

Aloe-emodin suppresses esophageal cancer cell TE1 proliferation by inhibiting AKT and ERK phosphorylation

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Abstract. Aberrant AKT and extracellular signal-regulated kinase (ERK) activation is often observed in various human cancers. Both AKT and ERK are important in the phosphoinositide 3-kinase/AKT and mitogen-activated protein kinase/ERK signaling pathways, which play vital roles in cell proliferation, differentiation and survival. Compounds that are able to block these pathways have therefore a promising use in cancer treatment and prevention. The present study revealed that AKT and ERK are activated in esophageal cancer TE1 cells. Aloe-emodin, an anthraquinone present in aloe latex, can suppress TE1 cell proliferation and anchor-independent cell growth. Aloe-emodin can also reduce the number of TE1 cells in S phase. Protein analysis indicated that aloe-emodin inhibits the phosphorylation of AKT and ERK in a dose-dependent manner. Overall, the present data indicate that aloe-emodin can suppress TE1 cell growth by inhibiting AKT and ERK phosphorylation, and suggest its clinical use for cancer therapy.

Introduction

Esophageal cancer (EC) is one of the most common cancers in the world (1). Esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) are the two main types

of EC (2), with ESCC being the main form of EC in Asian countries, and EAC being the most common type of EC in Western countries (3). The incidence of EC is increasing worldwide (4). In the USA in 2012, 17,460 patients were diagnosed with EC, and 15,070 patients succumbed to the disease (5). Upon initial diagnosis, the majority of EC patients already present with metastasis, which results in poor prognosis (6,7).

Accumulating evidence indicates that numerous molecular changes are associated with EC tumorigenesis, including epidermal growth factor receptor (EGFR) amplification, phosphoinositide 3-kinase, catalytic subunit alpha (PIK3CA) amplification and mutation (8-10), and phosphatase and tensin homolog (PTEN) mutation or loss (11,12). Alteration of these molecular events contributes to downstream pathway activation (8-12). The phosphoinositide 3-kinase (PI3K)/AKT and mitogen-activated protein kinase kinase (MEK)/ERK signaling pathways are two important pathways that can be activated by EGFR amplification and PTEN loss, which may ultimately lead to tumorigenesis (13). ERK is a downstream component of an evolutionarily conserved signaling module that is activated by Raf serine/threonine kinases (14). Raf is activated by growth factor stimulation, eventually leading to the activation of ERK (14). ERK then can mediate cell proliferation and oncogenesis through downstream targeting (15). ERK can activate the pro-apoptotic protein B-cell lymphoma-2 (Bcl-2)-associated death promoter (BAD) at Ser112 and phosphorylate the transcription factor cyclic adenosine monophosphate response element-binding protein (CREB) at Ser133 to promote cell survival by activating the ribosomal S6 kinase (RSK) family of serine/threonine kinases (16). Previously, inhibitors targeting this pathway were developed and tested in clinical trials (15,17) with certain success.

Tumorigenesis frequently occurs during PI3K/AKT signal pathway activation following EGFR amplification and PTEN loss (18). Activation of AKT results from Thr308 phosphorylation by 3-phosphoinositide-dependent kinase 1 (PDK1) and Ser473 phosphorylation by mechanistic target of rapamycin complex 2 (mTORC2) (18). AKT regulates CREB activity (19,20) by phosphorylating CREB at Ser133, which

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induces the binding of accessory proteins, thereby regulating anti-apoptotic genes, including Bcl-2 and myeloid cell leukemia 1 (21,22). AKT can suppress mouse double minute 2 homolog (MDM2) self-ubiquitination, thereby inhibiting p53-mediated apoptosis (23). AKT also promotes the cell cycle by phosphorylating p21^{Waf1/Cip1}, also known as cyclin-dependent kinase inhibitor 1 or CDK-interacting protein 1, at Thr145 (24). In addition, AKT directly controls the activation of glycogen synthase kinase (GSK)3 β , which phosphorylates cyclin D1 at Thr286 (25) and Myc at Thr58 (26). GSK3 β promotes the nuclear export and ubiquitin-pathway degradation of AKT, which could in turn regulate the cell cycle by regulating GSK3 β activity (25). Therefore, blocking the activation of PI3K/AKT signaling may be a promising strategy for cancer treatment.

Aloe-emodin has anti-proliferative effects and induces cellular apoptosis (27-30). Aloe-emodin has anti-cancer activity in neuroectodermal tumors (31), nasopharyngeal carcinoma (32), lung squamous cell carcinoma (33), hepatoma cells (34), gastric cancer (35) and prostate cancer (36). Aloe-emodin induces apoptotic cell death by oxidative stress and sustained c-Jun N-terminal kinase (JNK) activation (37). Previous studies have demonstrated that aloe-emodin induces cell death through S-phase arrest in human tongue squamous cancer SCC-4 cells (38). A previous study by the present authors also indicated that mTORC2 is a target of aloe-emodin, and aloe-emodin can strongly inhibit the AKT activation caused by PTEN loss (36). Aloe-emodin is a natural compound from aloe or *Rheum palmatum* (39,40). The present study aimed to determine the efficacy of aloe-emodin in the treatment of EC.

The present results demonstrate that both ERK and AKT are activated in EC cells. Aloe-emodin can suppress the proliferation and anchor-independent cell growth of the EC cell line TE1. Western blot data revealed that aloe-emodin inhibits both AKT and ERK phosphorylation and their downstream activation. The inhibition of these pathways results in cell cycle arrest at S phase and decreased cyclin D1 transcription. These results suggest that aloe-emodin could prevent and even reverse the development of EC, thus identifying it as a candidate compound for EC chemoprevention.

Materials and methods

Materials. Aloe-emodin (>95% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemical reagents, including Tris, NaCl, acrylamide, glycine and sodium dodecyl sulfate (SDS), were purchased from Sigma-Aldrich or Fluka (Sigma-Aldrich). AKT [rabbit polyclonal immunoglobulin G (IgG); sc-8312], p-AKT (mouse monoclonal IgG; sc-293125), ERK (rabbit polyclonal IgG; sc-94) and p-ERK (rabbit polyclonal IgG; sc-23759) primary antibodies, and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (sc-2004) and HRP-conjugated rabbit anti-mouse IgG (sc-358914) secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). α -tubulin (rabbit polyclonal; #2148) antibody was obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture. The esophageal cancer cell lines TE1 and KYSE140 were purchased from Nanjing KeyGen Biotech

Co., Ltd. (Nanjing, China). Eca109 and EC9706 cells were provided by the State Key Laboratory of Molecular Oncology, Chinese Academy of Medical Science (Shanghai, China). TE1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-high glucose (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 10 IU penicillin/ml and 10 IU streptomycin/ml at 37°C in a humidified incubator with 5% CO₂.

Cell counting kit-8 (CCK-8) assay. TE1 cells (2x10⁴) were seeded into 96-well plates in 100 μ l of 10% FBS-DMEM, and incubated in a 37°C, 5% CO₂ incubator overnight. The cells were treated with increasing doses of aloe-emodin (1, 5, 10, 25 and 50 μ M), and cytotoxicity was analyzed at 24 h and 48 h using a CCK-8 (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. TE1 cells (5x10³) were seeded in 96-well plates in 100 μ l of 10% FBS-DMEM and cultured in a 37°C, 5% CO₂ incubator. After 12 h, the medium was changed for medium containing different concentrations of aloe-emodin (0, 2.5, 5, 10 and 20 μ M). Cells were cultured for an additional 24, 48, 72 and 96 h, and 10 μ l of CCK-8 was then added to each well. The cells were incubated for 2 h and the absorbance was measured at 450 nm.

Soft agar assay. The soft agar assay was performed in 6-well plates containing two layers of agar (Bacto Agar; BD Biosciences, Franklin Lakes, NJ, USA) and different concentrations of aloe-emodin (0, 2.5, 5, 10 and 20 μ M). The bottom layer consisted of 0.5% agar in 1 ml of basal medium Eagle (BEM; Sigma-Aldrich) with 10% FBS. The top layer consisted of 0.33% agar in 1 ml of BEM with 10% FBS containing 8x10³ TE1 cells. The TE1 cells embedded in agar were incubated in a 37°C humid incubator for 14 days, and colonies were imaged using a microscope with the aid of Image-Pro Plus software (version 6; Media Cybernetics, Inc., Rockville, MD, USA) and MicroPublisher 5.0 RTV camera (Olympus Corporation, Tokyo, Japan).

Cell cycle analysis. For flow cytometric analysis of cell cycle, TE1 cells (1.5x10⁶) were seeded into a 60-mm dish. After 12 h of culture, the cells were washed three times with Dulbecco's phosphate-buffered saline (DPBS, pH 7.2). The cells were next cultured for 48 h in DMEM without FBS. Then, the FBS-free DMEM was removed, and different concentrations of aloe-emodin (0, 2.5, 5, 10 and 20 μ M) in DMEM containing 10% FBS were added to each dish. After culturing for additional 48 h, the cells were trypsinized, washed with ice-cold DPBS and fixed with ice-cold 70% ethanol at -20°C overnight. Cells were then washed twice with DPBS, incubated with 0.5 mg/ml RNase A and 200 μ g/ml propidium iodide in DPBS at room temperature for 30 min in the dark, and subjected to flow cytometry using a FACSCalibur flow cytometer (BD Biosciences). The percentages of cells in different cell cycle phases (G0/G1, S or G2/M) were calculated.

Western blotting. A total of 1.5x10⁶ TE1 cells were seeded and cultured in a 10-cm dish for 24 h. The cells were treated with various concentrations of aloe-emodin (0, 2.5, 5, 10 and

20 μ M) for additional 24 h. The cells were then harvested, and cell lysates were collected in modified radioimmunoprecipitation assay buffer (50 mM Tris base, 1% NP-40, 0.25% superoxide dismutase, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid and 0.1% SDS). A total of 50 μ g proteins were subjected to 12% SDS-polyacrylamide gel electrophoresis. The nitrocellulose membranes (Hybond-c pure; GE Healthcare Life Sciences, Chalfont, UK) with transferred protein were incubated with a specific primary antibody [anti-AKT (1:200), p-AKT (1:200), ERK (1:200), p-ERK (1:200) or α -tubulin (1:1,000)] at 4°C overnight, followed by incubation with the appropriate secondary antibody (1:1,000) for 2 h at room temperature. Protein bands were detected with an enhanced chemiluminescence (ECL) kit (Beyo ECL Plus; Beyotime Institute of Biotechnology) after hybridization with a specific secondary antibody.

Cyclin D1 luciferase assay. TE1 cells (600×10^3 cells/well) were seeded and cultured in 24-well plates for 12 h, prior to be transfected with 400 ng of the pGL4.29/Luc/Cyclin D1 reporter gene plasmid (generously donated by Dr Chris Albanese, Department of Developmental and Molecular Biology, Albert Einstein Cancer Center, Albert Einstein College of Medicine, New York, NY, USA) via a DNA transfection method (jetPRIME[®], Polyplus-transfection[®] SA, Illkirch, France). This reporter gene was constructed from the -1,715 to +134 region of the human cyclin D1 promoter (41). The medium was replaced with medium containing different aloe-emodin concentrations (0, 2.5, 5, 10 and 20 μ M) after 6 h transfection. For luciferase detection, the cells were incubated for another 24 h. Luciferase assays were performed with the Dual-Luciferase[®] Reporter (DLR[™]) Assay System (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. Detection was conducted with the Centro LB 960 microplate luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany), and the data were calculated as the mean of three independent experiments.

Statistical analysis. All the data were reported as means \pm standard error or standard deviation, as calculated with the statistical software SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Single factor analysis of variance was used for statistical analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

AKT and ERK are activated in EC cell lines. Since the MEK/ERK and PI3K/AKT signaling pathways are important in tumorigenesis, the level of activation of these pathways in EC cells was investigated. Cell lysates of different human EC cell lines were collected and subjected to western blot analysis to detect AKT and ERK phosphorylation. AKT phosphorylation at Ser473 was detected in Eca109, TE1 and KYSE140 cells. ERK phosphorylation was detected in Eca109, TE1 and KYSE140 cells. Phosphorylation could not be detected in either AKT or ERK in the EC9706 cell line (Fig. 1).

Aloe-emodin suppresses TE1 cell proliferation and anchor-independent cell growth. Aloe-emodin is a substance

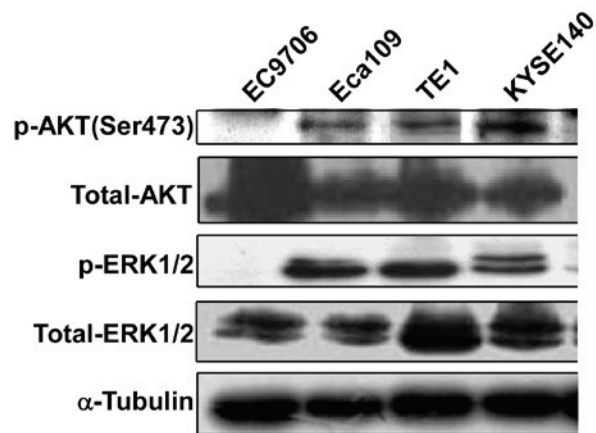


Figure 1. AKT and ERK were activated in EC cell lines. Four human EC cell lines were cultured, and cell lysates were harvested. A total of 50 μ g of cell lysates of each cell line were subjected to western blot analysis. The phosphorylation of AKT and ERK was evaluated. Representative data of three independent experiments are shown. ERK, extracellular-signal regulated kinase; EC, esophageal cancer; p-, phosphorylated.

derived from aloe or *Rheum palmatum* that is effective at suppressing cancer cell growth in gastric cancer, prostate cancer and colon cancer cells (Fig. 2A) (42,43). To investigate the degree to which aloe-emodin can suppress EC cell proliferation, TE1 cell growth and anchor-independent cell growth was examined. In the cytotoxicity assay, 90% of the cells survived after treatment with 20 μ M aloe-emodin for 48 h (Fig. 2B). To investigate the level to which aloe-emodin can inhibit TE1 cell proliferation, 2.5, 5, 10 and 20 μ M aloe-emodin was added to the medium of TE1 cells, and CCK-8 assay was performed. The data indicated that aloe-emodin suppressed TE1 cell proliferation in a dose-dependent manner (Fig. 3A). An anchor-independent cell growth assay was performed on TE1 cells in the presence of aloe-emodin. The results indicated that aloe-emodin could suppress colony formation of TE1 cells in a dose-dependent manner (Fig. 3B).

Aloe-emodin inhibits AKT and ERK activity. Aloe-emodin was used to inhibit the ERK and AKT-related signaling pathways activated in TE1 cells. The western blot data indicated that aloe-emodin inhibited the phosphorylation of AKT at Ser473 (Fig. 4A). Downstream of AKT, Ser9 phosphorylation of GSK3 β also decreased in a dose-dependent manner. In addition, the phosphorylation of ERK and its downstream target, RSK2, were also investigated. The results indicated that the phosphorylation of ERK at Thr202/Tyr204, RSK2 at Ser360 and CREB at Ser133 was also inhibited by aloe-emodin treatment (Fig. 4B).

Aloe-emodin inhibits TE1 cell growth by decreasing the number of cells in S phase. To investigate the extent to which the aloe-emodin-mediated TE1 cell growth was associated with cell cycle arrest, cell cycle analysis was performed. The data revealed that treatment with increasing concentrations of aloe-emodin for 48 h resulted in a dose-dependent decrease in the number of cells in S phase (Fig. 5A).

Aloe-emodin inhibits cyclin D1 expression in TE1 cells. AKT and its downstream kinase GSK3 β regulate cyclin

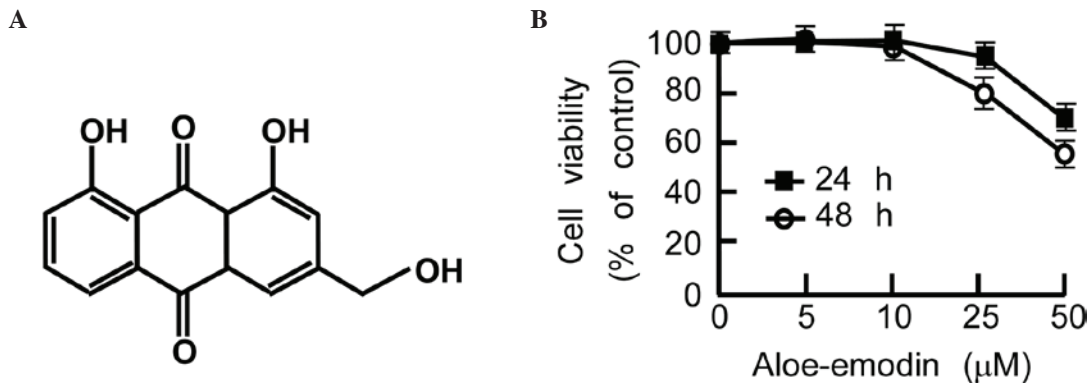


Figure 2. (A) Chemical structure of aloe-emodin. (B) Toxicity of aloe-emodin in TE1 cells. TE1 cells (2×10^4) were seeded into 96-well plates in 100 μ l of 10% fetal bovine serum-Dulbecco's modified Eagle medium, and incubated in a 37°C, 5% CO₂ incubator overnight. The cells were treated with increasing doses of aloe-emodin (1, 5, 10, 25 and 50 μ M), and cytotoxicity was analyzed at the indicated times using a Cell Counting Kit-8 (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol.

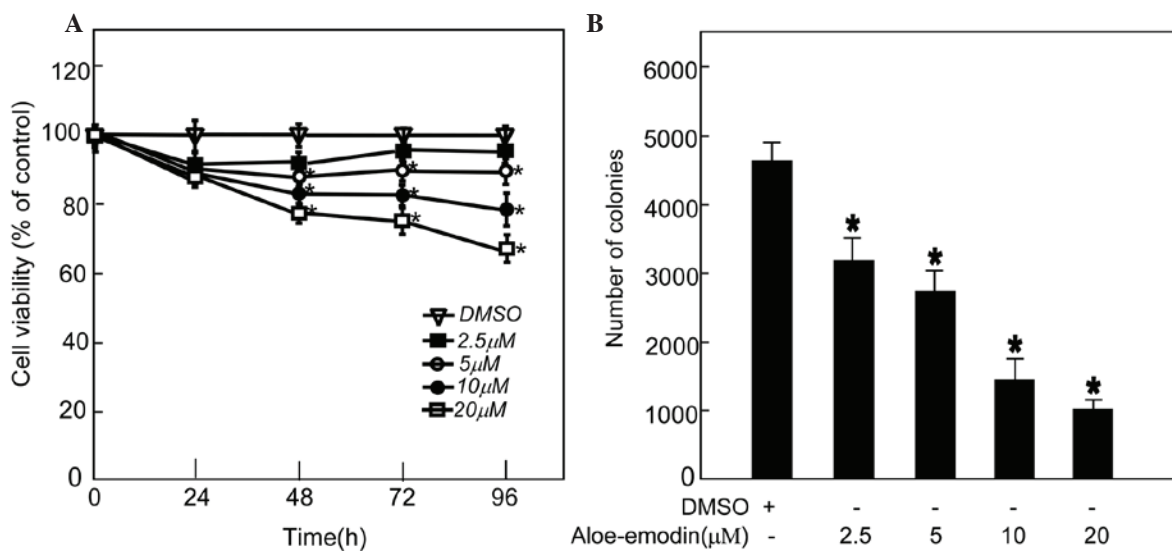


Figure 3. AE suppresses TE1 cell proliferation and anchor-independent cell growth. TE1 cells (5×10^3) were treated with different concentrations of AE. (A) AE significantly inhibited cell proliferation. Absorbance was measured at 24, 48, 72 and 96 h by Cell Counting Kit-8, as described in Materials and methods. Data are shown as means \pm SD (* $P < 0.05$ vs. untreated control, $n = 3$). (B) AE significantly suppressed anchor-independent cell growth of TE1 cells. Colonies were counted, and data are shown as means \pm SD (* $P < 0.05$ vs. untreated control, $n = 3$). DMSO, dimethyl sulfoxide; AE, aloe-emodin; SD, standard deviation.

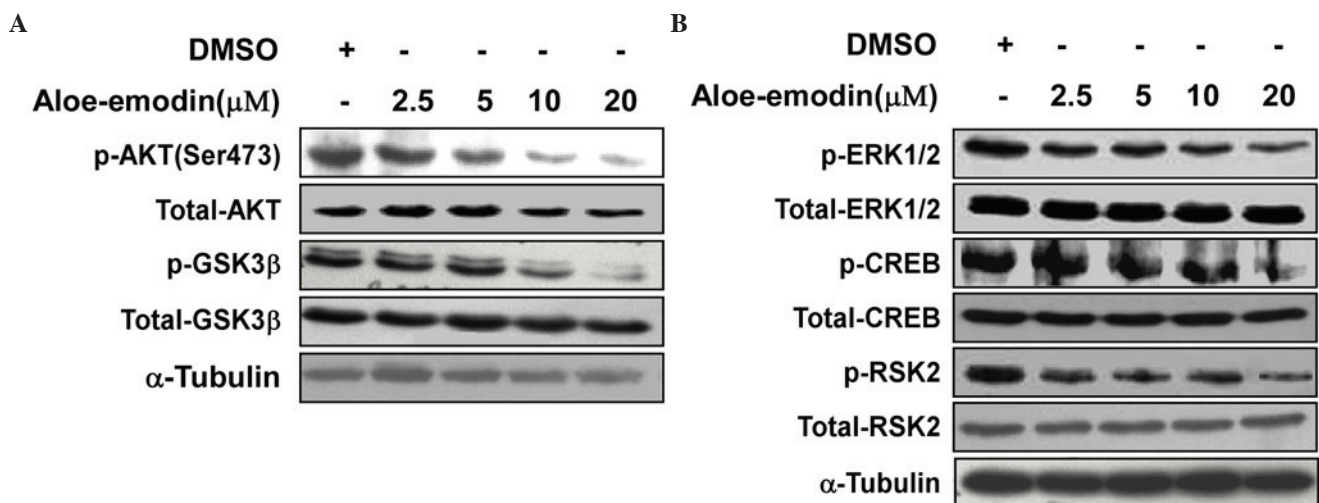


Figure 4. Aloe-emodin inhibits (A) AKT-glycogen synthase kinase 3 β and (B) extracellular-signal regulated kinase-ribosomal S6 kinase activity. Western blot analysis of TE1 cells exposed to increasing concentrations of aloe-emodin was performed. Representative data of three independent experiments are shown. DMSO, dimethyl sulfoxide; ERK, extracellular-signal regulated kinase; p-, phosphorylated; CREB, cyclic adenosine monophosphate response element-binding protein; RSK, ribosomal S6 kinase; GSK3 β , glycogen synthase kinase.

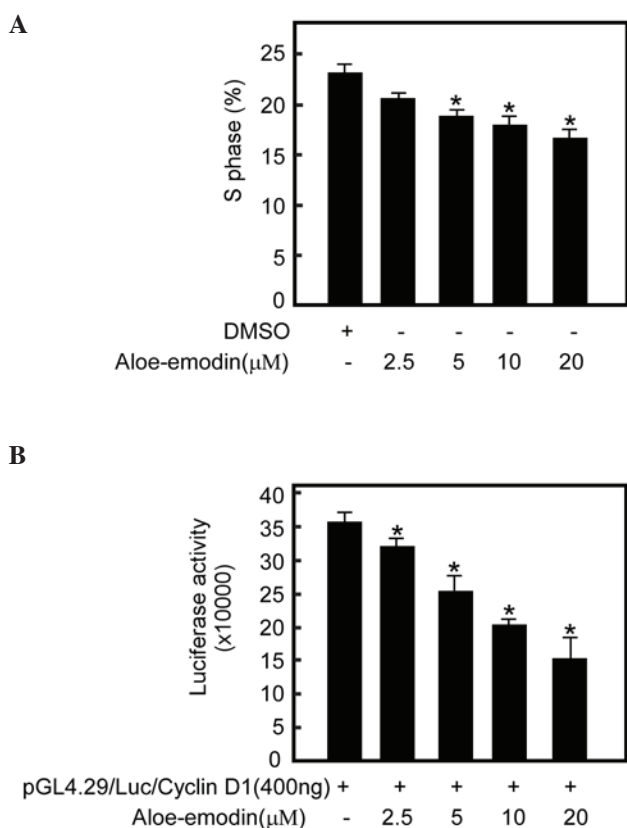


Figure 5. Aloe-emodin effects on the cell cycle. (A) Aloe-emodin significantly decreased the number of TE1 cells in S phase ($P < 0.05$ vs. untreated, $n = 3$). (B) Aloe-emodin significantly inhibited cyclin D1 transcription activity in TE1 cells in a dose-dependent manner ($P < 0.05$ vs. untreated, $n = 3$). DMSO, dimethyl sulfoxide; Luc, luciferase.

D1 transcription, which regulates cell transition from G1 to S phase (44). To investigate the degree to which aloe-emodin-mediated S phase reduction is associated with cyclin expression, a cyclin D1 reporter gene assay was performed with aloe-emodin treatment. The cyclin D1 reporter gene assay demonstrated that aloe-emodin could inhibit cyclin D1 transcription activity in a dose-dependent manner (Fig. 5B).

Discussion

Signal transduction pathways have an important role in tumorigenesis (45). Both AKT and ERK are important molecules in the MEK/ERK and PI3K/AKT signal transduction pathways (46,47). In the present study, ERK and AKT were activated in EC cell lines, including TE1, Eca109 and KYSE 140, which implies that these two activation pathways are important in esophageal tumorigenesis and development. Previous studies have also indicated that both MEK/ERK and PI3K/AKT signaling are activated in ESCC (8,48,49). Therefore, blocking these two pathways is a promising strategy for EC treatment and chemoprevention.

Previous research on cancer cells has revealed that aloe-emodin has anti-proliferative effects and can induce apoptosis at high doses (50). Aloe-emodin suppresses prostate cancer by targeting mTORC2 and inhibiting growth in a dose-dependent manner, with a maximal inhibitory effect at a concentration of 15 μM (36). By contrast, other studies have

indicated that aloe-emodin has anti-proliferative effects at 75 μM and induces apoptosis of human hepatoma Huh-7 cells via downregulation of calpain-2 and ubiquitin-protein ligase E3A (51). In the present study, aloe-emodin had a cytotoxic effect on EC cells. Thus, at lower doses than those previously reported ($< 20 \mu\text{M}$), aloe-emodin inhibited TE1 cell proliferation and anchor-independent cell growth in a dose-dependent manner. Cell cycle analysis indicated that aloe-emodin inhibited cell entry into S phase. These data demonstrated that aloe-emodin is a promising compound for suppressing the growth of EC cells.

Aloe-emodin has been reported to inhibit tumor cell proliferation through various mechanisms (35,50). Aloe-emodin induced G2/M arrest and differentiation of cervical cancer cells (52), while induced apoptosis of retina ganglion cells in glaucomatous patients through regulation of ERK phosphorylation (53). Aloe-emodin induced apoptosis, autophagy and differentiation of glioma cells by inhibiting the action of ERK (54). In the current study, aloe-emodin at low doses inhibited AKT and ERK phosphorylation, which is consistent with a previous study by the present authors on prostate cancer cells (PC3) (36). However, PTEN loss caused strong activation of AKT, which, in turn, attenuated the phospho-ERK signal. This may be the explanation for the lack of an effect of ERK (55). In the current study, GSK3 β and RSK2, the downstream kinases of AKT, were also inhibited, as was the phosphorylation of the transcription factor CREB. This suggests that aloe-emodin can suppress the MEK/ERK and PI3K/AKT signaling pathways. However, further investigation is required to determine if this is caused by one or several targets. Furthermore, aloe-emodin inhibited cyclin D1 transcription in the present study, which may be associated with AKT and ERK pathway inhibition.

In conclusion, the present study demonstrated that AKT and ERK were activated in EC cells. Aloe-emodin suppressed EC TE1 cell proliferation through the inhibition of AKT, ERK and their downstream molecules, thus regulating cyclin D1 transcription. Further studies on aloe-emodin and its potential use as a therapeutic agent for EC should be conducted.

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