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Original Article

Euscaphic acid inhibits proliferation and promotes apoptosis of nasopharyngeal carcinoma cells by silencing the PI3K/AKT/mTOR signaling pathway

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Abstract: *Rubus alceaefolius* Poir. has been used for the treatment of nasopharyngeal carcinoma (NPC) in China for many years. Euscaphic acid is an active component of *Rubus alceaefolius* Poir. However, the mechanism of action of euscaphic acid in NPC remains unclear. In this study, Euscaphic acid inhibited the proliferation of NPC cells, induced apoptosis, and led to cell cycle arrest in the G1/S phase. In addition, euscaphic acid inhibited the expression of phosphatidylinositide 3-kinases (PI3K), phosphorylated protein kinase B (p-AKT), and phosphorylated mammalian target of rapamycin (p-mTOR) p-mTOR in NPC cells. The activation of the PI3K/AKT/mTOR signaling pathway by IGF-1 promoted cell proliferation, inhibited apoptosis, and cell cycle arrest in NPC cells. In conclusion, we demonstrated that euscaphic acid reduced cell proliferation and induced apoptosis and cell cycle arrest in NPC cells by suppressing the PI3K/AKT/mTOR signaling pathway.

Keywords: Euscaphic acid, nasopharyngeal carcinoma, PI3K, AKT, mTOR

Introduction

Nasopharyngeal carcinoma (NPC) is an uncommon disease in most countries, with and age-adjusted incidence for both sexes of less than one person per population of 100000 [1]. However, the disease much more frequently in southern China, northern Africa, and Alaska. The people living in the province of Guangdong are especially prone to the disease [2]. Chemotherapy and radiotherapy are still the most common therapies for NPC: radiotherapy is the main treatment, but the 5-year survival rate is only approximately 50% [3]; and chemotherapy still has an important role in treatment, especially in advanced cases. However, these therapies may lead to side-effects [4, 5]. Therefore, it is necessary to look for more effective and less toxic drugs for NPC.

Traditional Chinese medicines (TCMs) have a long history of use for cancer treatment in China. In recent years, their use has become more accepted worldwide and the body of evidence supporting the anticancer effect of TCMs increased [6-9]. Previous studies have reported demonstrating the anticancer effects of celastrol, oridonin, and cinnamic acid through the promotion of apoptosis and the inhibition of proliferation of NPC cells [10-12]. *Rubus alceaefolius* Poir. is a traditional Chinese medicine compound found in Jiangsu, Fujian, Taiwan, Guangdong, and the Guangxi Province. It has wide range of pharmacological activities, including antibiotic [13, 14], anti-inflammatory [15], antitumor [16, 17], hepatoprotective [18, 19] effects. Traditionally, it is used in the treatment of NPC in southern China [20]. Euscaphic acid is a triterpene from the root of the *R. alceaefolius* Poir. It has been shown that euscaphic acid has DNA polymerase inhibitory activity and inhibits cell growth [21]. However, the role and mechanism of euscaphic acid against NPC remain unclear.

The PI3K/AKT/mTOR pathway is an important intracellular signal transduction pathway. It has already been reported to be related to cell activ-

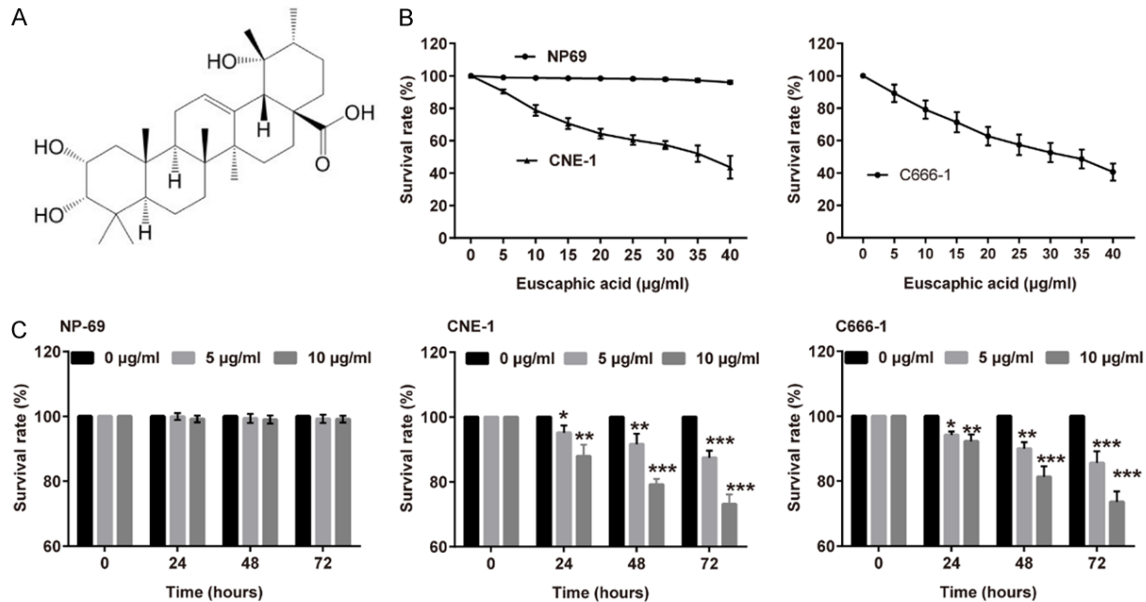

Figure 1. Euscaphic acid inhibited the proliferation of CNE-1 and C666-1 cells. A. The chemical structural formula of Euscaphic acid. B. CNE-1, C666-1, and NP69 cells were analyzed in the CCK8 assay after exposure to different concentrations of euscaphic acid. C. NP69 cells, CNE-1 cells, and C666-1 cells were analyzed in the CCK8 assay after treatment with euscaphic acid for 24, 48, and 72 hours. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ vs 0 μg/ml group.

ities such as proliferation, migration, and invasion [22-24]. Signaling through the PI3K/AKT/mTOR pathway can be initiated by several mechanisms, all of which increase the activation of the pathway in cancer cells. We aimed to identify the relationship between euscaphic acid and the PI3K/AKT/mTOR signaling pathway.

In this study, we investigated the effects of euscaphic acid on the proliferation, cell cycle, and apoptosis of NPC cells. Subsequently, we analyzed the potential regulatory mechanism of the PI3K/AKT/mTOR signaling pathway in NPC cells.

Materials and methods

Cell cultures and drugs treatment

NP69 (non-transformed nasopharyngeal epithelial cells derived from the human nasopharynx), C666-1, and CNE-1 human NPC cell lines were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The three cell lines were cultured in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO BRL, NewYork, USA). All NPC cells were cultured at 37°C in a humidified incubator with at atmosphere containing 5% CO_2. Euscaphic acid was purchased from PUSH BIO-TECHNOLOGY (http://www.push-herbchem.com/, $C_{30}H_{48}O_5$, Chengdu, Sichuan, China; Chemical structural formula was showed in **Figure 1B**) and was dissolved in absolute ethanol. A euscaphic acid stock solution (1 mg/mL) was prepared with a final ethanol concentration of ≤2% and sterilized by passing through a membrane filter with a pore size of 0.22 μm.

Proliferation assays

Cell Counting Kit-8 (CCK-8; Beyotime, Shanghai, China) was used to evaluate the growth of CNE-1, C666-1, and NP69 cells were incubated with different concentrations of euscaphic acid (0, 5, 10, 15, 20, 25, 30, 35, and 40 μg/mL) in accordance with the manufacturer's instructions. CNE-1, C666-1, and NP69 cells (4×10^3 cells/well) were seeded into 96-well plates in a final volume of 100 μL medium and cultured for 24, 48, or 72 h. At the end of the incubation period, CCK-8 solution (10 μL) was added to each well, mixed gently, and incubated for 4 h at 37°C. Subsequently, the optical density at 450 nm was measured by using a microplate reader. The survival rate of the non-treatment cells (0 μg/ml Euscaphic acid) was defined as

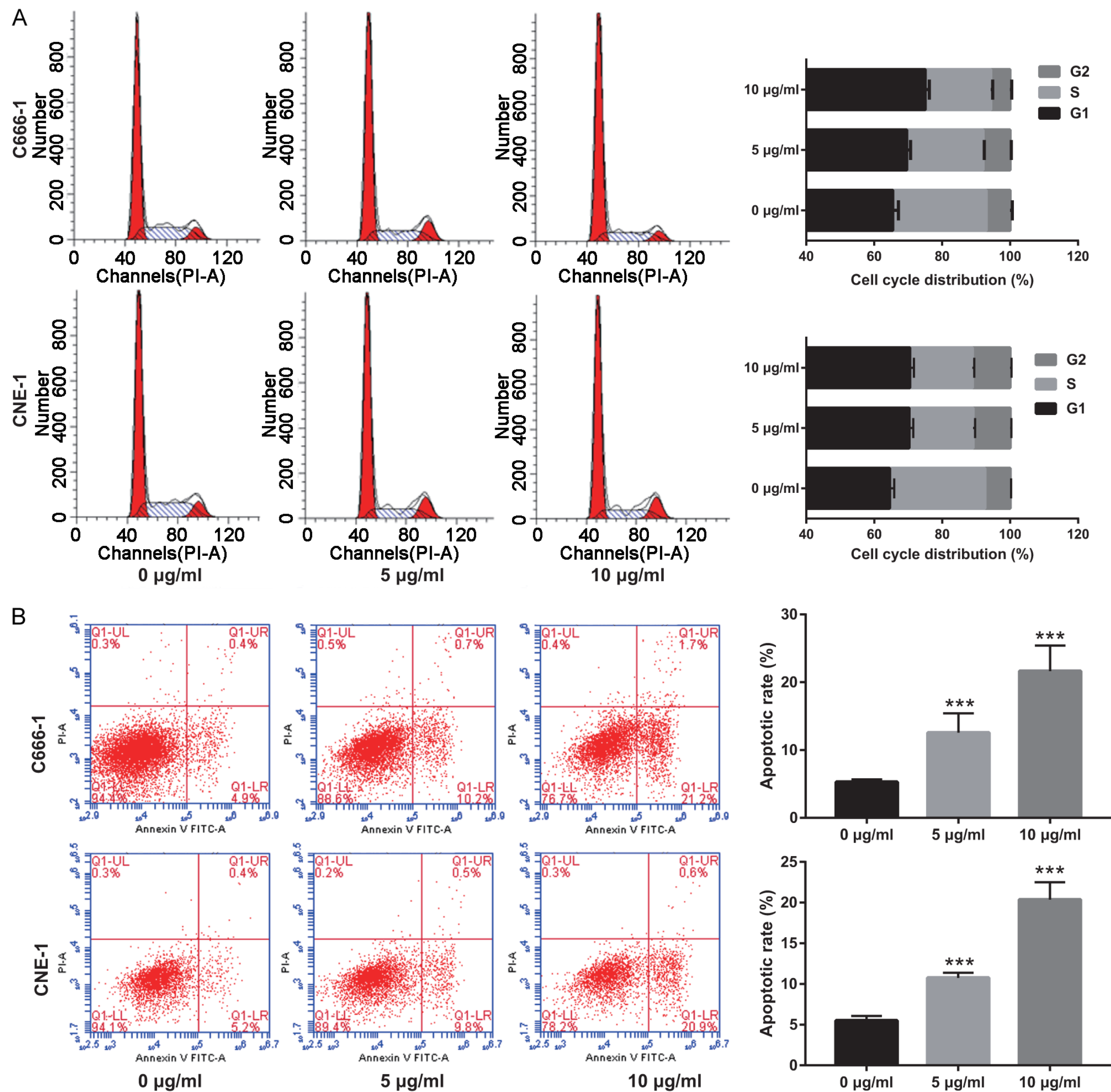

Figure 2. Euscaphic acid (0, 5, and 10 μg/mL) induces apoptosis and cell cycle arrest in CNE-1 and C666-1 cells. A. The effect of euscaphic acid on the cell cycle of CNE-1 and C666-1 cells was detected by flow cytometry. B. The effect of euscaphic acid on apoptosis in CNE-1 and C666-1 cells was detected by flow cytometry. ***$P<0.001$.

100%, and the survival rate of cells from all other concentrations of Euscaphic acid was calculated separately from that of the control group. All assays were performed in triplicate.

Cell cycle analysis

The cell cycle was detected by using flow cytometry. Briefly, the cells were treated with euscaphic acid at various concentrations (0, 5, and 10 μg/mL) for 48 h, and then harvested, washed twice with 1× PBS, and re-suspended in 200 μL 1× PBS. The cells were fixed in 4 mL ice-cold 75% ethanol at 4°C overnight and stained with 200 μL propidium iodide (50 μg/mL, Sigma-Aldrich), 20 μL RNase (1 mg/mL, Sigma-Aldrich) was added, and the cells were incubated in a 37°C water bath for 15-20 min to remove RNA. The cells were then analyzed by using a flow cytometer (Cytomics FC 500 MPL, Beckman Coulter). All assays were performed in triplicate.

Apoptosis assays

The cells were incubated with euscaphic acid at different concentrations (0, 5, and 10 μg/mL) for 48 h and assayed to determine the degree of induction of apoptosis. After incubation, the cells were harvested and washed twice with

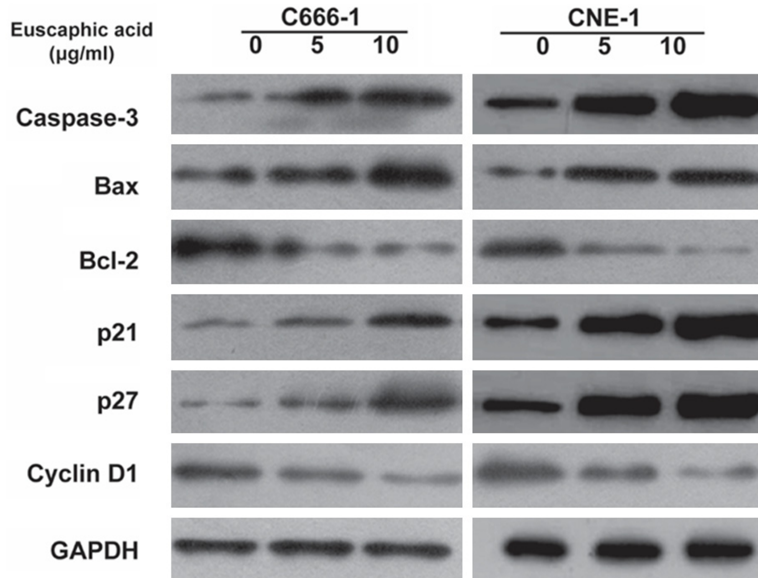

Figure 3. Western blotting of apoptosis-associated and cell cycle-associated proteins after euscaphic acid treatment.

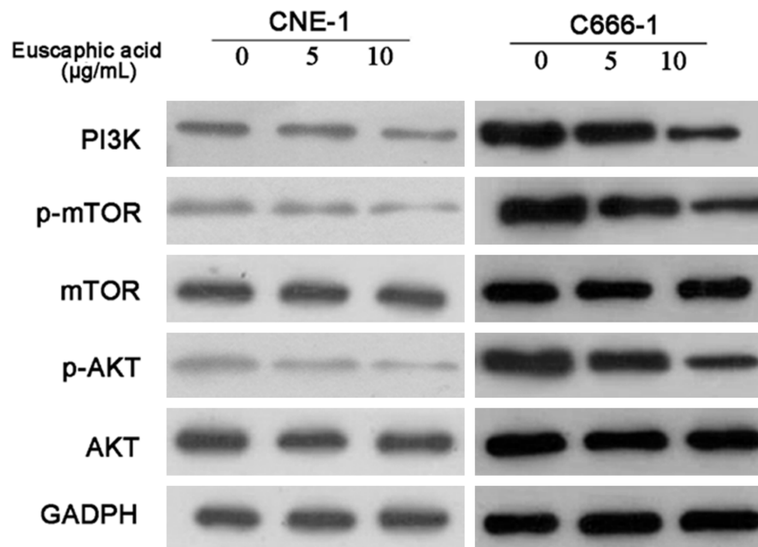

Figure 4. Western blotting of PI3K, p-AKT, AKT, p-mTOR, and mTOR after euscaphic acid treatment.

cold 1× PBS, after which 1×10^5 cells/mL were re-suspended in 200 μL binding buffer, stained with 5 μL Annexin-V and PI (BD Biosciences) for 15 min in the dark at 25°C, and analyzed by using a flow cytometer (Cytomics FC 500 MPL, Beckman Coulter).

Western blotting

Total cellular protein was extracted by using pre-chilled RIPA buffer (Beyotime) containing protease inhibitors. Equal amounts of protein samples were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. Non-specific binding to the membrane was blocked by incubation in PBS with 0.1% Tween-20 (PBST) containing 5% non-fat milk at 25°C for 2 h. For the protein analysis, the membrane was incubated with the corresponding primary antibody overnight at 4°C, washed with PBST, and then incubated with the secondary the horseradish peroxidase (HRP)-conjugated antibody (1:10,000) at 25°C for 2 h. The membranes were rinsed and the protein bands were visualized by the application of enhanced chemiluminescence reagent (Thermo Scientific). GAPDH was used as the loading control. The sections were incubated with the primary antibodies (anti-human phosphorylated PI3K (p-PI3K) polyclonal antibody (1:10,000; Santa Cruz), anti-human total PI3K (t-PI3K) polyclonal antibody (1:4,000; Santa Cruz), anti-human phosphorylated AKT (p-AKT) polyclonal antibody (1:10,000; Santa Cruz), anti-human total AKT (t-AKT) polyclonal antibody (1:4,000; Santa Cruz), anti-human mTOR polyclonal antibody (1:5,000; Santa Cruz), and anti-human GAPDH polyclonal antibody (1:1,0000; Abcam), respectively.

Statistical analysis

The SPSS 19.0 software package (IBM, Chicago, IL, USA) was used to calculate statistical analyses. All data were presented as the mean ± SD, and Student's t-test or one-way analysis of variance (ANOVA) was used to compare the difference between two or more groups. *P* values of <0.05 were considered statistically significant. Graphs were created by using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). IC50 was calculated using GraphPad Prism 7.

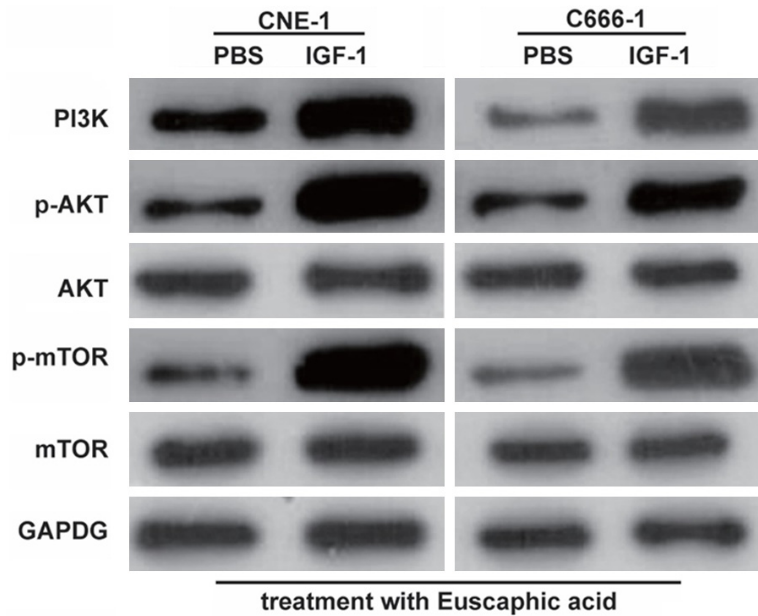

Figure 5. Activation of the PI3K/AKT/mTOR pathway by IGF-1. The effect of euscaphic acid treatment supplemented with IGF-1 on the expression of PI3K, p-AKT/AKT, and p-mTOR/mTOR in CNE-1 and C666-1 cells as measured by western blotting.

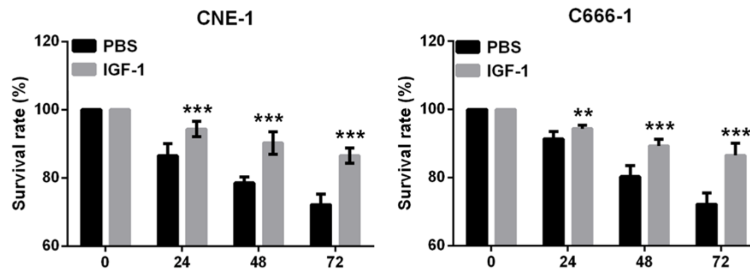

Figure 6. Activation of the PI3K/AKT/mTOR pathway reversed the effect of euscaphic acid on cell proliferation. The effect of euscaphic acid supplemented with IGF-1 on the ability of cell proliferation in CNE-1 and C666-1 cells that were detected by CCK8 analysis. ***P*<0.01, ****P*<0.001.

Results

Euscaphic acid inhibited the proliferation of CNE-1 and C666-1 cells

CNE-1, C666-1, and NP69 cells were treated with euscaphic acid (0, 5, 10, 15, 20, 25, 30, 35, 40 μg/mL) for 48 h, and cell viability was determined using the CCK8 assay (**Figure 1A**). The CCK8 assay showed that proliferation was significantly suppressed by an increase in the concentration of euscaphic acid in both CNE-1 and C666-1 cells compared to that in NP69 cells. Additionally, result showed that for C666-1 and CNE-1 cells, the Euscaphic acid concentration to reduce cell viability to 50% was about 36.86 μg/ml (IC50=36.86) and 33.39 μg/ml (IC50=33.39), respectively. We chose 0, 5, and 10 μg/mL euscaphic acid for the subsequent study as these concentrations exerted only low cytotoxicity against the cells, thereby excluding the anti-proliferation effect of high-dose euscaphic acid in cancer cells. To investigate whether euscaphic acid inhibited the proliferation of CNE-1, C666-1, and NP69 cells, the CCK8 assay was performed significantly indicated that proliferation was inhibited in CNE-1 and C666-1cells arrest in a dose- and time-dependent manner, but not in NP69 cells (**Figure 1B-D**).

Euscaphic acid induces apoptosis and cell cycle arrest in CNE-1 and C666-1 cells

To investigate the effect of euscaphic acid on the induction of apoptosis and cell cycle arrest in CNE-1 and C666-1 cells, flow cytometric analysis was used after treatment with different concentrations of euscaphic acid (0, 5, and 10 μg/mL). At 0 μg/mL, no effects on the cell cycle of NPC cells were observed. At 5 or 10 μg/mL, the proportion of cell cycle arrest in the G1/S phase was altered (**Figure 2A**). In addition, the results indicated that an increase in euscaphic acid promoted the induction of apoptosis in CNE-1 cells (**Figure 2B**). The proportion of apoptotic cells increased with an increase in euscaphic acid concentration. Similar results were found in the C666-1 cells. The results confirmed that euscaphic acid significantly promoted apoptosis and inhibited the progression of the cell cycle in CNE-1 and C666-1 cells in a dose-dependent manner. Apoptotic and cell cycle-associated proteins were measured by western blotting. The results clearly illustrated that Bax, caspase-3, p21, and p27 were increased, whereas Bcl-2 and cyclin D1

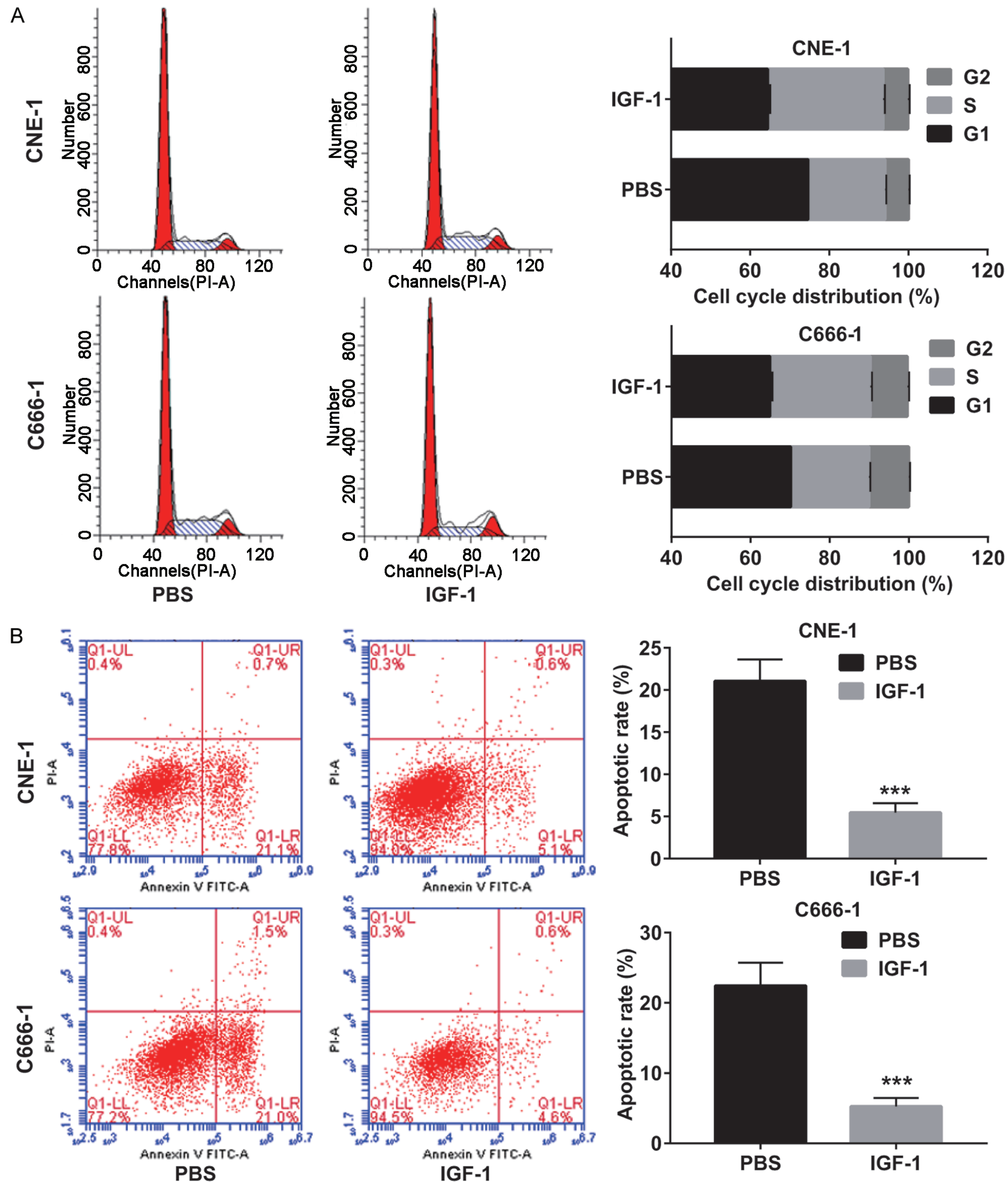

Figure 7. Activation of the PI3K/AKT/mTOR pathway reversed the effect of euscaphic acid on cell cycle and apoptosis. A. The effect of euscaphic acid supplemented with IGF-1 on the cell cycle arrest of CNE-1 and C666-1 cells as detected by flow cytometry. B. The effect of euscaphic acid supplemented with IGF-1 on apoptosis in CNE-1 and C666-1 cells was detected by flow cytometry. ***P<0.001.

were decreased after euscaphic acid treatment (**Figure 3**).

Euscaphic acid inhibited the PI3K/AKT/mTOR signaling pathway

The PI3K/AKT/mTOR pathway is an important signaling pathway for the regulation of cell proliferation and the induction of apoptosis in cancer cells. To determine the effects of euscaphic acid, we detected the proteins in this pathway after treatment of cells with different concentrations of euscaphic acid (0, 5, and 10 μg/mL) by western blotting. As shown in **Figure 4**, euscaphic acid clearly suppressed the protein expression of PI3K, p-AKT/AKT, and p-mTOR/

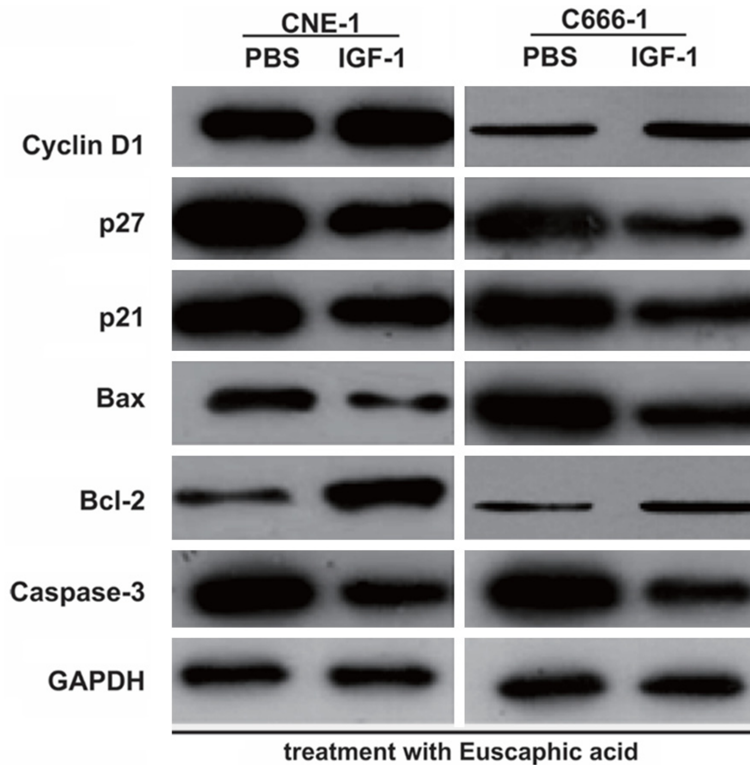

Figure 8. Effect of euscaphic acid supplemented with IGF-1 on the expression of apoptosis-associated and cell cycle regulatory proteins in CNE-1 and C666-1 cells as measured by western blotting after activation of the PI3K/AKT/mTOR pathway.

mTOR in a dose-dependent manner in CNE-1 and C666-1 cells. Euscaphic acid inhibited the NPC through the PI3K/AKT/mTOR signaling pathways. The expression of the components of the PI3K/AKT/mTOR pathways in both CNE-1 and C666-1 cell lines was detected by western blotting after treatment with different concentrations of euscaphic acid (0, 5, and 10 μg/mL).

Activation of the PI3K/AKT/mTOR pathway to reverse the effect of euscaphic acid

To confirm if euscaphic acid induced apoptosis and cell cycle arrest in CNE-1 and C666-1 cells through the suppression of the PI3K/AKT/mTOR signaling pathway, we further assessed whether the activation of the PI3K/AKT/mTOR signaling pathway by insulin-like growth factors-1 (IGF-1), a PI3K/AKT/mTOR signaling pathway activating agent, could reverse the effects of euscaphic acid in CNE-1 and C666-1 cells. First, we measured the expression of PI3K, p-AKT/AKT, and p-mTOR/mTOR in CNE-1 and C666-1 cells after treatment with euscaphic acid (10 μg/mL) and when supplemented with IGF-1 or PBS. The results clearly showed that supplementation with IGF-1 increased the expression of PI3K, p-AKT/AKT, and p-mTOR/mTOR in CNE-1 and C666-1 cells compared to that when supplemented with PBS (**Figure 5**). The CCK8 assay suggested that supplementation with IGF-1 significantly promoted cell proliferation in both CNE-1 and C666-1 cells compared to that when supplemented with PBS (**Figure 6**). The flow cytometry assay indicated that supplementation with IGF-1 significantly promoted cell cycle progression and inhibited cell apoptosis both in CNE-1 and C666-1 cells (**Figure 7**). The results of the western blot analysis clearly showed that the expression of Bcl-2 and Cyclin D1 in the cells supplemented with IGF-1 was significantly increased, whereas the expression of Bax, caspase-3, p21, and p27 was decreased, compared to that in cells supplemented with PBS (**Figure 8**).

Discussion

The antitumor activity of euscaphic acid has been previously reported. Banno *et al.* [25] confirmed the inhibitory effect of euscaphic acid on *in vivo* two-stage mouse skin carcinogenesis. Kim *et al.* [26] reported that tumor cells treated with different concentrations of euscaphic acid exhibited a significant decrease in proliferation in a dose-dependent manner. Rocha *et al.* [27] confirmed the antitumor activity of euscaphic acid in different types of cancer and showed that it induced apoptosis in a caspase-dependent manner. Nonetheless, there has been little research on the effects of euscaphic acid on NPC. In this study, we found that euscaphic acid inhibited the proliferation of CNE-1 and C666-1 cells, and induced apoptosis and cell cycle arrest, unlike in NP69 cells, in a dose- and time-dependent manner. We also found that euscaphic acid treatment clearly increased the expression of Bax, caspase-3, p21, and p27,

whereas the expression of Bcl-2 and cyclin D1 was reduced. The results suggested that euscaphic acid exerts anticancer effects through the inhibition of proliferation and the induction of apoptosis in NPC.

Subsequently, we analyzed the antitumor effects of euscaphic acid in the NPC cells through the changes in the expression of PI3K, p-AKT, and p-mTOR to evaluate changes in the PI3K/AKT/mTOR signaling pathway. The PI3K/AKT/mTOR signaling pathway has an important role in NPC, promoting cell proliferation and inhibiting apoptosis [28]. In recent studies, many mechanisms of silencing the PI3K/AKT/mTOR signaling pathway by traditional Chinese medicine have been reported to be related to anticancer effects [29, 30]. We found that the activation of the PI3K/AKT/mTOR signaling pathway by IGF-1 reversed the effect of euscaphic acid on proliferation, the cell cycle, and apoptosis. These results showed that euscaphic acid regulated the PI3K/AKT/mTOR signaling pathway to exert anticancer effects in NPC cells.

In summary, we demonstrated the euscaphic acid inhibits the proliferation and promotes apoptosis in NPC cells through the silencing of the PI3K/AKT/mTOR signaling pathway. We believed that euscaphic acid has the potential for development as an anticancer drug for NPC. However, before euscaphic acid can be developed as an anticancer agent, its antitumor activity should be investigated *in vivo* and in clinical settings.

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Disclosure of conflict of interest

None.

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