

Antiproliferation effect of evodiamine in human colon cancer cells is associated with IGF-1/HIF-1 α downregulation

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Received July 3, 2015; Accepted August 18, 2015

DOI: 10.3892/or.2015.4309

Abstract. Colon cancer is one of the most common malignancies. Although the current treatment regimes for colon cancer have been well-developed in the past decades, the prognosis remains still undesirable. It is still urgent to explore new treatment strategies for colon cancer. Natural products is one of the most useful sources for anticancer agents, although some of them have serious side-effects. Evodiamine (Evo) is a quinolone alkaloid from the traditional herb medicine *Evodia rutaecarpa*. In the present study, we investigated the anticancer effect of Evo in human colon cancer cells. We found that Evo exhibits prominent antiproliferation and apoptosis inducing effects in LoVo cells. Evo leads to apparent downregulation of HIF-1 α either *in vitro* or *in vivo*; exogenous expression of HIF-1 α can attenuate the antiproliferation effect of Evo in LoVo cells, while HIF-1 α knockdown potentiates this effect greatly. Further analysis indicated that Evo can also inhibit the phosphorylation of Akt1/2/3 and decrease greatly the expression of IGF-1. Thus, our findings strongly suggested that the anticancer effect of Evo in human colon cancer may be partly mediated by downregulating HIF-1 α expression, which is initiated by inactivating PI3K/Akt signaling transduction though decreasing the expression of IGF-1 in colon cancer cells. Therefore, Evo may be used alone or in combination as a potential anticancer agent for colon cancer treatment.

Introduction

Colon cancer, one of the most common malignancies, leads to major cancer morbidity and mortality. Although the current

diagnoses and treatment for colon cancer have greatly improved in the last decades, the prognosis is still poor. The challenges for colon cancer clinical treatment are the serious side-effects of the current chemotherapy drugs and the metastasis of colon cancer cells. Thus, there is a great need to develop new clinical treatment regimens for colon cancer therapy.

Some natural products and their essential derived bioactive components, including semi-synthetic and synthetic analogs, have been used as anticancer agents for a few decades (1-4), such as taxol, vincristine, camptothecin, vinblastine, teniposide and etoposide (5). Hence, natural products play a major role in cancer chemotherapy. Evodiamine (Evo), a quinolone alkaloid from the traditional herb medicine *Evodia rutaecarpa* (6), may be used to treat many diseases, such as obesity, inflammation and cardiovascular diseases, due to its versatile pharmacological functions (7). Increasing evidence supports that Evo processes anticancer activity in various cancer types, such as lung, colon and breast cancer (8-10). This effect of Evo may be associated with downregulating ERK (8), activating JNK (9) or degrading estrogen receptor (11). However, the exact molecular mechanism underlay this effect of Evo in cancer remains unclear.

Hypoxia-inducible factors (HIFs) are special transcriptional factors that respond to the decrease of oxygen in the cellular environment (12). HIF-1 is composed of HIF-1 α and HIF-1 β subunits, and HIF-1 α level determines the transcriptional activity of HIF-1 since it has an extremely short half-life (12). HIF-1 α forms a dimer with HIF-1 β to regulate the expression of downstream target genes associated with metabolism, proliferation, apoptosis, inflammation, immunity, survival and angiogenesis (13). Consequently, HIF-1 α is very important, not only for homeostasis, but also for blood vessel formation in embryos and tumorigenesis (13,14). HIF-1 α has already been reported as a critical target to block angiogenesis for cancer treatment (14), Evo can decrease the expression of HIF-1 α in cancer cells (15), but it remains unknown whether Evo-induced downregulation of HIF-1 α could mediate this function in colon cancer cells, and how Evo decreases the expression of HIF-1 α .

Herein, we investigated the antiproliferation effect of Evo in colon cancer cells, and dissected the possible mechanism underlying this function.

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Key words: evodiamine, colon cancer, antiproliferation, HIF-1 α , IGF-1

Materials and methods

Chemicals and drug preparations. Evo was purchased from Hao-Xuan Bio-Tech Co., Ltd. (Xi'an, China). LoVo cell line was purchased from the American Type Culture Collection (ATCC). Evo was dissolved in dimethylsulfoxide (DMSO) for *in vitro* test or prepared with 0.4% carboxymethylcellulose sodium (CMC-Na) as suspension for *in vivo* experiments. All antibodies were purchased from Santa Cruz Biotechnology. Cells were maintained in the Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μ g/ml of streptomycin at 37°C in 5% CO₂.

Crystal violet viability assay. Crystal violet staining was conducted as reported (16). Briefly, LoVo cells were seeded into a 24-well plate and treated with different concentrations of Evo. Cells were washed carefully with cold (4°C) phosphate-buffered saline (PBS) and stained with 0.5% crystal violet formalin solution at room temperature to visualize the cell viability at the scheduled time points. For quantification, crystal violet was extracted with 1 ml 20% acetic acid at room temperature for 20 min with gentle shaking. Absorbance at 570 nm was measured. Each assay was carried out in triplicate.

Clone formation assay. The clone formation assay was conducted as reported (16). Briefly, sub-confluent LoVo cells were treated with Evo at the indicated concentrations for 24 h. The cells were reseeded at 12-well plate without Evo treating, and maintained in culture for 14 days. The initial cell number was 100 or 200/well. Colonies were subjected to crystal violet staining. Each assay condition was carried out in duplicate.

Construction of recombinant adenovirus. The recombinant adenoviruses expressing human HIF-1 α (AdHIF-1 α), RFP (AdRFP) and small interference RNA fragments targeting HIF-1 α (AdsiHIF-1 α) were generated using the AdEasy technology, as described (17,18).

Flow cytometry analysis for apoptosis. Sub-confluent LoVo cells were seeded in 6-well plates and treated with different concentrations of Evo for 48 h. Then, cells were harvested and washed with cold PBS (4°C), followed by incubating with Annexin V-EGFP and propidium iodide (PI) according to kit instructions (KeyGen Biotech, Nanjing, China). The stained cells were analyzed by fluorescence-activated cell sorting (FACS). Each assay was carried out in triplicate.

Annexin V-EGFP staining for apoptosis assay. Sub-confluent LoVo cells were seeded into 24-well plates and treated with different concentrations of Evo for 24 h. Cells were washed with PBS twice and incubated with 500 μ l of binding buffer and 2 μ l of Annexin V-EGFP fusion protein each well for 5 min, followed by washing with PBS twice. Images were captured under a fluorescence microscope. Each assay was carried out in triplicate.

Western blot assay. Sub-confluent LoVo cells were seeded into a 6-well plate and were treated with different concentrations of

Evo or DMSO. At the scheduled time points, cells were washed with cold PBS and lysed in 300 μ l lysis buffer. The lysates were boiled for 10 min, and then subjected to SDS-PAGE electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were immunoblotted with corresponding primary antibodies, and followed by incubating with horseradish peroxidase (HRP)-conjugated secondary antibodies. The target proteins were visualized with the SuperSignal West Pico substrate (Pierce, Rockford, IL, USA). Each assay was carried out in triplicate.

Reverse transcription and polymerase chain reaction analysis (RT-PCR). Sub-confluent LoVo cells were seeded in T25 flasks and treated with different concentrations of Evo or DMSO. At the scheduled time point, total RNAs were extracted with TRIzol reagents (Invitrogen, Carlsbad, CA, USA) and used to generate cDNA templates by RT reaction. Then, the cDNAs were used as templates for PCR to detect the expression level of the genes of interest. The primers used were available upon request. Each assay was carried out in triplicate.

Xenograft tumor model of human colon cancer. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Chongqing Medical University. Athymic nude mice (female, 4-6 weeks old, 5/group) were purchased from the Animal Center of Chongqing Medical University (Chongqing, China). LoVo cells were collected and resuspended in cold PBS to a final density of 2 \times 10⁷ cells/ml. Cells in 50 μ l of cold PBS were injected into the flanks of athymic mice. At 3 days after injection, animals were treated with different doses of Evo (5, 10 and 20 mg/kg) or the same volume of solvent by intragastric administration once a day. The tumor size was measured once a week and the tumor volume (V, mm³) was calculated as the following equation: $V = \pi/6 \times (R_{\max} \times 6R_{\min})^2 \times R$ (R, is the tumor diameter). Five weeks after injection, the animals were sacrificed and the tumor samples were retrieved for histological evaluation.

Luciferase reporter assay. Sub-confluent LoVo cells were seeded into T25 flask and transfected with 2 μ g HIF-1 responsive element luciferase reporter plasmid (pBGluc-HIF-1 α) per flask using Lipofectamine (19), and replacing the medium 4 h later with fresh complete medium. After incubating for 12 h, cells were reseeded into a 24-well plate and treated with different concentrations of Evo or DMSO. Twenty-four hours after treatment, cells were lysed and subjected to luciferase assays using luciferase assay kit (E1500; Promega). Each assay was carried out in triplicate.

Histological evaluation and immunohistochemical staining. Retrieved tumor masses were fixed in 10% formalin and embedded in paraffin. Serial sections of the embedded specimens were stained with hematoxylin and eosin. For immunohistochemical staining, slides were deparaffinized and then rehydrated in a graduated fashion. The deparaffinized slides were subjected to antigen retrieval and probed with primary antibody, or isotype IgG as control, followed by incubation with biotinylated secondary antibody and streptavidin-conjugated HRP. The target proteins were visualized by DAB staining and imaged under a microscope (20).

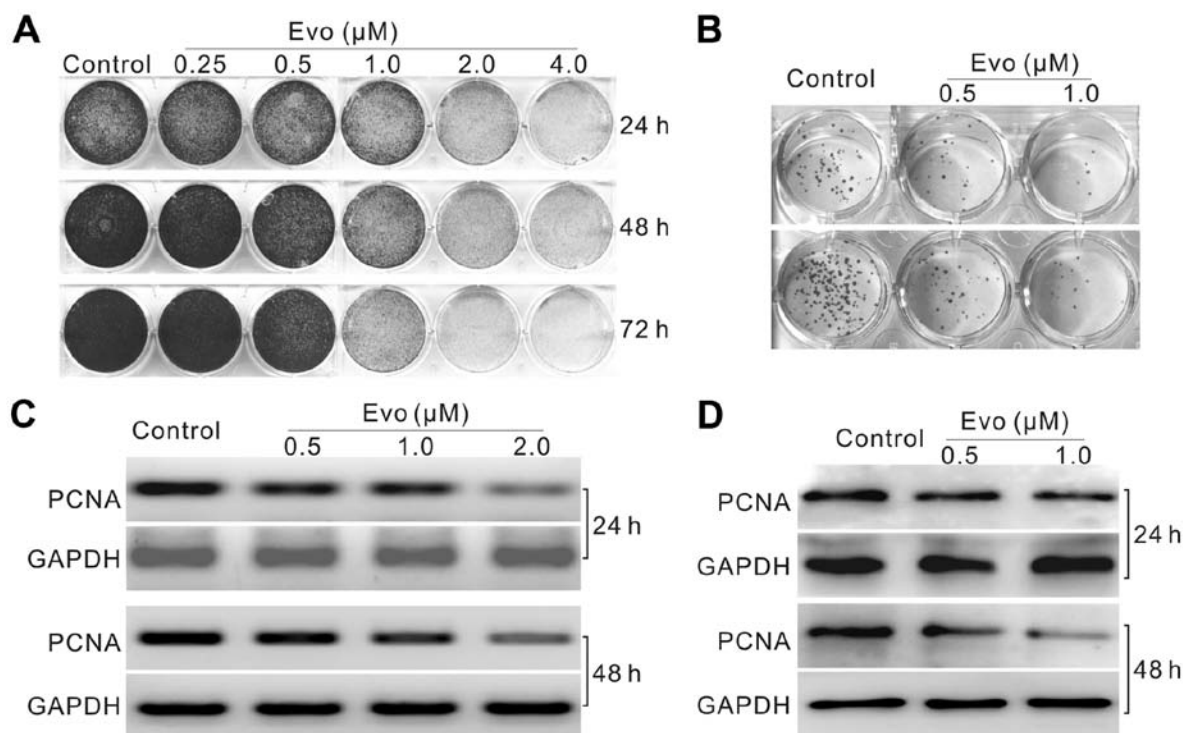


Figure 1. Effect of Evo on proliferation in LoVo cells. (A) Crystal violet staining results show the antiproliferation effect of Evo in LoVo cells. (B) Clone formation assay results show the antiproliferation effect of Evo in LoVo cells (upper panel was initialized with 100 cells/well and the lower panel was 200). (C) RT-PCR results show the effect of Evo on PCNA expression in LoVo cells, GAPDH was used as loading control. (D) Western blot results show the effect of Evo on PCNA expression in LoVo cells, GAPDH was used as a loading control.

Statistical analysis. All quantitative experiments were performed in triplicate. Data are expressed as mean \pm SD. Statistical significances between vehicle treatments vs. drug treatment were determined by the Student's t-test. A p-value of <0.05 was considered to indicate a statistically significant result.

Results

Evo shows antiproliferation effect on LoVo cells. We initiated the investigation by analyzing whether Evo could inhibit the proliferation in LoVo cells. Crystal violet staining results showed that Evo inhibits the proliferation of LoVo cells concentration- and time-dependently (Fig. 1A and B). PCR and western blot results showed that Evo suppresses significantly the expression of proliferating cell nuclear antigen (PCNA) (Fig. 1C and D). These results suggested that Evo can inhibit the proliferation of LoVo cells.

Evo induces apoptosis in LoVo cells. We next analyzed whether Evo could induce LoVo cells to undergo apoptosis. Annexin V-EGFP staining results showed that Evo can effectively induce apoptosis even at concentration of $0.5 \mu\text{M}$ (Fig. 2A). FACS assay results showed that the percentage of apoptotic cells increased concentration-dependently (Fig. 2B). Caspase-3 as an executor of apoptosis, and often used as a definite marker for apoptosis detection. Western blot results showed that Evo increases the level of caspase-3 (Fig. 2C). Thus, these data indicated that Evo can induce apoptosis in LoVo cells.

Evo inhibits tumor growth in a xenograft tumor model of human colon cancer. Next, we injected LoVo cells subcutaneously into the flanks of athymic nude mice, and then treated the mice with different doses of Evo (5, 10 and 20 mg/kg) or solvent by intragastric administration to assess the anticancer activity of Evo. The results showed that Evo suppresses the tumor growth in a dose-dependent manner, compared with the solvent control group (Fig. 3A and B). Hematoxylin and eosin (H&E) staining result showed that more necrotic cells were found in Evo treated groups than that of control group (Fig. 3C). These results showed that Evo can inhibit the proliferation of colon cancer cells.

Evo downregulates the expression of HIF-1 α in LoVo cells. We next sought to investigate the possible mechanism underlying the antiproliferation effect of Evo in human colon cancer cells. With HIF-1 α responsive element reporter assay, we found that Evo can significantly decrease the transcriptional activity of the reporter (Fig. 4A). This suggested that HIF-1 α may be involved in the antiproliferation effect of Evo in colon cancer cells. Using PCR and western blot assay, we found that Evo inhibited the expression of HIF-1 α in LoVo cells (Fig. 4B and C), which was confirmed by immunohistochemical staining for the tumor tissues retrieved from the *in vivo* experiment (Fig. 4D). These results implied that HIF-1 α may be associated with the antiproliferation effect of Evo in LoVo cells.

HIF-1 α affects the anticancer activity of Evo in LoVo cells. We next scheduled to establish whether HIF-1 α could affect the anticancer activity of Evo in LoVo cells. We constructed red

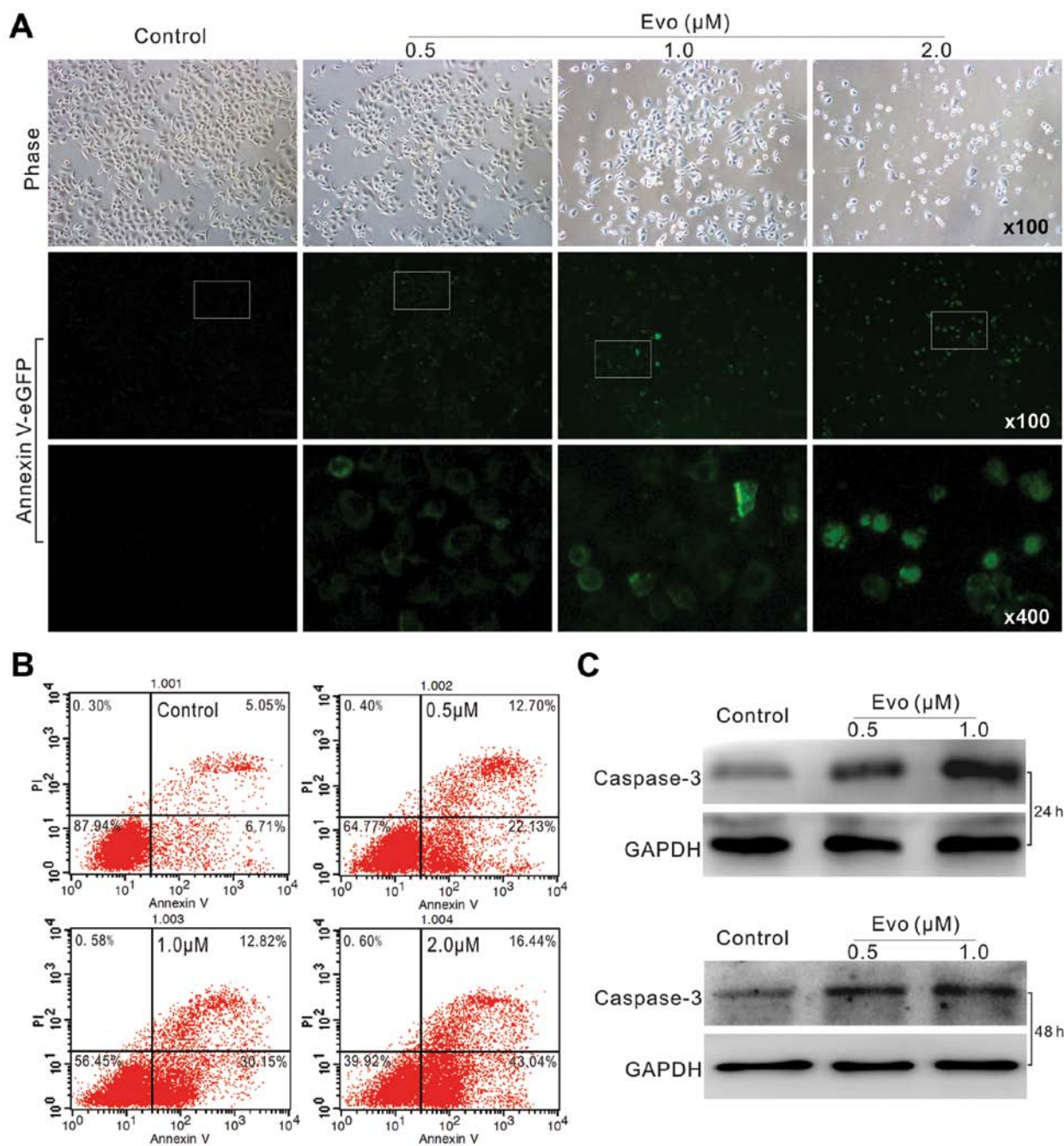


Figure 2. Effect of Evo on apoptosis in LoVo cells. (A) Annexin V-EGFP staining results show the apoptosis induced by Evo in LoVo cells (the lower panel shows the detail for the rectangle part of the middle panel). (B) Flow cytometric assay results show the effect of Evo on apoptosis in LoVo cells. (C) Western blot results show the effect of Evo on caspase-3 in LoVo cells.

fluorescent protein (RFP) tagged recombinant adenoviruses for HIF-1 α overexpression or siRNA fragments. Western blot analysis showed that both recombinant adenoviruses were function well (Fig. 5A). The crystal violet staining results showed that exogenous expression of HIF-1 α can reverse Evo-induced proliferation inhibition partly, but HIF-1 α knockdown enhances prominently this effect (Fig. 5B). Exogenous expression of HIF-1 α can notably reverse the decrease of PCNA induced by Evo, while HIF-1 α knockdown can substantially promote the inhibitory effect of Evo on PCNA (Fig. 5C). For apoptosis, exogenous expression of HIF-1 α reduces the level of caspase-3 induced by Evo, while it is increased by HIF-1 α knockdown (Fig. 5D). Flow cytometric

assay results showed that the percentage of apoptotic cells induced by Evo was decreased by exogenous expression of HIF-1 α , but increased by HIF-1 α knockdown (Fig. 5E). These results suggested that the anticancer activity of Evo is associated with downregulation of HIF-1 α .

Evo may decrease HIF-1 α expression though IGF-1/PI3K/Akt signaling in LoVo cells. Our results have demonstrated that the antiproliferation effect of Evo in LoVo cells maybe partly mediated by downregulating HIF-1 α . However, the possible mechanism of this process remains unknown. PI3K/Akt is important for cell survival signaling, and HIF-1 α is an important downstream target of this signaling. Thus, the effect of

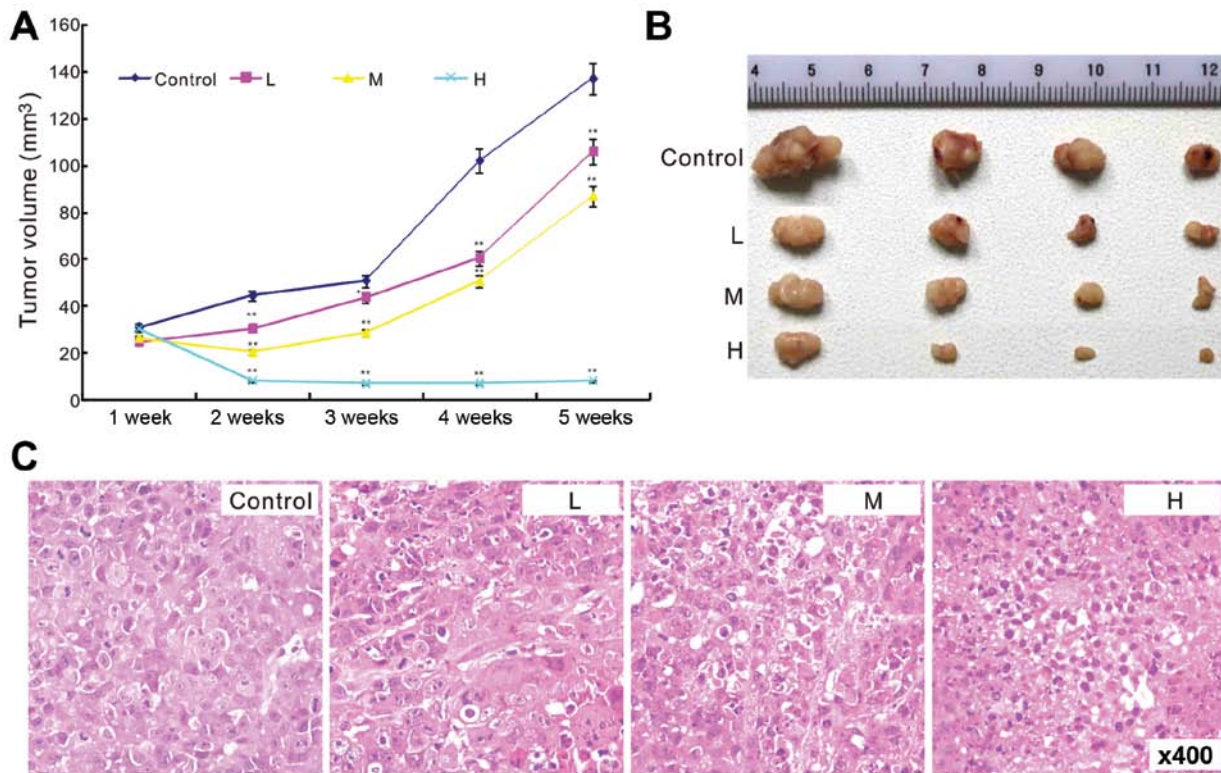


Figure 3. Effect of Evo on tumor growth in xenograft tumor model of human colon cancer. (A) The effect of Evo on colon cancer tumor growth (* $p < 0.05$ and ** $p < 0.01$, compared with control group). (B) Representative tumor masses show the effect of Evo on colon cancer tumor growth (L, low-dose, 5 mg/kg; M, middle-dose, 10 mg/kg; H, high-dose, 20 mg/kg). (C) H&E staining results show the antiproliferation effect of Evo in colon cancer (L, low-dose, 5 mg/kg; M, middle-dose, 10 mg/kg; H, high-dose, 20 mg/kg).

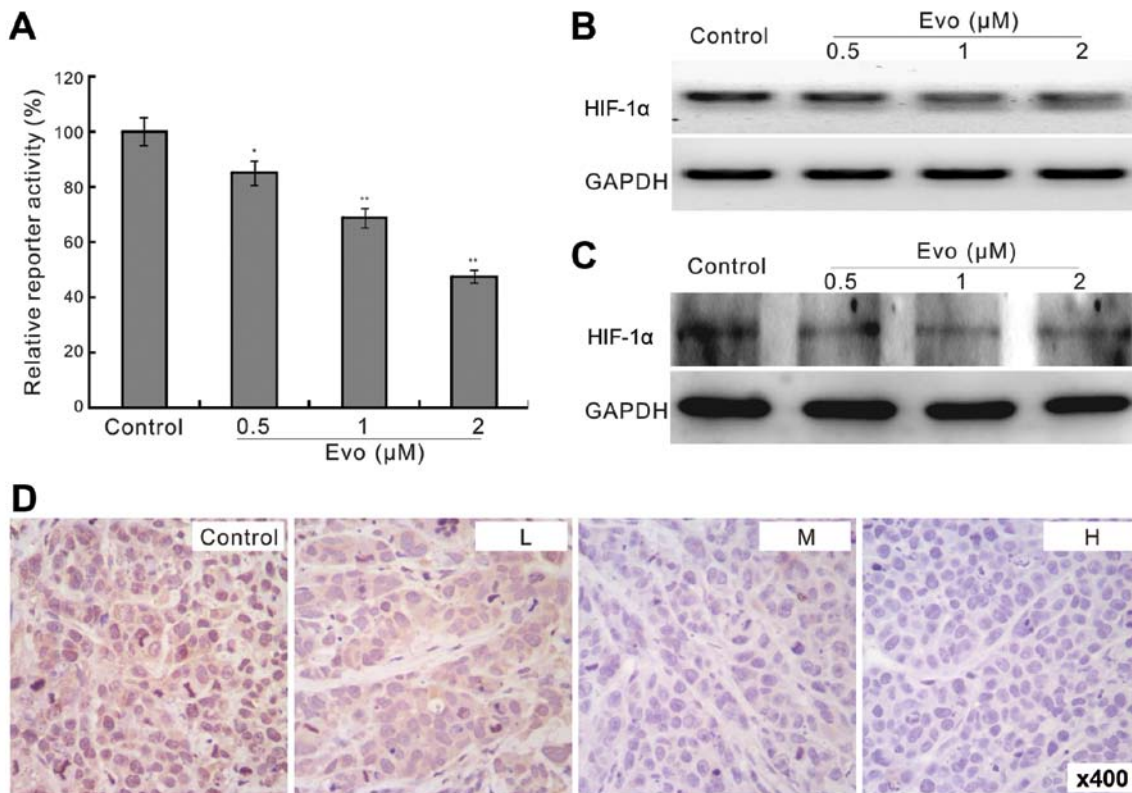


Figure 4. Effect of Evo on the expression of HIF-1 α in LoVo cells. (A) Luciferase reporter assay results show the effect of Evo on the transcriptional activity of HIF-1 in LoVo cells (* $p < 0.05$ and ** $p < 0.01$, compared with control group). (B) RT-PCR assay results show the effect of Evo on the expression of HIF-1 α in LoVo cells (24 h), GAPDH was used as loading control. (C) Western blot results show the effect of Evo on the expression of HIF-1 α in LoVo cells (24 h), GAPDH was used as loading control. (D) Immunohistochemical staining results show the effect of Evo on the expression of HIF-1 α in tumor masses (L, low-dose, 5 mg/kg; M, middle-dose, 10 mg/kg; H, high-dose, 20 mg/kg).

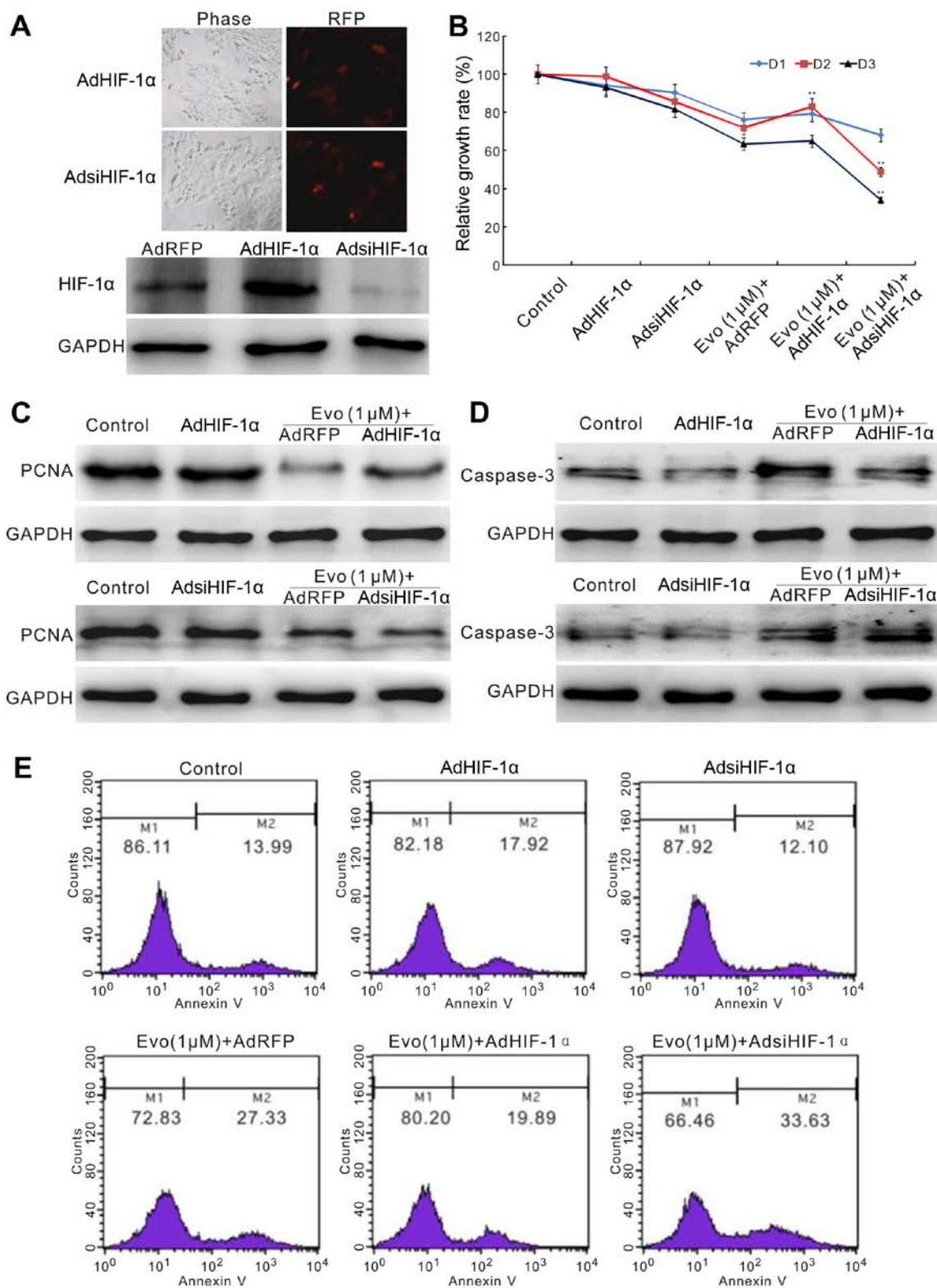


Figure 5. Effect of HIF-1 α on the anticancer activity of Evo in colon cancer cells. (A) Phase and fluorescent images show the infection of the recombinant adenoviruses in LoVo cells (upper panel), western blot results show the effect of different recombinant adenoviruses on the expression of HIF-1 α (lower panel). (B) Quantification results of crystal violet staining show the effect of recombinant adenoviruses on the antiproliferation activity of Evo in LoVo cells (** p <0.01, compared with Evo plus AdRFP group). (C) Western blot results show the effect of exogenous expressed HIF-1 α (upper panel) or HIF-1 α knockdown (lower panel) on PCNA level in LoVo cells, GAPDH was used as loading control. (D) Western blot results show the effect of exogenous expression of HIF-1 α (upper panel) or HIF-1 α knockdown (lower panel) on caspase-3 level in LoVo cells, GAPDH was used as loading control. (E) Flow cytometric assay results show the effect of adenovirus mediated HIF-1 α overexpression or knockdown on Evo-induced apoptosis in LoVo cells.

Evo on HIF-1 α may be associated with the inhibition of PI3K/Akt signaling. Using western blot analysis, we found that

Evo can decrease the phosphorylation of Akt1/2/3, as well as HIF-1 α (Fig. 6A). PI3K/Akt signaling can be regulated

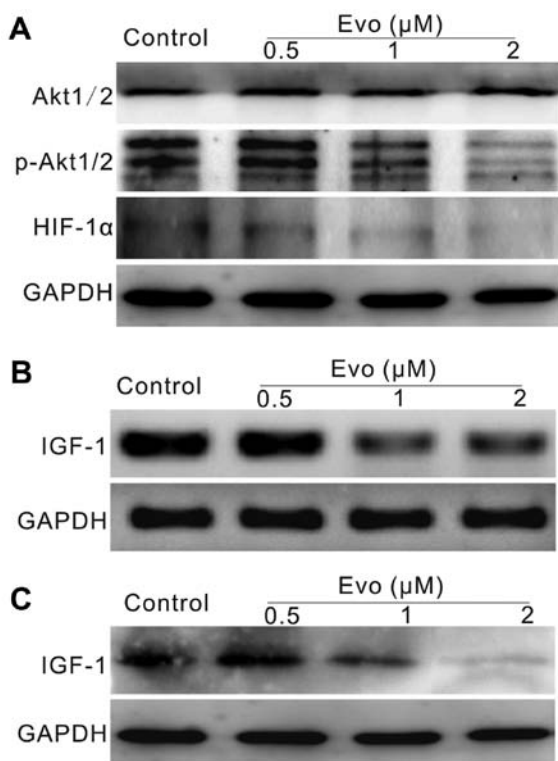


Figure 6. Effect of Evo on Akt1/2 phosphorylation and IGF-1 expression in colon cancer cells. (A) Western blot results show the effect of Evo on Akt1/2 phosphorylation and HIF-1 α expression in LoVo cells (24 h), GAPDH was used as loading control. (B) RT-PCR results show the effect of Evo on the expression of HIF-1 α in LoVo cells (24 h), GAPDH was used as loading control. (C) Western blot results show the effect of Evo on the expression of HIF-1 α in LoVo cells (24 h), GAPDH was used as a loading control.

by certain cytokines or growth factors. IGF-1 is one of the most important activators of this signaling, thus we detected whether Evo could affect the expression of IGF-1. The PCR and western blot results showed that Evo can decrease the expression of IGF-1 notably in LoVo cells (Fig. 6B and C). Taken together, these data suggested that the effect of Evo on HIF-1 α may be related with the inhibition of IGF-1 in human colon cancer cells.

Discussion

Colon cancer is one of the major causes of cancer morbidity and mortality. Although the diagnosis and treatment of colon cancer have greatly improved in the last decades, the prognosis is still poor for most patients. Thus, it is urgent to develop new regimens for colon cancer treatment. In the present study, we investigated the anticancer activity of evodiamine (Evo) in human colon cancer cells. We found that Evo can effectively inhibit the proliferation and promote apoptosis in LoVo cells. Mechanistically, we found that the antiproliferation activity of Evo in colon cancer may be mediated by downregulating HIF-1 α through inactivating IGF-1/PI3K/Akt signaling.

Although colon cancer is one of the most common malignancies, it is still treatable and often curable if localized to the bowel. The metastasis or recurrence is often the major causes of death. At the late stage or recurrence of colon

cancer, chemotherapy or targeted therapy are the primary choices. Natural products and their derived active components, including semi-synthetic and synthetic analogs, have served as the major source for anticancer agents (1-4). Several plant-derived compounds have been used as anticancer drugs for some years (5). Evo, a quinolone alkaloid, was extracted from the traditional herbal medicine *Evodiae fructus* (6). Evo shows pharmacological actions and can be used to treat many diseases, such as obesity, inflammation and cardiovascular diseases (7). Expanding pool of evidence supports that Evo possesses anticancer activities either *in vitro* or *in vivo* by inhibiting proliferation, metastasis, and inducing apoptosis in various tumor cell lines, such as lung, prostate, breast and colon cancer cells (8-10). For colon cancer, the antiproliferation effect of Evo has been well demonstrated (9,21,22). However, the exact molecular mechanism underlay this function remains unknown, although JNK and caspase have been reported associated with the anticancer activity of Evo in colon cancer cells (9,21).

In this investigation, we analyzed the antiproliferation effect of Evo in colon cancer cells, and then tried to unveil the possible mechanism underlying this effect. Our results from crystal violet and clone formation assay demonstrated that Evo is a very potent proliferation inhibitor in LoVo cells (Fig. 1A and B). Proliferating cell nuclear antigen (PCNA) is an important factor for proliferation (23), and can be used as a potential target for cancer treatment (24). We found that Evo could decrease the level of PCNA in a concentration dependent manner (Fig. 1C and D). As most of the current anticancer agents are capable of inducing apoptosis, we analyzed whether Evo also possesses this ability. Phosphatidylserine, usually locates on the inner side of cell membrane, and can specifically bind with Annexin V. At the early stage of apoptosis, phosphatidylserine translocates to the outer side of cell membrane. Thus, it is widely used for early apoptosis detection (25). Caspase-3 has been thought as the executor for apoptosis (26). Our results showed that Evo can increase the Annexin V-EGFP signal greatly, as well as the protein level of caspase-3 (Fig. 2A-C). These data supported that Evo can induce the colon cancer cells to undergo apoptosis. The *in vivo* xenograft tumor assay results showed that Evo can inhibit tumor growth (Fig. 3A-C). Taken together, these data confirmed that Evo may be used as a potential antiproliferation agent for colon cancer treatment alone or as an adjuvant.

Hypoxia-inducible factors (HIFs), including HIF-1, HIF-2 and HIF-3, are special transcriptional factors that respond to the decrease of oxygen in cellular environment. For this reason, HIFs are vital to development (27,28). HIF-1 consists of HIF-1 α and HIF-1 β subunits, which form heterodimers to regulate the downstream targets. HIF-1 α and HIF-1 β are constitutively expressed, but HIF-1 α has an extremely short half-life, so HIF-1 α level determines the transcriptional activity of HIF-1 (12). HIF-1 α is critical for angiogenesis and new vascular formation, which is very important for tumor growth. Therefore, HIF-1 α and other angiogenesis-related factors have been thought as potential targets for cancer treatment, such as vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) (14,29,30). HIF-1 α can regulate not only vascular formation, but also proliferation, glucose metabolism, inflammation, survival and

apoptosis (13). In fact, metabolism regulation is the principal function of HIF-1 α (27,31). Inhibition of HIF-1 α has shown anticancer activity (14), and the level of HIF-1 α is associated with increasing metastasis and/or patient mortality (14,32,33). With HIF-1 responsive element luciferase reporter, we found that Evo can decrease the transcriptional activity of this reporter (Fig. 4A), which suggested that Evo may downregulate the expression of HIF-1 α . With further analysis, we confirmed that Evo can apparently decrease the expression of HIF-1 α in LoVo cells (Fig. 4B and C), this was consistent with the immunohistochemical staining results for the tumor masses (Fig. 4D). Thus, it is definite that HIF-1 α is a target of Evo in colon cancer, but its role in the anticancer activity of Evo in colon cancer remains unknown. We introduced recombinant adenoviruses to mediate overexpression or knock down of HIF-1 α . The results showed that exogenous expression of HIF-1 α have no apparent effect on the proliferation of colon cancer cells, but it can partly reverse the proliferation inhibitory effect of Evo; knockdown of HIF-1 α can enhance the antiproliferation effect of Evo in colon cancer cells (Fig. 5B). Exogenous expression of HIF-1 α can reverse the effect of Evo on PCNA, while knockdown of HIF-1 α can potentiate this effect (Fig. 5C). Similar results were found in Evo-induced apoptosis in LoVo cells (Fig. 5D and E). Taken together, all these data suggested that the antiproliferation function of Evo in colon cancer may be mediated by decreasing the expression of HIF-1 α .

HIFs are special transcriptional factors that respond to hypoxia, and are vital for development, and in cancer therapy (31). Growth factors and cytokines can also induce HIF-1 α accumulation, such as vascular endothelial growth factor A (VEGF-A), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) and tumor necrosis factor α (TNF- α) (33,34). Several signaling pathways, such as PI3K/Akt and NF- κ B (35), are strongly involved in mediating response of these factors to regulate the expression of HIF-1 α . PI3K/Akt signaling pathway is very important for differentiation, proliferation and survival. Therefore, we hypothesized that the Evo-induced decrease of HIF-1 α may result from the inhibition of PI3K/Akt signaling transduction. Western blot analysis supported our hypothesis that Evo can decrease the phosphorylation level of Akt1/2/3 concentration-dependently (Fig. 6A). PI3K/Akt signaling can be regulated by various factor, such as insulin-like growth factor 1 (IGF-1) and PTEN (36,37). IGF-1 is one of the most potent natural activators for PI3K/Akt signaling, while PTEN is the negative regulator for this signaling (38,39). Thus, we tested whether Evo can affect the expression of IGF-1, an important component of IGFs signaling. IGF signaling is another very complex system, comprising two ligands (IGF-1 and IGF-2), two receptors (IGF-1R and IGF-2R), seven binding proteins (IGF binding proteins, IGFBP-1 to IGFBP-7) with high affinity to IGFs, and IGFBPs degrading enzymes. The IGF signaling is vital for regulating the development and homeostasis, including differentiation, proliferation and apoptosis (40-43). Thus, the aberrant IGF signaling are implicated with the development of cancer (44-47), including human colon cancer (48). We found that Evo can downregulate the expression of IGF-1 in a concentration-dependent manner (Fig. 6B and C). Taken together, these data demon-

strated that Evo-induced downregulation of HIF-1 α may be the result of inhibition of the expression of IGF-1.

In summary, our investigation elucidated that Evo can efficiently inhibit the proliferation of colon cancer cells and may be used alone or combination as a potential anticancer agent; the antiproliferation effect of Evo in colon cancer cells may be mediated by downregulating HIF-1 α at least, followed by partly decreasing the expression of IGF-1. Future investigations will focus on how Evo affect the IGFs signaling in colon cancer cells.

Acknowledgements

We thank Dr T.-C. He (University of Chicago Medical Center, USA) for generously providing all the recombinant adenoviruses and hypoxia responsive element reporter plasmid (pBGluc-HIF-1). The present study was supported in part by research grants from the Natural Science Foundation of China (NSFC 81372120 to B.-C.H.).

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