and then treated with different concentrations (0, 20, 50, 100 and 150 μM) of magnolol when the cells reached 70–80% confluence. After 24 h or 48 h of incubation, the media was removed and 100 μL CCK-8 buffer was added per well and incubated for an additional 4 h. Absorbance of each well was measured at 450 nm.

Apoptosis analysis

Apoptosis was measured using flow cytometry. FITC-Annexin V/PI detection kit was used to quantify the percentage of cells in different stages of apoptosis. KYSE-150 cells were seeded into 6-well plates, and then treated with PBS (control) or magnolol (20 and 100 μM) for 48 h. Then, 1×10⁵ cells were re-suspended in 100 μL 1x binding buffer. After addition of FITC-Annexin V and PI, the cell suspension was incubated for 15 min in the dark. Subsequently, 400 μL 1x binding buffer was added to the cells for flow cytometry analysis.
Caspase-3 and caspase-9 activity assay

KYSE-150 cells were seeded into 96-well plates at 1×10⁴ cells per well and cultured in complete medium overnight. Cells were then treated with magnolol (0, 20, 50, 100 and 150 μM). Caspase-3 and caspase-9 activity was then measured by adding 50 μL Caspase-Glo® 3/7 or Caspase-Glo® 9 to each well. After a 2 h incubation at 37 °C, luminescence was measured.

Transwell migration assay

Cells were treated with DMSO or 20 μM magnolol for 24 h, and then 1×10⁵ cells were loaded onto a migration chamber. Media containing 10% FBS was placed in the lower chamber. After 12 hours, the cells that had migrated through the membrane were stained using crystal violet. The number of cells that migrated were quantitated using a fluorescence microscope.

Western blotting

Cells were treated with magnolol for 48 h and lysed in RIPA buffer as previously described (26). Lysates were mixed with sample loading buffer and heated to 100 °C for 10 min. After proteins were separated by SDS-PAGE, the samples were transferred to PVDF membranes. Membranes were then blocked with 5% skim milk in TBST and then incubated with the specified primary antibodies overnight at 4 °C. Membranes were then incubated with secondary antibodies for 2 h at room temperature. Protein bands were visualized using enhanced chemiluminescent detection reagent.

In vivo antitumor activity

KYSE-150 cells (1×10⁶) were subcutaneously injected on the right side of the dorsal area of 4-week old female nude mice. Tumor growth was observed daily. Mice bearing tumors were selected and divided into two groups (n=5 each): control group and magnolol (30 mg/kg) group. Magnolol was injected intraperitoneally every other day. Tumor size was measured using calipers, and the tumor volume was calculated using the following formulae; V = 1/2 × (length × width²). All animal procedures were approved by the Institutional Animal Care Committee of Shanghai Changzheng Hospital.

Statistical analysis

Data was expressed as mean ± SEM. Statistical significance was determined using the Student’s t-test or ANOVA analysis. P value of less than 0.05 was considered statistically significant. For all graphs: *P<0.05, **P<0.01, ***P<0.001. All calculations were performed using the SPSS 22.0 software.

Results

Magnolol inhibits the proliferation of TE-1, Eca-109 and KYSE-150 cells

CCK-8 cell proliferation assay was used to determine the effect of magnolol on esophagus cancer cell proliferation. TE-1, Eca-109 and KYSE-150 cells were treated with different concentrations of magnolol (0, 20, 50, 100 and 150 μM) for 24 h. Magnolol inhibited the proliferation of all three esophagus cancer cell lines in a dose-dependent manner, and had the greatest toxicity on KYSE-150 cells (Figure 1A). To determine whether the inhibitory effect of magnolol on proliferation was time-dependent, we tested the effect using similar concentrations of magnolol for 48 h. The inhibition of proliferation was similar with the 24 h treatment. We observed that the KYSE-150 cell line was the most sensitive to magnolol (Figure 1B), and was
Magnolol induces apoptosis in KYSE-150 cell lines

We further investigated the effect of magnolol on esophagus cancer cell apoptosis using Annexin V-FITC/PI staining. As shown in Figure 2A, a dose-dependent increase in apoptotic cells were observed in magnolol-treated KYSE-150 cells. 20 μM magnolol showed no obvious effect on apoptosis, while 100 μM magnolol treatment significantly increased apoptosis of KYSE-150 cells, P<0.001 (Figure 2B).

Effect of magnolol on KYSE-150 cell migration

Transwell migration assays were used to measure tumor cell migration. Cells were treated with low dose (20 μM) of magnolol for 24 h, and then transferred (1×10⁵ cells) onto migration chambers. Magnolol treatment significantly reduced cell migration. As shown in Figure 3, 20 μM magnolol markedly inhibited the migration of KYSE-150 cells. Western blot analysis demonstrated that magnolol treatment reduced MMP-2 expression in KYSE-150 cells in a dose-dependent manner. The expression of matrix metalloproteinases (MMPs) family of proteins was closely associated with the migration ability of tumor cells. Magnolol treatment reduced the expression of MMP-2 to affect the migration ability of KYSE-150 cells.

Magnolol activates caspase-3 and caspase-9 in KYSE-150 cells

Caspase-3 and caspase-9 activity was measured using the Caspase-Glo® 3/7 and Caspase-Glo® 9 assay systems. As shown in Figure 4, after 24 h treatment, low-dose magnolol had no obvious effect on the activation of both caspase-3 and caspase-9. However, after a high-dose of magnolol...
(150 μM) treatment, caspase-9 activity was increased by 2.2-fold and caspase-3 activity was increased by 1.6-fold. After 48 hours, low-dose magnolol (50 μM) could significantly activate both caspase-3 and caspase-9. These results indicate that high dose (150 μM) or 48 hours of low-dose (50 μM) magnolol treatment could induce the activation of the caspase pathway. Activation of the caspase pathway is the major mechanism of cell apoptosis induced by magnolol on KYSE-150 cells.

**Magnolol regulates the MAP Kinase pathway**

We evaluated the expression of apoptosis-related proteins (cleaved caspase-3, cleaved caspase-8, cleaved caspase-9, Bcl-2, Bax). The results of Western blot analysis showed that magnolol treatment reduced Bcl-2 expression and increased the expression of Bax in KYSE-150 cells in a dose-dependent manner. Furthermore, 50 μM magnolol treatment induced the activation of cleaved caspase-3 and cleaved caspase-9 in KYSE-150 cells while cleaved caspase-8 showed no significant change (Figure 5).

MAPK signaling pathway plays an important role in the regulation of cell apoptosis. To determine whether MAPKs are involved in magnolol-induced apoptosis, we measured the phosphorylation of p38, ERK and JNK by western blot analysis. 50 and 100 μM magnolol treatment increased the phosphorylated form of ERK and p38 as shown in Figure 6. However, phosphorylated JNK was decreased after 25 and 50 μM magnolol treatment. These results indicated that ERK and p38 may play an important role in magnolol induced apoptosis.

**Magnolol inhibits the growth of KYSE-150 tumor xenografts**

To evaluate the tumor-suppressing effect of magnolol in vivo, a xenograft tumor model using KYSE-150 cells in nude mice was established. KYSE-150 cells (1×10⁶) were subcutaneously injected into 4-week old female nude mice. The effect of magnolol (30 mg/kg) on tumor xenografts was then examined (Figure 7). Magnolol treatment significantly reduced tumor size compared to the controls. Intraperitoneal administration of magnolol (30 mg/kg) reduced tumor volumes by over 50%. These results demonstrated that magnolol treatment effectively reduced growth of KYSE-150 xenografts in vivo.
Esophagus cancer is categorized into esophagus squamous cell carcinoma (ESCC) and esophagus adenocarcinoma (EADC). Both subtypes have a high incidence, especially in Eastern Asia, Western America and Oceania (27). Smoking, alcohol and nutrient deficiency are the main risk factors for ESCC, and are commonly observed in developing countries. However, the incidence of EADC are generally observed in developed countries. The risk factors for EADC are age, smoking, obesity and a diet deficient in vegetables (28,29). Overall, the number of ESCC patients are much higher compared to the number of EADC patients worldwide (30).

Regardless of the type of esophagus cancer, patients have a poor prognosis and a low quality of life. With the advances in medicine, there are several treatment strategies for esophagus cancer such as surgical resection, chemotherapy, and radiation therapy. However, these treatment methods are inadequate due to their serious side effects, lack of efficacy and tumor recurrence. The development of alternative and safer therapeutic options is vital. Natural compounds are safe, inexpensive, have good efficacy and fewer side effects and are gradually gaining interest and acceptance in the medical community.

Magnolol, a natural compound isolated from the root of magnolia officinalis, has been reported to have anti-oxidative, anti-cancer, anti-inflammatory, and anti-bacterial effects (31-34). However, the effect and mechanism underlying the anti-cancer activity of magnolol for esophageal cancer remains to be deciphered. In this study, we investigated the efficacy of magnolol treatment in human esophagus cancer KYSE-150 cells both in vitro and in vivo. We showed that cellular proliferation decreased significantly after magnolol treatment in a dose- and time-dependent manner.

Caspases are a family of cysteine proteases that play a critical role in apoptosis. When a cell receives an apoptotic signal, cytochrome c interacts with Apaf-1 and procaspase-9 to activate caspase-9. This in turn cleaves and activates caspase-3 and other caspases (35). The cleaved caspase-3 serves as a convergence signal for cell apoptosis. In this study, we found that Magnolol activated caspase-9 and capase-3, and significantly increased their protein expression. However, the levels of cleaved caspase-8 were unchanged.

The Bcl-2 family is composed of multiple members that play an important role in the regulation of apoptosis. We found that magnolol induced the up-regulation of Bax, cleaved caspase-3 and -9 expression.
and down-regulated Bcl-2. This is consistent with several previous studies demonstrating the regulation of Bcl-2 family proteins are involved in the apoptosis process (36-38).

The MAPK pathway consists of the p38, ERK and JNK pathway. Our data demonstrated that magnolol induced apoptosis in parallel with the activation of p-ERK and p-38, and the inactivation of p-JNK in esophagus cancer KYSE-150 cells. In xenograft tumor nude mouse models, in vivo treatment with magnolol significantly suppressed tumor growth. Treatment with 30mg/kg of Magnolol reduced tumor volume by over 50%.

Although magnolol had the inhibitory effect on KYSE-150 cell lines, we are unclear on the specific molecular mechanisms of this inhibitory effect. How magnolol exerts its apoptosis function through the MAPK pathway is yet to be deciphered. Magnolol activated both ERK and p38, while it decreased the phosphorylation of JNK. A MAPK pathway specific inhibitor should be used to investigate how magnolol exerts its apoptotic effect.

**Conclusions**

In summary, our data demonstrated that magnolol inhibited the growth of KYSE-150 cells both in vitro and in vivo by increasing apoptosis. These findings suggest that magnolol may have novel therapeutic benefits for the treatment of esophageal cancer.

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None.

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**Footnote**

**Conflicts of Interest:** The authors have no conflicts of interest to declare.

**Ethical Statement:** The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All animal procedures were approved by the Institutional Animal Care Committee of Shanghai Changzheng Hospital.

**References**


