



Evodiamine Inhibits the Proliferation of BGC-823 and SGC-7901 Cells by Inducing Cell Cycle Arrest and Apoptosis in Gastric Cancer

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Abstract

Gastric cancer represents a major cause of cancer-related death worldwide. Although various tactics and anti-tumor drugs have been used to improve curative effects, five-year survival rate of lung cancer patients remains poor. Evodiamine, a sophora alkaloid, has been demonstrated to exert antitumor effects on many types of cancer. However, the molecular mechanism of evodiamine against gastric cancer has not been clearly elucidated. In this study, we investigated the anti-tumor activity and the underlying mechanisms of EVO on gastric cancer cells, and found that it significantly inhibited the proliferation of BGC-823 and SGC-7901 cells by inducing cell cycle arrest at G2/M phase and cell apoptosis in a dose- and time-dependent manner. Its molecular mechanism may be that it reduces the expression of cell cycle-promoting protein Cdc25C and promotes the expression of cell cycle inhibitor p53, as well as prompts the activity of caspases pathways, such as the expression level of cleaved caspase-3 and cleaved caspase-8; cleaved caspase-9 and cleaved PARP-1 are up-regulated, treated with EVO (10 μ M) at different points in time (0, 3, 6, 9, 12, 24 h). Collectively, our data demonstrated that EVO was a potential anti-tumor agent against gastric cancer.

Subject Areas

Gastroenterology, Hepatology

Keywords

Evodiamine (EVO), BGC-823 Cells, SGC-7901 Cells, Proliferation, Cell Cycle, Cell Apoptosis

1. Introduction

Gastric cancer is one of the most commonly digestive system carcinoma and remains the major cause of cancer-related death with characteristics of rapid progression, poor curative effect, easy metastasis, and unfavorable prognosis in the domestic and overseas. According to reports, the global incidence and the mortality rate of gastric cancer respectively rank fifth and third in clinical diagnosed malignant tumors [1]. In China, it has been ranking from the second among all cancers with 15.8% annual incidence ratio and 17.6% mortality ratio [2]. At present, the major treatment methods for gastric cancer mainly remain surgical resection, chemotherapy and targeted therapy, even though new treatment approaches are emerging [3]. Therefore, to search for safer and more effective therapy is an urgent problem in the treatment of gastric cancer.

Evodiamine (EVO) ($C_{19}H_{17}N_3O$) is one of the main active components in dried roots and ripe fruits of *Evodia rutaecarpa*, which has a wide range of pharmacological effects and has few obviously side effects or toxicity [4] (Figure 1). Recently, it has been extensively studied for its chemopreventive potential against various cancers, for instance, hepatocellular carcinoma [5] [6], breast cancer [7], colon cancer [8], lung cancer [9], prostatic cancer [10] and osteosarcoma [11]. The data have certificated that EVO exerts its anticancer activities through inhibiting cancer cell proliferation, accelerating apoptosis, inducing cell cycle arrest, suppressing invasion and metastasis, and reducing chemotherapy-induced toxicity [12]. The related research has shown that evodiamine has inhibited the effective proliferation of gastric cancer in SGC-7901 cells [13], but its specific anti-tumor molecular mechanism is still unclear. In this study, we examined the mechanism of anti-tumor effects of EVO in BGC-823 and SGC-7901 cells, finding that it exerted its anti-proliferation effects by inducing cell cycle arrest at G2/M phase and cell apoptosis in gastric cancer cells, and tried to clarify its associated molecular mechanisms.

2. Materials & Methods

2.1. Cell lines and Culture

The Human gastric cancer cell lines (BGC-823, SGC-7901) were purchased from the National Cell Resource Center (Beijing, China). All cell lines were propagated in DME/F-12 Medium (HyClone, USA), supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (HyClone, USA) in a humidified atmosphere with 5% CO_2 at 37°C. The cells with 80% confluence were treated by EVO (National Vaccine and Serum Institute, Beijing, China) of different concentrations.

2.2. Cell Viability Assay

The Cells were seeded into 96-well plates at a density of 3000 cells/well overnight to allow their adhesion to the plate, then treated with EVO at different concentrations (0, 5 μ M, 7.5 μ M, 10 μ M, 12.5 μ M, 15 μ M) for 24 h, 48 h, and 72 h,

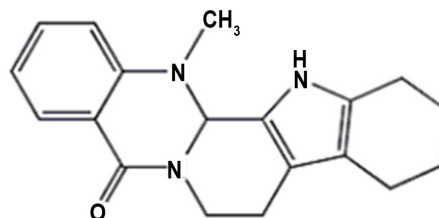


Figure 1. Chemical structures of evodiamine and its molecular formula ($C_{19}H_{17}N_3O$).

respectively. Five parallel wells for each concentration. At each time point, Cell Counting Kit 8 (CCK-8) agent (Dojindo, Japan) was added to each well and incubated at 37°C for 2 h. The numbers of viable cell were calculated by detecting the optical density (OD) at 450 nm using the microplate autoreader (Bio-Rad, CA, USA). IC_{50} was determined using the trimmed Spearman-Kärber method. Cell viability (%) = $\text{OD}_{\text{treated}}/\text{OD}_{\text{control}} \times 100$.

2.3. Cell Morphology Observation

The Cells were seeded into 6-well plates at a density of 3×10^5 cells/well overnight. Then, allowing their adhesion to the plate, the cells were treated with EVO ($10 \mu\text{M}$) for 48 h. The morphological changes of the cells were observed under a microscope.

2.4. Cell Colony Formation Assay

The cells were seeded at 500 cells/well in 6-well plates overnight, and then treated with EVO (the concentrations: $10 \mu\text{M}$) for 5 days. Discarding the supernatant, in every well fixed with 4% paraformaldehyde for 20 minutes and stained with 0.1% Giemsa for 15 minutes at room temperature. The numbers of colony were scanned and counted with the microscope. The colony formation rate that colonies contained more than 50 cells was calculated according to the following equation “Colony formation rate (%) = (colony counts/number of seeded cells) $\times 100\%$ ”.

2.5. Cell Cycle Analysis

The cell cycle was detected by using flow cytometry (FCM) with propidium iodide (PI)/RNase staining solution (BD Biosciences, San Jose, CA, United States). Cells were seeded in 6-well plates at 3×10^5 cells per well and treated with EVO (the concentrations: $10 \mu\text{M}$) for 48 h. Following by collecting cells, fixed in ice-cold 70% ethanol at 4°C overnight in darkness. Then washed with cold PBS for two times, and added with $100 \mu\text{L}$ RnaseA for 30 min at 37°C , the cells were suspended in PI Staining Buffer at 4°C for 20 min, finally analyzed on a flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

2.6. Annexin V-FITC/PI Staining

The apoptotic rate of BGC-823 and SGC-7901 cells were quantified with An-

nexin V-FITC/PI double staining solution (BD Biosciences, San Jose, CA, United States) by FCM. Cells were planted into 6-well plates at 3×10^5 cells per well and treated with EVO (10 μM) for 48 h. Then digested by trypsinization, washed with cold PBS for two times, and fixed cell suspension with $1 \times$ Binding Buffer. The cells were then stained with Annexin V-FITC/PI according to the manufacturer's instruction. After incubation for 10 min at room temperature in darkness, the apoptotic cells were detected with flow cytometry.

2.7. Western Blot Analysis

The cells were planted into T25 flask at 5×10^5 cells per flask and treated with EVO (10 μM) for 48 h, washed twice with PBS and then lysed with 300 μL of RIPA buffer for 30 min in ice. After centrifuged at 12,500 rpm for 20 min at 4°C , the supernatants were transferred to clean microcentrifuge tubes. The total protein concentration was determined using the bicinchoninic acid (BCA) (Beyotime, China) method. Equal amount of protein (30 μg) from each sample was separated by 10% or 12% SDS-PAGE and transferred onto PVDF membranes. After being blocked in defatted milk (5% in Tris-buffered saline with Tween-20 buffer) at 37°C for 1 h, the membrane was incubated with various primary antibodies overnight at 4°C and then with appropriate secondary antibodies for 1 h at room temperature. After each incubation period, the membrane was washed three times with TBST (Tris buffered saline with Tween-20). Signals were visualized by ECL detection reagents (Bio-Rad, CA, USA). The protein quantitative analysis was conducted by using the Image J software.

2.8. Statistical Analysis

Data are presented as the mean \pm SD, every experiment was performed at least 3 times. The difference between the groups was assessed using a one-way analysis of variance (ANOVA) or student's t-examination by the SPSS 22.0 software. A *P*-value of less than 0.05 indicates a statistical significance.

3. Results and Discussion

3.1. EVO Can Significantly Inhibit the Proliferation of Human Gastric Cancer Cells

The proliferation activity of the gastric cancer cells, which were treated with EVO at different concentration (0, 5 μM , 7.5 μM , 10 μM , 12.5 μM , 15 μM) for 24 h, 48 h, and 72 h, respectively, was evaluated by CCK-8 cell viability assay. The results were demonstrated that EVO can obviously decrease the viability of BGC-823 and SGC-7901 cells, with the increase of EVO concentration during the treatment time, compared with the control group. And it can inhibit the proliferative activity of BGC-823 and SGC-7901 cells in a dose- and time-dependent manner (**Figure 2(a)**). Similarly, the plate colony-formation assay showed that EVO can inhibit the colony formation of BGC-823 and SGC-7901 cells in a dose-dependent manner (**Figure 2(b)**). Meanwhile, the IC_{50} values of EVO were

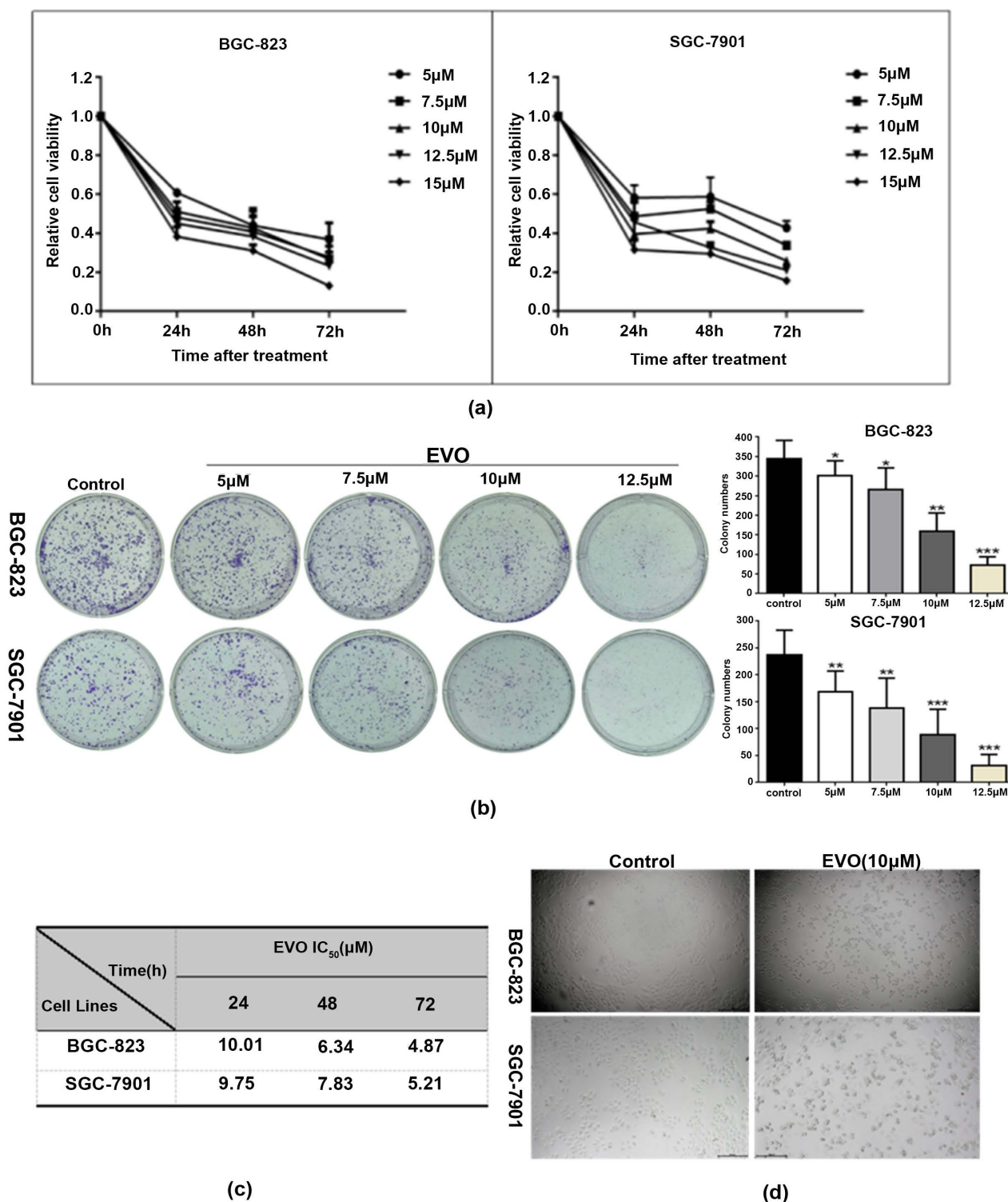


Figure 2. The effects of EVO on cell proliferation in gastric cancer cell lines BGC-823 and SGC-7901 cells. (a) Exponentially growing cells of BGC-823 and SGC-7901 cells were treated with EVO at the indicated concentrations (0 - 15 μM) for 24, 48, and 72 h; then, the percentages of viable cells were determined using CCK-8 assay. (b) The effect of EVO on the colony formation ability of BGC-823 and SGC-7901 cells. Data were shown as mean \pm SD from at least three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs. the Control group (culture medium only). (c) The IC₅₀ value of EVO for BGC-823 and SGC-7901 cells. (d) The morphological changes of BGC-823 and SGC-7901 cells which treated with EVO (10 μM) for 24 h were observed under the inverted microscope.

respectively calculated by Graph-pad Prism7.0 software. The IC_{50} values of EVO were respectively 10.01 μ M and 9.73 μ M in BGC-823 and SGC-7901 cells after intervention for 24 h (**Figure 2(c)**), so the follow-up experiments were used 10 μ M as the working concentration. The microscopic observation was shown that, the cell bodies were not reduced, rounded and shrunk, even separated from each other, but also there were a small amount of particulate matter appeared and more cell debris in the culture solution after 24 h, compared with the control group (**Figure 2(d)**). The results indicated that EVO has a better anti-gastric cancer activity.

3.2. EVO Induces Gastric Cancer Cell Cycle Arrest at the G2/M Checkpoint

We have verified that uncontrolled cell mitosis represents one of the hallmarks of cancer. Thus, we used the PI staining to inspect the effects of EVO on the cell cycle distribution upon BGC-823 and SGC-7901 cells by FCM. Luckily, cell cycle analysis revealed that the proportion of gastric cancer in G2/M phase was significantly increased after treatment for 24 h. Specifically, after all cells were treated with 10 μ M EVO for 24 h, the G0/G1 checkpoint ratio of BGC-823 and SGC-7901 cells were respectively decreased to $8.57\% \pm 2.83\%$ ($t = 10.681$, $P < 0.001$) and $23.11\% \pm 4.84\%$ ($t = 5.376$, $P < 0.01$); the S-phase ratio were respectively rose up to $19.31\% \pm 4.34\%$ ($t = 2.121$, $P < 0.05$) and $16.24\% \pm 10.23\%$ ($t = -0.196$, $P > 0.05$); the cell cycle ratio in G2/M checkpoint were increased to $54.13\% \pm 6.81\%$ ($t = -11.552$, $P < 0.001$) and $47.93\% \pm 9.18\%$ ($t = -10.776$, $P < 0.001$), compared with the control group (**Figure 3(a)** and **Figure 3(b)**). Thus, EVO mainly induces gastric cancer cell cycle impeded at the G2/M checkpoint.

3.3. EVO Reinforces the Apoptosis of Gastric Cancer Cells

To determine if EVO could synergistically aggravate the apoptosis of gastric cancer cells, Annexin V-FITC/PI staining and FCM method were applied to detect the apoptotic events. After treatment with EVO (10 μ M) for 24 h, the total apoptotic percentages were respectively $18.93\% \pm 5.78\%$ ($t = -12.728$, $P < 0.001$) and $17.24\% \pm 5.07\%$ ($t = -12.956$, $P < 0.001$), much higher than the $4.88\% \pm 1.96\%$ and $3.74\% \pm 2.49\%$ of the control group. Among them, EVO obviously induced the late apoptosis of cells, the apoptotic percentages were $16.13\% \pm 4.53\%$ ($t = -10.844$, $P < 0.001$) and $10.87\% \pm 5.67\%$ ($t = -8.854$, $P < 0.01$), respectively, in EVO-treated BGC-823 and SGC-7901 cells (**Figure 4(a)** and **Figure 4(b)**). Together, the findings indicated that EVO inhibits the malignant proliferation of gastric cancer cells by inducing apoptosis.

3.4. EVO Mediates the Activity of Apoptosis-Related Proteins and Cell Cycle-Related Proteins in Gastric Cancer Cells

Based on the above results, we found that the mechanism of EVO inhibiting the proliferation of gastric cancer cells may be that EVO can block the development of cell cycle and induce apoptosis. Moreover, we detected the level of p 53 signaling and apoptosis-related proteins as well as caspases activation which were

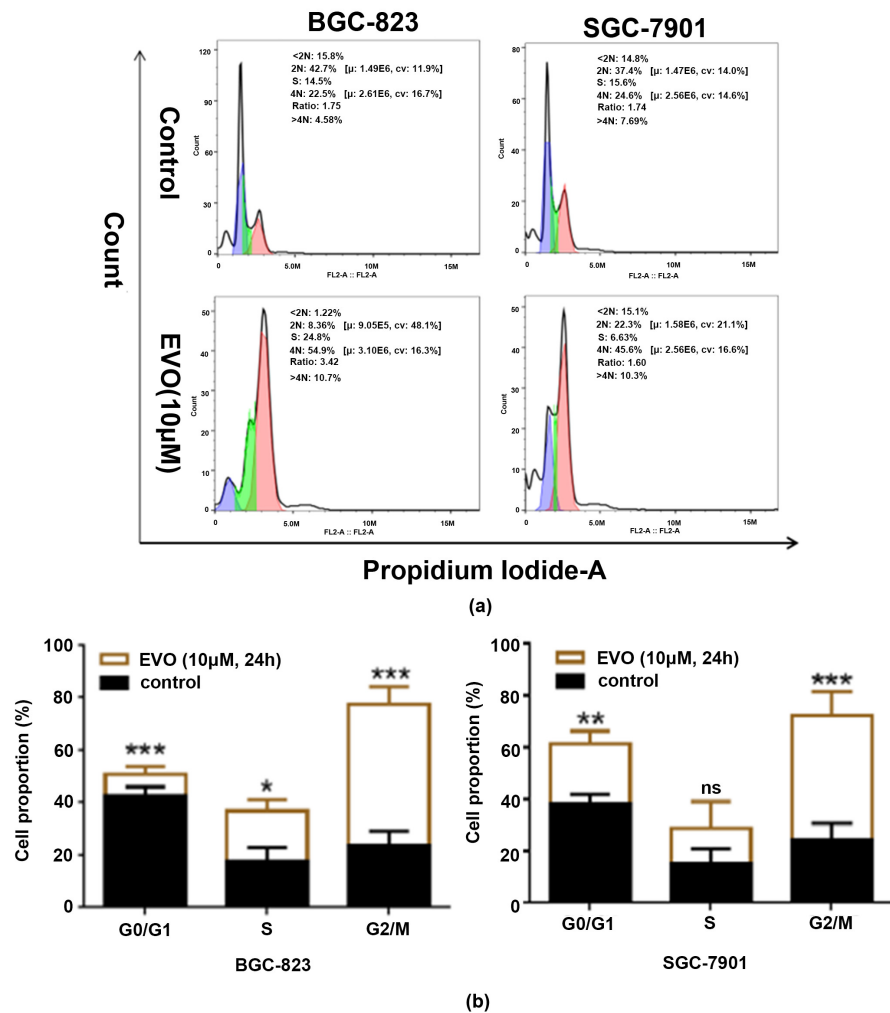


Figure 3. EVO induced gastric cancer cell cycle arrest at the G2/M phase. The length of each cell cycle phase was calculated and compared from three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. the Control group.

examined by western-blot analysis. After treatment with EVO (10 μM) at different points in time (0, 3, 6, 9, 12, 24 h), the results showed that the expression levels of p53, cleaved-caspase-3, cleaved-caspase-8, cleaved-caspase-9 and cleaved-PARP-1 were significantly up-regulated, but the expression level of cdc25c was markedly reduced, in the EVO-treated BGC-823 and SGC-7901 cells in a time-dependent manner compared with their control groups (Figure 5(a) and Figure 5(b)). Therefore, we concluded that EVO can induce gastric cancer cells apoptosis by regulating the activity caspases pathways, and accelerate cell cycle arrested at the G2/M checkpoint by changing the expression levels of p53 and cdc25c.

4. Conclusions

Evodiamine, extracted from dried roots and ripe fruits of *Evodia rutaecarpa*, has been demonstrated to exhibit various anticancer activities in a variety of tumor treatments. In this study, we assessed the anti-tumor effect of EVO and found

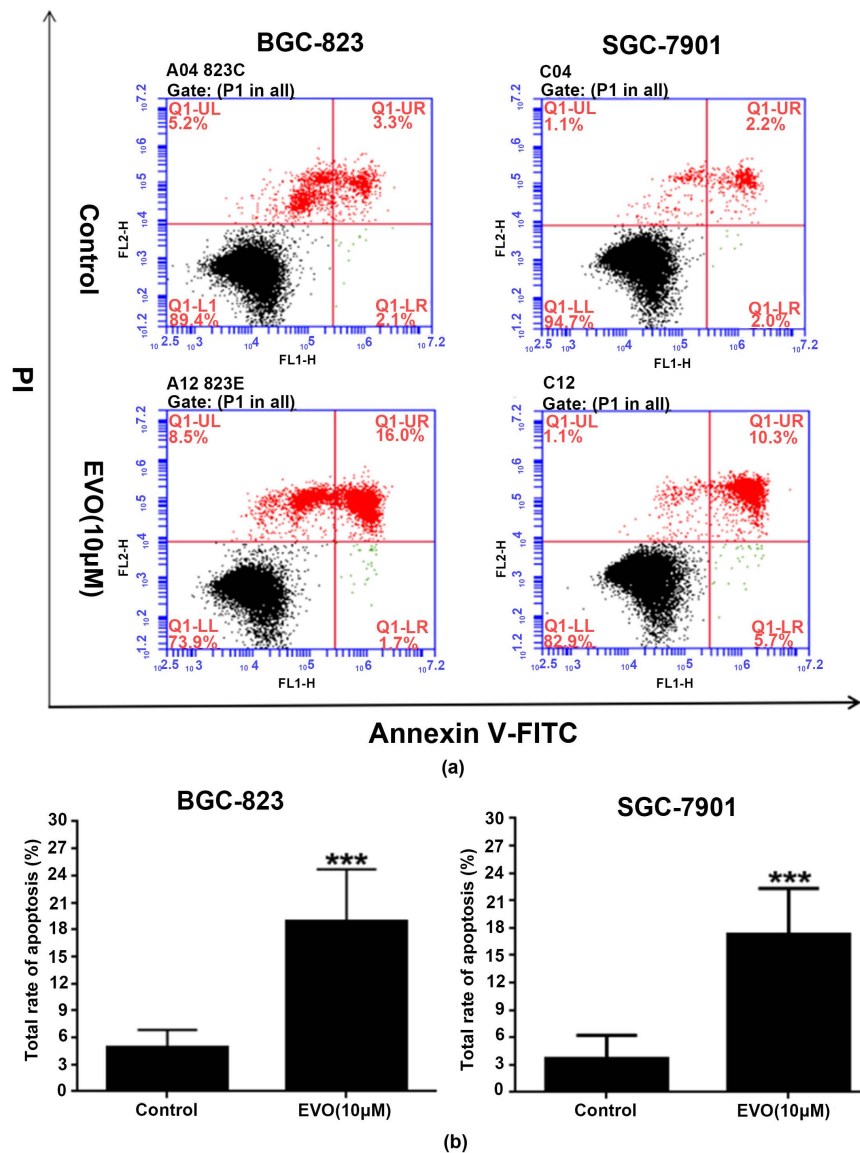


Figure 4. Apoptosis-induced effect of EVO was evaluated by FCM in BGC-823 and SGC-7901 cells, with Annexin V-FITC/PI staining. The apoptotic percentages from three independent experiments were analyzed and compared.

that it significantly inhibited the proliferation of BGC-823 and SGC-7901 cells by inducing cell cycle arrest at G2/M phase and cell apoptosis in a dose- and time-dependent manner.

Based on these preliminary observations, the molecular mechanisms underlying the anti-carcinogenic effects of EVO were further evaluated in gastric cancer cells. Abnormal cell cycle progression is the core link of malignant proliferation of tumor cells [14], so the process of regulating cell cycle is one of the effective ways to prevent abnormal proliferation of tumor cells. After the eukaryotic cells successfully passed the G1/S checkpoint, the periodic protein CyclinB1 began to accumulate and form a complex with CDC2 (CDK1) to propel the cells into the M phase [15]. The periodic protein *cdc25c* is very important to participate in the

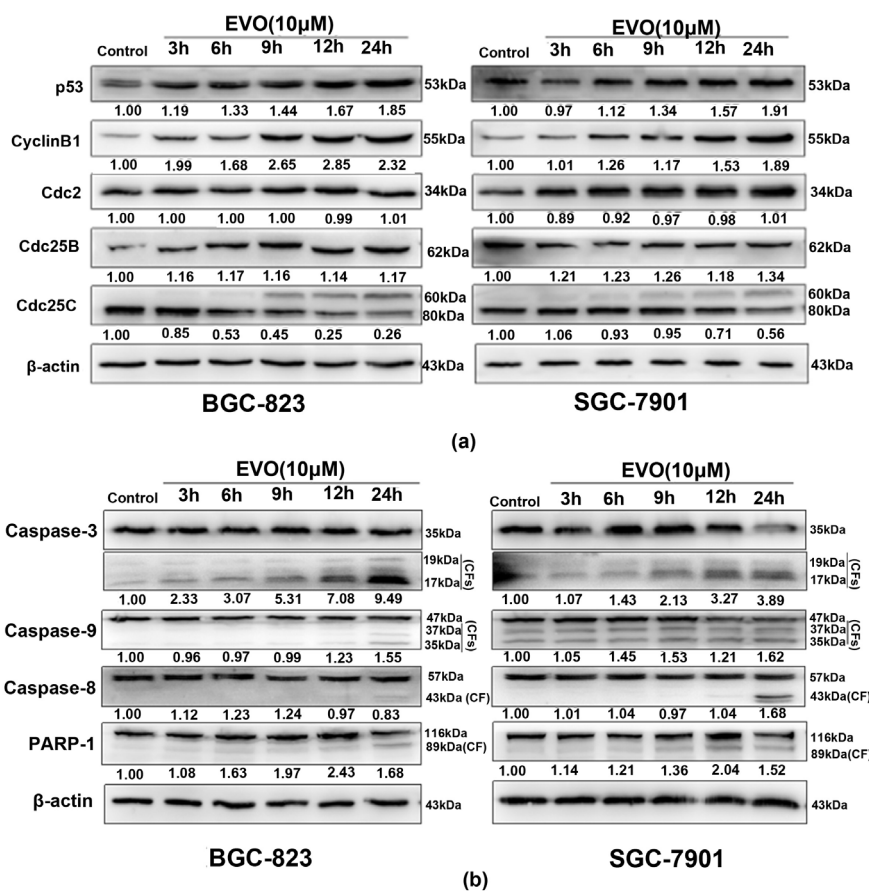


Figure 5. EVO suppressed the activity of p 53 signaling and caspases pathways in gastric cancer cells. BGC-823 and SGC-7901 cells were cultured in T25 flask and treated with EVO (10 μ M) at different points in time (0, 3, 6, 9, 12, 24 h), then the expression of the indicated factors was examined by Western blot analysis. β -actin was used as the loading control. The densitometry analysis of every factor was performed, normalized with the corresponding β -actin content.

activation of CDC2, which can inhibit the activation of the CDC2/CyclinB1 complexes, inducing cancer cell cycle arrest at the G2/M checkpoint [16] [17]. At the same time, the expression of cdc25c is regulated by cell cycle inhibition protein p53. It can combine with cdc25c promoter to inhibit its transcription and maintain the smooth operation of cell cycle [18] [19]. Similarly, the results have shown that EVO can increase the expression of p 53 and reduce the expression of cdc25c in BGC-823 and SGC-7901 cells after treatment for 24 h.

On the other way, apoptosis is the autonomous and procedural death process of cells regulated by genes, in which caspases pathways and p 53 play an important role in regulation of cell death [20] [21]. The results show that mitochondrial membrane potential change, death receptor pathway activation and others can cause caspases signal cascade activation to induce and amplify the effect of apoptosis cell [22] [23]. Caspase-3, as the most important effect factor of apoptosis, can induce the activation of shear death substrate PARP-1 [24] [25]. Importantly, our research also finds that EVO can raise the expression of cleaved

caspase-3, cleaved caspase-8, cleaved caspase-9 and cleaved PARP-1 in BGC-823 and SGC-7901 cells, treated with EVO at different time, thus having prompted the activity of caspases pathways, which induces gastric cancer cells apoptosis.

Above all, EVO may induce gastric cancer cell arrest at G2/M checkpoint by promoting the expression of cell cycle inhibitor p53 and raising p53 expression and reducing the expression of cell cycle-promoting protein Cdc25C, as well as prompting the activity of caspases pathways to induce the apoptosis of gastric cancer cells. It provides new thoughts for our future research in which EVO is a new potential anticarcinogen for treatment of gastric cancer.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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