

# Psoralidin inhibits proliferation and enhances apoptosis of human esophageal carcinoma cells via NF- $\kappa$ B and PI3K/Akt signaling pathways

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**Abstract.** Esophageal cancer is the most common gastrointestinal cancer. Psoralidin exhibits antioxidant, anti-apoptotic, anti-inflammatory and antitumor effects, which result in the inhibition of cancer formation. The present study aimed to investigate the effect of psoralidin on esophageal carcinoma proliferation and growth, and to elucidate its underlying mechanism of action. The effect of psoralidin on cell proliferation was investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Using an annexin V-fluorescein isothiocyanate/propidium iodide apoptosis detection kit and 4',6-diamidino-2-phenylindole staining assay, the present study demonstrated that psoralidin significantly enhanced apoptosis of human esophageal carcinoma Eca9706 cells. In addition, caspase-3 activity was analyzed with a caspase-3 colorimetric assay kit, while nuclear factor (NF)- $\kappa$ B activity and protein phosphatidylinositol 3-kinase (PI3K)/Akt expression were measured with an NF- $\kappa$ B enzyme-linked immunosorbent assay kit and western blot analysis, respectively. Eca9706 cells were treated with a PI3K agonist in order to investigate the mechanism of action of psoralidin. It was observed that psoralidin was able to decrease the proliferation and promote the cellular apoptosis of Eca9706 cells in a dose-dependent manner. Furthermore, psoralidin was also able to inhibit the caspase-3 activity of Eca9706 cells in a dose-dependent manner. In addition, psoralidin inhibited NF- $\kappa$ B activity and reduced PI3K and Akt protein expression in Eca9706 cells. Notably, the PI3K agonist was able to reverse the effect of psoralidin on Eca9706 cells. The results of the present study

demonstrated that psoralidin was able to inhibit proliferation and enhance apoptosis of human esophageal carcinoma cells via the NF- $\kappa$ B and PI3K/Akt signaling pathways.

## Introduction

Esophageal cancer is the most common gastrointestinal cancer, and is ranked sixth in terms of worldwide cancer-associated mortality rate (1). According to the World Health Organization histological classification, esophageal cancer includes squamous cell carcinoma (SCC) and adenocarcinoma of the esophagus, which account for 90 and 5% of cases, respectively (2). The remaining 5% of cases are rare types of esophageal cancer, including epidermal mucinous carcinoma, small cell carcinoma, leiomyosarcoma and others (3). In the past recent years, the incidence of esophageal adenocarcinoma in the United States and Europe has increased significantly (4). The majority of esophageal cancer cases are SCC, which is ranked fourth in terms of cancer-associated mortality, and the current average annual incidence rate of SCC is 17/100,000 patients (5,6). The male incidence rate of SCC is generally higher, compared with that of females (7). However, there are large differences between different regions, and China is known to be an 'esophageal cancer-prone region' (8).

As an important nuclear transcription factor, nuclear factor (NF)- $\kappa$ B is able to mediate environmental stimuli, and exerts numerous biological functions via control of gene transcription, including regulation of cell proliferation and apoptosis-mediated immunity, inflammation and tumor formation (9). Han *et al* (10) indicated that neuregulin 1 was able to promote the progression of gastric cancer via NF- $\kappa$ B inactivation. In addition, Dai *et al* (11) demonstrated that Golgi phosphoprotein 3 was able to promote hepatocellular carcinoma cell aggressiveness via the NF- $\kappa$ B signaling pathway. Wang *et al* (12) reported that andrographolide was able to induce apoptosis of esophageal cancer cells via suppression of the NF- $\kappa$ B signaling pathway.

Abnormal apoptosis is directly associated with the occurrence and development of a number of diseases, including cancer, viral diseases and a variety of degenerative diseases (13). Previous studies have revealed that constitutively activated phosphatidylinositol 3-kinase (PI3K)/Akt signaling

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may lead to the development of disorders classified within the human tumor spectrum, including ovarian, breast, endometrial and nasopharyngeal cancer, as well as glioblastoma, medulloblastoma and myeloproliferative abnormal syndrome (14). Furthermore, Wang *et al.* (15) reported that hypomethylation of the catalytic subunit alpha of PI3K has a significant role in the activation of the PI3K/Akt signaling pathway in esophageal cancer. Li *et al.* (16) demonstrated that inhibitor of DNA binding 1, dominant negative helix-loop-helix protein promoted metastasis of human esophageal cancer cells via activation of the PI3K/Akt signaling pathway.

Previous studies have identified that psoralidin contains a variety of compounds, including coumarin, flavonoids, monoterpenes and phenols, which may have immunomodulatory, anti-inflammatory, antioxidant and antitumor effects (17-19). Yang *et al.* (17) reported that psoralidin inhibited the proliferation of androgen-independent prostate cancer cells via PI3K-mediated Akt signaling. Furthermore, Hao *et al.* (20) reported that psoralidin was able to inhibit the proliferation of human lung cancer A549 cells. The present study aimed to provide the first evidence of the anticancer effect of psoralidin on esophageal cancer, and render mechanistic insights into the antitumor action of this compound against human esophageal carcinoma Eca9706 cells.

## Materials and methods

**Reagents.** Psoralidin (purity,  $\geq 98\%$ ), the chemical structure of which is shown in Fig. 1, was purchased from Sigma-Aldrich (St. Louis, MO, USA). 4',6-diamidino-2-phenylindole (DAPI) was also obtained from Sigma-Aldrich. RPMI-1640 was obtained from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). Fetal bovine serum (FBS) was obtained from HyClone (GE Healthcare Life Sciences, Logan, UT, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit was obtained from BestBio Biotechnology Co., Ltd. (Shanghai, China). Caspase-3 colorimetric assay kit and NF- $\kappa$ B enzyme-linked immunosorbent assay (ELISA) kit were acquired from Beyotime Institute of Biotechnology (Nanjing, China).

**Cell culture.** The Eca9706 human esophageal carcinoma cell line was acquired from the Department of Oncology of Jingzhou Central Hospital (Jingzhou, China). Cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS, 100 U/ml penicillin (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 100 mg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.), at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. The culture medium was replaced every 2-3 days, and fresh complete medium was added to cells.

**MTT assay.** The viability of Eca9706 cells (2.0x10<sup>4</sup> cells/well) was investigated following incubation with psoralidin (0, 5, 10 and 20  $\mu$ M). Cells were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> for 0, 1, 2 and 3 days in 96-well plates (Thermo Fisher Scientific). Eca9706 cells were washed twice using phosphate-buffered saline (PBS; Sangon Biotech Co.,

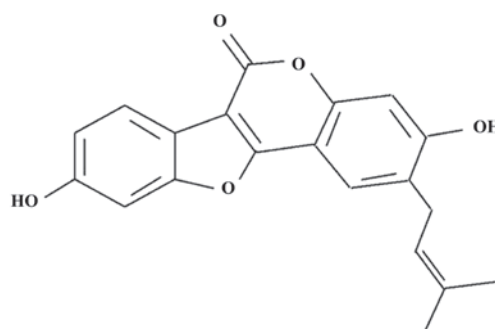


Figure 1. Chemical structure of psoralidin.

Ltd.), and 10  $\mu$ l MTT was added to each well. Subsequently, Eca9706 cells were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> for 4 h. Following incubation, the culture medium was removed, and 150  $\mu$ l dimethyl sulfoxide (Invitrogen; Thermo Fisher Scientific, Inc.) was added into each well. Eca9706 cells were incubated for 20 min at room temperature. The absorbance of Eca9706 cells at 570 nm (iMark microplate reader; Bio-Rad Laboratories, Inc., Hercules, CA, USA) was determined by MTT assay, as previously described (21).

**Flow cytometric detection of cellular apoptosis.** Eca9706 cells (2x10<sup>6</sup> cells/well) were cultured in 6-well plates (Thermo Fisher Scientific) with psoralidin (0, 5, 10 and 20  $\mu$ M) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> for 2 days. Following incubation, Eca9706 cells were collected and washed twice with cold PBS. Subsequently, annexin V binding buffer was used to resuspend Eca9706 cells (1x10<sup>6</sup> cells/ml) in a test tube. A total of 10  $\mu$ l annexin V-FITC was added to the resuspended Eca9706 cells, and incubated for 30 min in the dark. Following incubation, 5  $\mu$ l PI was added to the resuspended Eca9706 cells, and incubated for 10 min in the dark. Apoptosis of Eca9706 cells was immediately measured using flow cytometry (COULTER® EPICS® ALTRA™ Flow Cytometer; Beckman Coulter, Inc., Brea, CA, USA).

**DAPI staining assay.** Eca9706 cells (2x10<sup>6</sup> cells/well) were cultured in 6-well plates with psoralidin (0, 5, 10 and 20  $\mu$ M), at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> for 2 days. PBS was used to wash the cells, prior to the addition of 0.5 ml/well 4% paraformaldehyde (Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China). Cells were fixed for 30 min at 4°C, washed twice with PBS, and incubated for 5 min at 4°C in the presence of sodium citrate (0.1%; Xinfan Biological Technology Co., Ltd., Shanghai, China) containing 0.1% Triton X-100 (Biosharp, St. Louis, MO, USA). DAPI (5  $\mu$ g/ml) was added to each well, and incubated for 10-15 min at 4°C in the dark. Eca9706 cells were observed and photographed under a fluorescence microscope (Axio Observer A1; Zeiss AG, Oberkochen, Germany) at excitation/emission  $\sim 340/450$  nm.

**Detection of caspase-3 activity.** Eca9706 cells (2x10<sup>6</sup> cells/well) were cultured in 6-well plates with psoralidin (0, 5, 10 and 20  $\mu$ M) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> for 2 days. Caspase-3 activity was detected at a wavelength of 405/650 nm (excitation/emission) using a caspase-3 colorimetric assay kit, according to the manufacturer's protocol.

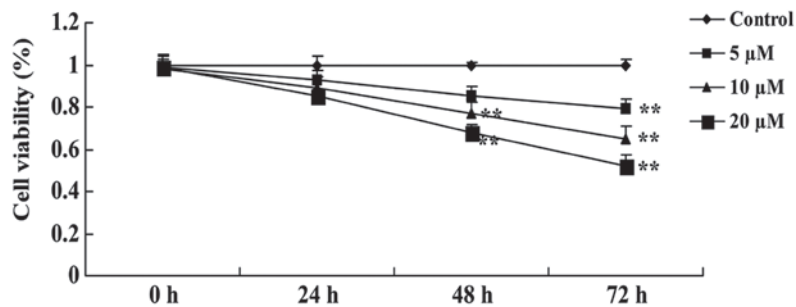


Figure 2. Psoralidin inhibits the viability of Eca9706 cells. Data are expressed as the mean  $\pm$  standard deviation of  $\geq 3$  independent experiments. \*\* $P < 0.01$  vs. control group.

**Measurement of NF- $\kappa$ B activity.** Eca9706 cells ( $2 \times 10^6$  cells/well) were cultured in 6-well plates with psoralidin (0, 5, 10 and 20  $\mu$ M) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> for 2 days. NF- $\kappa$ B activity was analyzed using an ELISA kit, according to the manufacturer's protocol.

**Western blot analysis.** Eca9706 cells ( $2 \times 10^6$  cells/well) were cultured in 6-well plates with psoralidin (0, 5, 10 and 20  $\mu$ M) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> for 2 days. Subsequently, Eca9706 cells were incubated on ice for 30 min with ice-cold lysis buffer (Biosharp), and centrifuged at 12,000  $\times$  g (Biosharp) for 10 min at 4°C. The supernatant was collected in order to determine the total protein concentration using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.). Equivalent amounts of total protein were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tiandz, Inc., Beijing, China) and subsequently transferred onto a polyvinylidene difluoride membrane (pore size, 0.22  $\mu$ m; Roche Diagnostics GmbH, Mannheim, Germany). The membrane was blocked with Tris-buffered saline (TBS; Tiandz, Inc.) containing 5% skimmed milk for 2 h at room temperature, and subsequently incubated overnight at 4°C with goat polyclonal anti-PI3K (catalog no., sc-48637; dilution, 1:2,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), mouse monoclonal anti-Akt (catalog no., sc-293125; dilution, 1:2,500; Santa Cruz Biotechnology, Inc.) or mouse monoclonal anti- $\beta$ -actin (catalog no., sc-8432; dilution, 1:500; Santa Cruz Biotechnology, Inc.; control) antibodies. The membrane was washed twice using TBS and 0.05% Tween 20 (Biosharp) for 2 h, and subsequently incubated at room temperature for 2 h with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G (catalog no., sc-358922; dilution, 1:1,000; Santa Cruz Biotechnology, Inc.). The specific protein bands were detected using enhanced chemiluminescence (Beyotime Institute of Biotechnology), FluorChem™ (ProteinSimple, Santa Clara, CA, USA) and AlphaEaseFC™ software (Alpha Innotech, San Leandro, CA, USA).

**Statistical analysis.** Statistical analysis was performed with SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as the mean  $\pm$  standard deviation of  $\geq 3$  independent experiments. Data was analyzed using the Student's t-test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Psoralidin inhibits the viability of Eca9706 cells.** In order to clarify the anticancer effect of psoralidin (0, 5, 10 and 20  $\mu$ M) on the viability of Eca9706 cells, cell viability was evaluated using an MTT assay. Treatment with psoralidin (5, 10 and 20  $\mu$ M) affected the viability of Eca9706 cells in a dose- and time-dependent manner. Notably, the anticancer effect of psoralidin at 10 and 20  $\mu$ M significantly inhibited the proliferation of Eca9706 cells at 48 and 72 h (Fig. 2). Therefore, 48-h exposure to 10  $\mu$ M psoralidin was selected as the standard pretreatment in subsequent experiments.

**Psoralidin induces apoptosis of Eca9706 cells.** In order to reveal the anticancer effect of psoralidin (0, 5, 10 and 20  $\mu$ M) on the apoptosis of Eca9706 cells, the apoptotic rate of Eca9706 cells exposed to psoralidin was analyzed with flow cytometry and DAPI staining assay. Treatment with psoralidin (5, 10 and 20  $\mu$ M) induced apoptosis of Eca9706 cells in a dose-dependent manner. Notably, the apoptotic rate of Eca9706 cells was significantly increased due to the anticancer effect of psoralidin (10 and 20  $\mu$ M) following 48 h of incubation (Fig. 3A). Furthermore, apoptosis of Eca9706 cells was augmented in psoralidin-treated (5, 10 and 20  $\mu$ M) cells, compared with the control group (Fig. 3B).

**Psoralidin induces caspase-3 activity in Eca9706 cells.** In order to investigate the anticancer effect of psoralidin (0, 5, 10 and 20  $\mu$ M) on the caspase-3 activity of Eca9706 cells, caspase-3 activity was measured using a colorimetric assay kit. Treatment with psoralidin (5, 10 and 20  $\mu$ M) induced caspase-3 activity in a dose-dependent manner. Notably, following treatment with psoralidin (10 and 20  $\mu$ M) for 48 h, caspase-3 activity was significantly augmented (Fig. 4).

**Psoralidin inhibits NF- $\kappa$ B activity in Eca9706 cells.** In order to investigate the effect on NF- $\kappa$ B activity caused by treatment with psoralidin (0, 5, 10 and 20  $\mu$ M) in Eca9706 cells, the activity of the p65 subunit of NF- $\kappa$ B was investigated using an ELISA kit. Treatment with psoralidin (5, 10 and 20  $\mu$ M) inhibited the activity of NF- $\kappa$ B in Eca9706 cells (Fig. 5).

**Psoralidin inhibits PI3K and Akt protein expression in Eca9706 cells.** In order to determine the effect of psoralidin (0, 5, 10 and 20  $\mu$ M) on the PI3K/Akt signaling pathway in

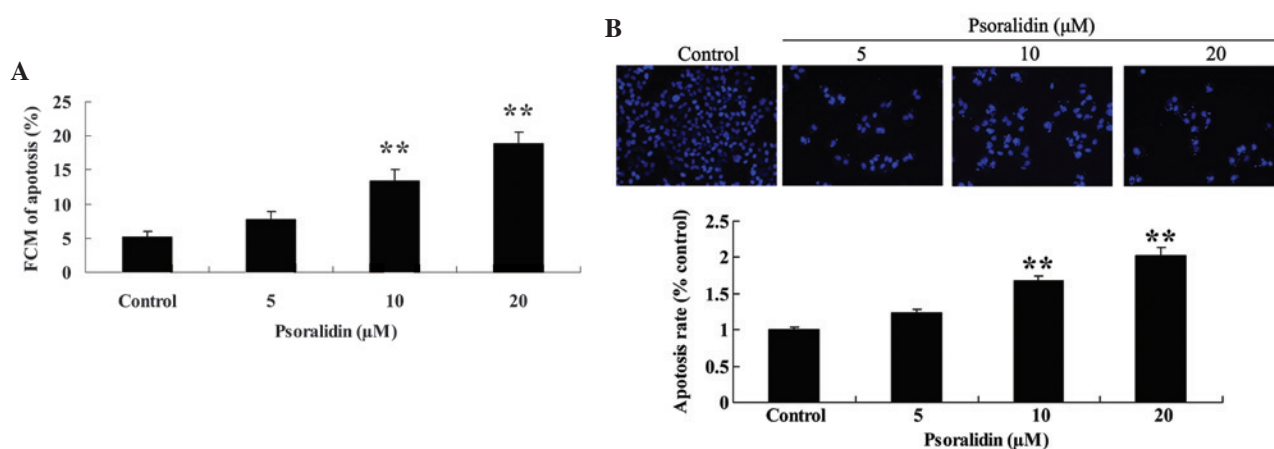


Figure 3. Psoralidin induces apoptosis of Eca9706 cells. The effect of psoralidin treatment on apoptosis of Eca9706 cells was assessed by (A) flow cytometry and (B) 4',6-diamidino-2-phenylindole staining. Data are expressed as the mean  $\pm$  standard deviation of  $\geq 3$  independent experiments. Magnification,  $\times 200$ . \*\* $P < 0.01$  vs. control group. FCM, flow cytometry.

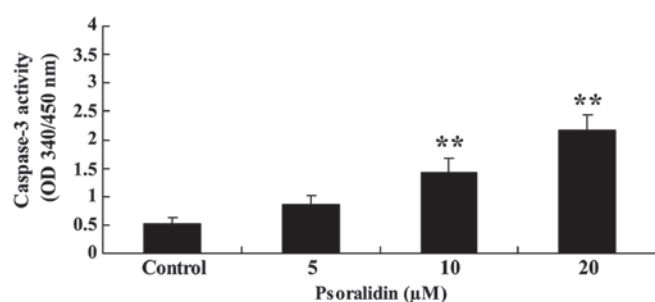


Figure 4. Psoralidin induces caspase-3 activity of Eca9706 cells. Data are expressed as the mean  $\pm$  standard deviation of  $\geq 3$  independent experiments. \*\* $P < 0.01$  vs. control group. OD 340/450 nm, excitation/emission wavelength.

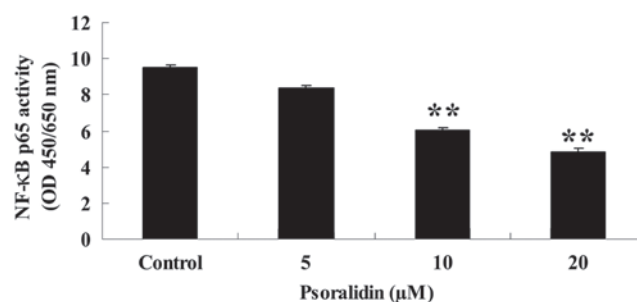


Figure 5. Psoralidin inhibits the activity of the p65 subunit of nuclear factor- $\kappa$ B in Eca9706 cells. Data are expressed as the mean  $\pm$  standard deviation of  $\geq 3$  independent experiments. \*\* $P < 0.01$  vs. control group. OD 450/650 nm, excitation/emission wavelength. NF- $\kappa$ B, nuclear factor- $\kappa$ B; OD, optical density.

Eca9706 cells, PI3K and Akt protein expression in Eca9706 cells was detected by western blot analysis. Treatment with psoralidin (5, 10 and 20  $\mu$ M) reduced the protein expression levels of PI3K and Akt in Eca9706 cells (Fig. 6).

*PI3K agonist is able to reverse the effect of psoralidin on Eca9706 cells.* In order to investigate the potential association between upregulation of PI3K protein expression and the effect of psoralidin on Eca9706 cells, Eca9706 cells were incubated with a PI3K agonist, namely insulin-like growth factor 1 (1  $\mu$ g/10  $\mu$ l) for 48 h. Notably, the PI3K agonist markedly increased PI3K protein expression in Eca9706 cells (Fig. 7A and B), and increased the viability of Eca9706 cells (Fig. 7C), compared with the psoralidin-treated (10  $\mu$ M) group.

## Discussion

Esophageal cancer is a common type of human cancer, and is ranked second and third in terms of cancer-associated mortality in men and women, respectively (22). The treatment of esophageal cancer may include surgery, radiation therapy and comprehensive treatment, among which, surgical treatment is preferred (23). However, only 1/4 of patients are able to tolerate radical surgery (24). Radiation therapy is a safe and effective method for the treatment of esophageal cancer (25). As cancer

possesses the characteristics of recurrence and metastasis, a review of the efficacy of radiation therapy for patients with esophageal cancer prior to discharge from hospital is important for consolidation and reduction of the recurrence rate (26). In the present study, the anticancer effect of psoralidin resulted in significant inhibition of cell proliferation and increased apoptosis of Eca9706 cells in a dose-dependent manner. Furthermore, psoralidin was additionally able to decrease the caspase-3 activity of Eca9706 cells in a dose-dependent manner. Hao *et al* (20) reported that psoralidin was able to inhibit the proliferation of A549 human lung cancer cells via generation of reactive oxygen species. Yang *et al* (17) reported that psoralidin was able to inhibit cell viability and induce apoptosis in human prostate cancer PC-3 and DU-145 cells. Das *et al* (27) suggested that psoralidin promoted growth arrest via activation of caspase-3 and caspase-9 in prostate cancer cells.

Loss of control of NF- $\kappa$ B activity is associated with the occurrence of mammalian tumors (28). Activation and abnormal expression of NF- $\kappa$ B is observed in numerous tumors (29). In esophageal cancer tissue, NF- $\kappa$ B gene amplification is common in the form of multiple internal and external factors acting on the body, which encode proteins that are expressed in the cytoplasm and nucleus (30). A number of intracellular signal transduction pathways are activated, and

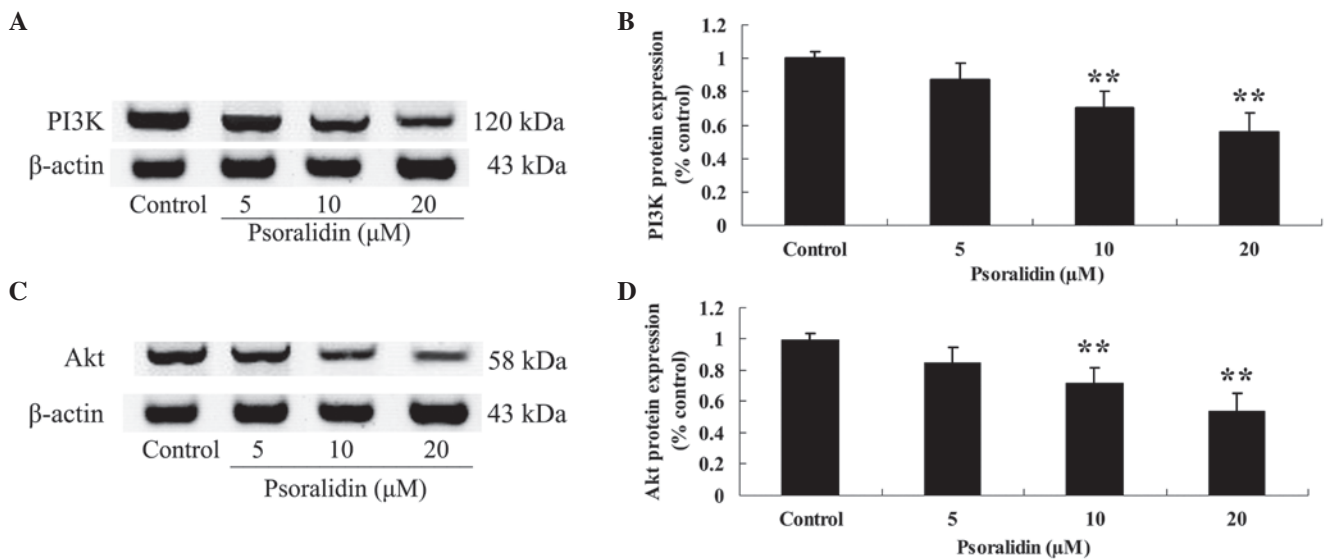


Figure 6. Effect of psoralidin treatment on PI3K and Akt protein expression in Eca9706 cells. (A) The effect of psoralidin on PI3K protein expression in Eca9706 cells was assessed by western blotting. (B) Statistical analysis of PI3K protein expression in Eca9706 cells. (C) The effect of psoralidin on Akt protein expression in Eca9706 cells was assessed by western blotting. (D) Statistical analysis of Akt protein expression in Eca9706 cells. Data are expressed as the mean  $\pm$  standard deviation of  $\geq 3$  independent experiments. \*\* $P < 0.01$  vs. control group. PI3K, phosphoinositide 3-kinase.

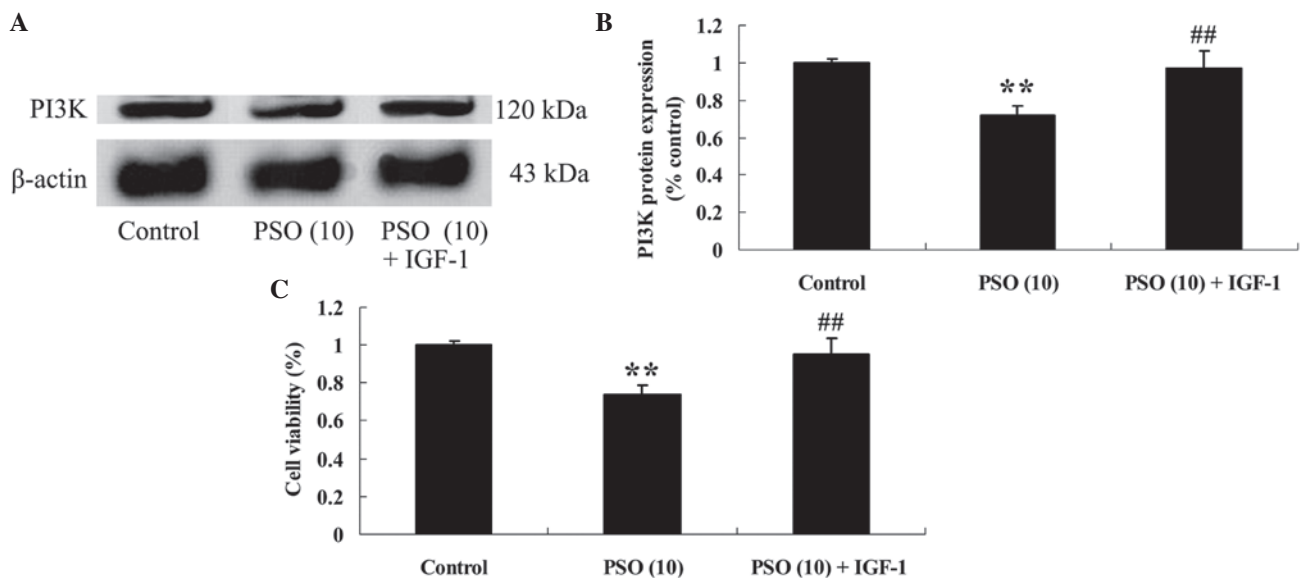


Figure 7. IGF-1, a PI3K agonist, is able to reverse the effects of psoralidin on Eca9706 cells. (A) The effects of IGF-1 on PI3K protein expression in Eca9706 cells were assessed by western blotting. (B) Statistical analysis of PI3K protein expression in Eca9706 cells. (C) Effects of IGF-1 on the viability of Eca9706 cells. Data are expressed as the mean  $\pm$  standard deviation of  $\geq 3$  independent experiments. \*\* $P < 0.01$  vs. control group; ## $P < 0.01$  vs. PSO (10) group. PI3K, phosphoinositide 3-kinase; PSO (10), psoralidin (10  $\mu$ M); IGF-1, insulin-like growth factor 1.

malignant cells abnormally proliferate, eventually leading to cancer (31). The present study demonstrated that treatment with psoralidin inhibited the NF- $\kappa$ B activity of Eca9706 cells. Chiou *et al* (32) reported that psoralidin inhibited lipopolysaccharide-induced expression of nitric oxide via the NF- $\kappa$ B signaling pathway. Yang *et al* (17) suggested that psoralidin regulated ionizing radiation-induced pulmonary inflammation via modulation of the NF- $\kappa$ B signaling pathway.

Previous studies have demonstrated that the PI3K/Akt signaling pathway has a significant role in the development of a number of tumors (33). PI3K/Akt signaling primarily exerts anti-apoptotic effects by affecting multiple downstream

effector molecules (34). Knockout of PI3K, Akt and associated genes by genetic intervention, or inhibition by small molecule drugs, which blocks the activation of downstream anti-apoptotic effector molecules and promotes apoptosis, has become a key focus of research on cancer treatment (35). The results of the present study suggested that psoralidin was able to reduce the protein expression levels of PI3K and Akt in Eca9706 cells; however, upregulation of PI3K protein expression reduced the viability of Eca9706 cells. Previous studies have demonstrated that psoralidin is able to regulate ionizing radiation-induced pulmonary inflammation via regulation of the PI3K/Akt signaling pathway (17), and is

also capable of inhibiting lipopolysaccharide-induced nitric oxide expression via the activation of PI3K/Akt-mediated signaling (32).

In conclusion, the results of the present study suggested that psoralidin may have significant therapeutic effects on esophageal cancer, via the NF- $\kappa$ B and PI3K/Akt signaling pathways. The results of the present study additionally suggest the potential benefits of the use of psoralidin in clinical practice.

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