Evodiamine inhibits the proliferation of human osteosarcoma cells by blocking PI3K/Akt signaling

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Abstract. Osteosarcoma (OS) is the most common non-hematologic primary malignancy of bone, and multiple chemotherapeutic agents have been applied in the treatment of OS for over 40 years. Nevertheless, due to the poor prognosis of OS, it is essential to develop a novel treatment strategy. Evodiamine (EVO), a quinolone alkaloid extracted from the fruit of Evodia rutaecarpa, has been demonstrated to inhibit tumor cell proliferation. Thus, the main aim of the present study was to investigate the anti-proliferative and apoptosis-inducing effects of evodiamine (EVO) on human OS 143B cells, but also the possible mechanisms underlying these effects. The results of crystal violet staining, flow cytometry, western blot analysis and an in vivo experiment demonstrated that EVO exhibits significant inhibitory effects on cell proliferation, exhibits apoptosis-inducing effects and arrests the cell cycle in 143B cells. According to our findings of polymerase chain reaction (PCR), western blot analysis and recombinant adenoviral transfection, we confirmed that EVO upregulates both the protein and gene levels of phosphatase and tensin homolog (PTEN) in a concentration-dependent manner in 143B cells. Overexpression of PTEN reinforced the anti-proliferative effect of EVO in the 143B cells, while knockdown of PTEN upregulated PI3K/Akt signaling transduction and reversed the inhibitory effect of EVO on 143B cell proliferation. Further analysis indicated that EVO upregulated the expression of PTEN by inactivating PI3K/Akt signaling by decreasing phosphorylated Akt1/2. Based on the above results, we conclude that PTEN/PI3K/Akt signaling is involved in the inhibitory effect on human OS 143B cell proliferation by EVO.

Introduction

The most common non-hematologic primary malignancy of bone, osteosarcoma (OS), mainly occurs in children and adolescents (1,2). In fact, OS is the third most common malignancy in adolescents and accounts for over 56% of all bone sarcomas. OS is a primary sarcoma of adolescents, but it is believed to present as secondary neoplasms attributed to malignant transformation of Paget disease in older individuals (3-5). OS is often located in the metaphysis of long bones, and the most common sites have been reported to be linked to rapid bone growth in the younger age group, including the distal femur, proximal tibia and proximal humerus (4,6,7). Due to the application of pre-operative and post-operative multiagent chemotherapy combined with gradually developed surgical techniques for OS management, the long-term survival rate for localized OS has improved to ~60% (8), still, ~33% of OS patients present with recurrent disease and OS patients rarely achieve recovery status (9). OS patients commonly succumb to respiratory system failure due to the highly pulmonary metastatic feature of OS (4). Therefore, more efficient anti-tumor drugs for OS are urgently needed. Recently, traditional Chinese medicine (TCM) has received increased attention. A high curative effect has been noted when combining TCM and chemotherapy agents for various types of cancers.

Evodiamine (EVO; 8,13,13b,14-tetrahydro-14-methylin-dolo[2'3'-3,4]pyrido[2,1-b]quinazolin-5-[7H]-one), a quinolone alkaloid isolated from a Chinese herbal medicinal plant Evodia rutaecarpa (10), plays multiple roles in biological physiological process, such as anti-inflammatory (11), anti-nociceptive (12) and uterotonic effects (13). Currently, evidence indicates that EVO possesses anticancer activities. EVO is reported to hinder tumor development by inhibiting cancer cell proliferation and inducing cell apoptosis in various types of cancer cells, such as lung (14), acute leukemia (15-17), prostate (18-20) and cervical (17). Yang et al reported that EVO can upregulate the expression of phosphatase shatterproof 1 (SHP-1) (21), which is the key gene of IL-6-induced signal transducer and activator of transcription signaling 3 (STAT3) signaling leading to the suppression of survival and proliferation in hepatocellular carcinoma cells. Yet, little is known concerning the possible molecular mechanisms underlying the EVO-induced apoptosis of OS cells.

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Phosphatidylinositol 3 kinase (PI3K)/Akt signaling dominates many aspects of cell growth, cell apoptosis and cell cycle progression (22). The hyperactivated PI3K/Akt pathway has been identified as the most pivotal impetus in tumor development (23). Although the components of PI3K/Akt signaling are regulated by a range of factors, phosphatase and tensin homologue (PTEN), considered as an anti-oncogene (24), is the only known lipid phosphatase attenuating PI3K signaling transduction (23). Mutations of PTEN occur frequently in many types of carcinomas, such as prostate cancer (25) and human glioma (26). PTEN serves as a phosphatase for the lipid-signaling second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3) (27). The 3’-phosphate on PIP3 is hydrolyzed to generate PIP2 by PTEN, thereby, PTEN can directly antagonize PI3K/Akt signaling transduction (28).

In the present study, we investigated the possible mechanisms involved in the anti-proliferative and apoptosis-inducing effects of EVO in OS cells. Our data demonstrated that EVO inhibits the proliferation of OS cells and the antitumor effect of EVO is mediated by attenuating the PI3K/Akt signaling pathway through downregulation of the expression of PTEN.

Materials and methods

Cell culture and agents. The human OS cell line 143B was purchased from the American Type Culture Collection (ATCC) and EVO was obtained from Hao-xuan Bio-tech Co., Ltd. (Xi’an, China). EVO was dissolved in dimethyl sulfoxide (DMSO) for the in vitro experiment. VO-OHpic was purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). EVO was prepared with 0.4% carboxymethylcellulose sodium (CMC-Na) as suspension for the in vitro experiments. All other reagents were purchased from Sigma-Aldrich or Fisher Scientific, unless otherwise indicated. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS), 100 µg/ml of penicillin and 100 µg/ml of streptomycin at 37°C in 5% CO2.

Viability assay of the cell cultures. Cell viability was determined using the crystal violet assay, and it was conducted as previously described (29). In brief, 143B cells were plated in a 24-well plate and treated with predesigned concentrations of EVO. The cells were washed carefully with 4°C phosphate-buffered saline (PBS) and stained with 0.5% crystal violet formalin solution to assess the cell viability at room temperature for 20-30 min. For quantification and imaging, a 1 ml aliquot of 20% acetic acid was added to the well to dissolve the crystal violet and the plate was shaken for 20 min at room temperature. The absorbance was estimated at 570 nm. Each test was conducted in triplicate.

Flow cytometric analysis of cell cycle distribution and apoptosis. The cells were plated into 6-well plates. For cell cycle analysis, the cells were treated with the indicated concentrations of EVO or DMSO for 48 h. Then, the cells were washed with PBS (4°C), washed and collected with cold 70% ethanol (4°C) followed by washing with 50% and 30% ethanol and PBS. After the above operation, the cells were incubated with 1 ml of 20 mg/ml propidium iodide (PI) which contained RNase (1 mg/ml) in PBS for 30 min followed by fluorescence-activated cell sorting (FACS) assay. For the apoptosis assay, the cells were collected after treatment with the indicated concentrations of EVO for 48 h. The cells were washed with cold (4°C) PBS, followed by incubation with PI and Annexin V-EGFP according to the kit procedures (KeyGen Biotech Co. Ltd., Nanjing, China). Then, the processed cells were inspected using FACS assay.

Construction of the recombinant adenoviruses. Recombinant adenoviruses expressing PTEN (Ad-PTEN), GFP (Ad-GFP) and small interfering RNA (siRNA) fragments transfection were performed for the detection of PTEN (Ad-siPTEN). All these recombinant adenovirus were generated previously using the AdEasy technology, as described (30).

Reverse transcription and polymerase chain reaction analysis (RT-PCR). Human OS 143B cells were seeded in T25 flasks and treated with the indicated concentrations of EVO or solvent for 24 and 48 h. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and used to obtain cDNA templates by RT reaction. Then, the cDNAs were used as templates for determining the expression of target genes by PCR. The primers were: GAPDH forward, 5’-CAACGAATTTGGCCTACAGCA-3’ and reverse, 5’-AGGGGAGATTCAGTGTGGTG-3’; PTEN forward, 5’-CAACGAATTTGGCCTACAGCA-3’ and reverse, 5’-AGGGGAGATTCAGTGTGGTG-3’; PTEN forward, 5’-TAAAGGCAAAAGGCGCCTA-3’ and reverse, 5’-CGC CACTGAAACATTGGAATA-3’.

Western blot analysis. Subconfluent 143B cells were plated in 6-well plates and treated with the indicated concentrations of EVO or DMSO. At the pre-designed time points (24 and 48 h), the cells were washed with cold (4°C) PBS followed by being lysed with 300 µl lysis buffer. Then, the lysates were boiled for 10 min. Extracted total proteins were separated by SDS-PAGE and then transferred onto polyvinylidene difluoride (PVDF) membranes. Bovine serum albumin (BSA) (10%) was used to block the membranes at room temperature for 1 h, and then the membranes were blotted with primary antibodies. Finally, the images of the target proteins were detected with SuperSignal West Pico Chemiluminescent substrate.

Xenograft tumor model of human osteosarcoma. All animal experiments abided by the guidelines of the Institutional Animal Care and Use Committee of Chongqing Medical University (IACUC, Chongqing, China) and were approved by the IACUC. Athymic nude mice (female, 4- to 6-weeks old, 5/group) were obtained from the Animal Center of Chongqing Medical University (Chongqing, China). The cells (143B) were washed and resuspended in cold (4°C) PBS. Then the cells were injected into the backs of nude mice by subcutaneous injection (2.5x107 cells/injection). Three days after injection, the athymic nude mice were treated with different doses of EVO (20 and 50 mg/kg) or solvent by intragastric administration once a day. After a 4-week intragastric administration, the mice were sacrificed and the sarcoma samples were retrieved for histological evaluation.

Immunohistochemical staining and histological evaluation. Formulin (10%) was used to stain the retrieved tumor samples.
and paraffin was used to embed the slides respectively (31). For the immunohistochemical staining, the processed slides were deparaffinized and then rehydrated in a graduated manner. The deparaffinized sections were subjected to antigen retrieval. Then the background was blocked and the slides were incubation with a primary anti-proliferating cell nuclear antigen (PCNA) antibody, or mouse IgG as the control group. Finally, the primary PCNA was contrasted by 3,3'-diaminobenzidine (DAB) staining (31).

Statistical analysis. All the experiments were performed at least twice independently and the results were repeated in triplicate. The data are represented as mean ± standard deviation. Statistics from Microsoft Excel software were analyzed using the Student’s t-test among different groups. A P<0.05 was judged to be statistically significant.

Results

Evodiamine inhibits the proliferation of human osteosarcoma cells. Previous research has reported the antitumor effect of EVO on digestive system tumors. To explore whether EVO has the potential to be a novel pharmacotherapy for OS patients, we used crystal violet staining to evaluate the inhibitory effect of EVO on OS cell proliferation. The results revealed that EVO inhibited the proliferation of 143B cells in a concentration- and time-dependent manner (Fig. 1A and B). As shown in Fig. 1C, the activity of PCNA, which plays a key role in the cell cycle (32), was obviously suppressed. Data clearly indicated that EVO is capable of inhibiting the proliferation of 143B cells.

Evodiamine induces apoptosis in human osteosarcoma cells. We aimed to explore whether EVO induces apoptosis in 143B cells. We first employed FACS and western blot analysis to estimate the effect of EVO on the apoptosis induction in 143B cells. The results demonstrated that EVO induced the apoptosis of the 143B cells in a time- and concentration-dependent manner (Fig. 2A and B). For further investigation, Annexin V-EGFP staining was performed. Cells (143B) were treated with the indicated concentrations of EVO for 24 h followed by staining with Annexin V-EGFP fusion protein. The results demonstrated that EVO induced the apoptosis of 143B cells in a concentration-dependent manner (Fig. 2C). These results strongly indicate EVO can induce apoptosis in human OS 143B cells.

Evodiamine upregulates the expression of PTEN and arrests the cell cycle at G1 phase. PTEN has been proven to possess antitumor activity in many carcinomas for its vital role in the inhibition of cell proliferation (33,34). Therefore, we investigated whether PTEN exhibited anti-proliferative activity in 143B cells. The experimental results of the PCR and western blot analyses (Fig. 3B and C) demonstrated that EVO concentration...
dependently suppressed the expression of PTEN in the treatment group compared to the control. To detect whether these characteristics are linked with the cell cycle arrest, we analyzed the cell cycle data in the presence of EVO and/or solvent, and found that the percentage of cells in the G1 phase was significantly increased (Fig. 3A) compared to this percentage in the control. To detect whether these characteristics are linked with the cell cycle arrest, we analyzed the cell cycle data in the presence of EVO and/or solvent, and found that the percentage of cells in the G1 phase was significantly increased (Fig. 3A) compared to this percentage in the control.
control. Through further investigation, we also found that overexpression of PTEN potentiated the anti-proliferative effect of EVO, while knockdown of PTEN attenuated this effect of EVO in the 143B cells (Fig. 5A and B). Furthermore, PTEN inhibitor (VO-OHpic) reversed the anti-proliferative effect of EVO in the 143B cells (Fig. 5C and D). Based on these results, PTEN is thought to be a key gene involved in the anti-proliferative effect by EVO on human OS cells.

Evodiamine attenuates PI3K/Akt signaling transduction in 143B cells via upregulation of PTEN. The PI3K/Akt signaling pathway, which is involved in many physiological and pathological processes of tumors, is consistently upregulated in various malignant diseases (35,36). We explored whether PI3K/Akt signaling transduction participates in the EVO-induced inhibition of proliferation in the 143B cells. The results demonstrated that EVO downregulated the phosphorylation level of Akt1/2 in a concentration-dependent manner (Fig. 4A). The phosphorylation level of Akt1/2 was further decreased when the 143B cells were treated with a combination of EVO and exogenous expression of PTEN (Fig. 4C). Notably, the effect of EVO on the phosphorylation of Akt1/2 was reversed following knockdown of PTEN in the 143B cells (Fig. 4B). These results indicate that PI3K/Akt signaling transduction was attenuated by PTEN during the the EVO-induced inhibition of 143B cell proliferation.

Evodiamine inhibits human osteosarcoma growth in a xenograft tumor model. The results of the in vitro experiments demonstrated the anti-osteosarcoma effects of EVO and the corresponding molecular mechanisms. We estimated the anti-osteosarcoma effects of EVO with an in vivo experiment. To construct the xenograft tumor model, 143B cells were injected into the backs of athymic nude mice by subcutaneous injection.

Three days after the injection, the athymic nude mice were treated with different doses of EVO (20 and 50 mg/kg) or solvent via intragastric administration once a day for four weeks. The result revealed that the treatment groups exhibited significant suppression of tumor growth in a dose-dependent manner, compared to the control group (Fig. 6A). Hematoxylin and eosin (H&E) staining results showed that the EVO-treated groups exhibited a decreased cellularity in the tumor masses. The H&E histologic evaluation confirmed that the EVO-treated groups showed more necrotic cells than that in the control group (Fig. 6C). In addition, we also determined the expression of PCNA (Fig. 6B), and the results revealed that EVO significantly inhibited the proliferation of 143B cells in the EVO treatment group. Thus, these in vivo experimental results further demonstrated that EVO effectively inhibits OS growth.

Discussion

OS is the most frequent cancer of the bone (37). OS has a bimodal distribution; the first presentation may occur in adolescents and the second occurrence is in the elderly. To date, combination therapy of multi-antitumor drugs has been
introduced for OS treatment for the past 40 years, yet ~80% of patients with local early stage tumors progress to a metastatic status (2,38), and recurrent and/or metastatic OS tumors are extremely invasive and even resistant to traditional antitumor strategies (2). Therefore, identification of a novel treatment modality with more efficacy for OS patients is vital.

EVO, (Chinese name, Wu-Chu-Yu), a quinolone alkaloid, is the essential component extracted from the fruit of Evodia rutaecarpa (39,40). The role of EVO in the inhibition of tumor cell proliferation has been well-documented in a number of studies (13-20), while our knowledge of the antitumor effects of EVO on OS cells remains rather sparse. To the best of our knowledge, this is the first study to explore whether EVO possesses anticancer activities in OS cells, and the possible molecular mechanism involved. Based on the data presented in the present study, we found that EVO significantly inhibited the proliferation of 143B cells in a time- and concentration-dependent manner, even at a concentration of 0.5 µM (Fig. 1A and B). More importantly, it has been reported that EVO exhibits low cytotoxicity to human peripheral blood cells (41) and increasing evidence suggests that EVO has strong specificity to tumor cells. Mechanistically, our results also showed that overexpression of PTEN is responsible for the anti-proliferation effect of EVO on 143B OS cells. Furthermore, PI3K/Akt signaling transduction is inhibited via EVO-induced upregulation of PTEN.

According to previous research, EVO was found to strongly inhibit tumor progression by reducing the proliferation rate and inducing apoptosis in a variety of tumor cells (13-20). A series of analyses attempted to explain the underlying molecular mechanisms. A large number of trials have shown that EVO can cause cell cycle arrest at the G2/M phase in a majority of cancer cells (42), yet research on the cell cycle regulation by EVO in OS cells has not been carried out. Kan et al found that EVO activated the Cdc2/cyclin B complex to regulate cycle cycle arrest (G2/M) in human prostate cancer cell lines Du145 and PC3 (18). Conversely, EVO developed atypical apoptosis in murine fibrosarcoma L929 cells by cell cycle arrest at the G0/G1 phase (15). Our research obtained similar results (Fig. 3A), suggesting that the cell cycle regulation by EVO in 143B cells may be different from that in other types of tumor cells. In addition, Takada et al found...
that EVO exerts apoptotic activity by regulating NF-κB activation, resulting in the inhibition of NF-κB-controlled downstream genes, including cyclin D1, c-Myc, survivin, X chromosome-linked IAP (XIAP), Bcl-2 and Bcl-XL (43). Numerous experiments have demonstrated that EVO activated initiator caspases (caspase-8 and 9) and/or effector caspases (caspase-3) to induce the apoptosis in a variety of tumor cell lines including human colorectal carcinoma COLO205 and HT-29 cells (44) and human thyroid cancer cell line ARO (45). In addition, another study showed that EVO promoted translocation of apoptosis-inducing factor (AIF) into the nucleus of human leukemia u 937 cells (46). Collectively, EVO was found to induce the apoptosis of many tumor cell lines via caspase-dependent and caspase-independent signaling pathways (47). Moreover, Liu et al proved that EVO-mediated autophagy leading to increased apoptosis and reduction in cell viability was via a calcium-JNK signaling pathway (48).

Further study suggested that the downregulation of PI3K/Akt/caspase and Fas-L/NF-κB signaling pathways may be responsible for the apoptosis of A375-S cells induced by EVO (49). Although a number of studies have analyzed the possible molecular mechanisms by which EVO exhibits inhibitory action on tumorigenesis in various cancer cells, the inhibitory effect of EVO on OS cells and the possible molecular mechanism of the signaling transduction involved in the anticancer activity of EVO remain unclear.

PTEN, a tumor suppressor, is commonly mutated in the majority of human epithelial cell-derived tumors (50). On account of its phospholipid phosphatase characteristic, PTEN regulates various cellular processes and potentially affects the transduction of many other signaling pathways. PTEN is able to catalyze 3'-phosphate on PIP3 to form PIP2, thereby, PI3K/Akt signaling transduction is directly abrogated by PTEN (28). Despite the high aberration rate of PTEN in many types of cancers, it is controversial that PTEN mutations are linked with OS tumorigenesis. For example, the biallelic and monoallelic mutation rate of PTEN in OS samples was found to reach ~15 and 33%, respectively (51). The present results indicated that both the protein and gene levels of PTEN were increased concentration-dependently in the EVO-induced 143B cells. Exogenous PTEN strengthened the effect of EVO in 143B cells, while VO-OHpic treatment or knockdown of PTEN reversed the inhibitory effect on proliferation caused by EVO. Further research showed that EVO upregulated the expression of PTEN through the deactivation of PI3K/Akt signaling transduction by the reduction of phosphorylated Akt1/2 in 143B cells. The in vivo experiment results showed that EVO inhibited tumorgenesis. Collectively, PTEN/
PI3K/Akt signaling was found to participate in the inhibition of proliferation of human OS 143B cells by EVO. The present results suggest that EVO may be a promising antitumor strategy against human OS. Further studies are warranted to elucidate additional targets of EVO and the specific molecular mechanisms of the anticancer activity of EVO. In addition, a number of pre-clinical assessments should be carried out for the analysis of drug safety.

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References


