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Original Article

Inhibition of ovarian cancer proliferation and invasion by pachymic acid

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Abstract: To determine the effect of pachymic acid (PA) on proliferation, cell cycle, and invasion in human ovarian carcinoma cell lines HO-8910 and explore some possible mechanisms, HO-8910 cells was treated with different concentrations of PA (0.5, 1, 2 μM). CCK-8 assay, propidium iodide staining, was applied to measuring the growth inhibiting rates of HO-8910 cells. Cell cycle was measured by flow cytometry. In addition, the activity of PA against HO-8910 cells invasion was evaluated in transwell assay. Western blot detected the proteins expression of E-cadherin, β-catenin and COX-2 of different groups treated with PA in different concentrations (0.5, 1, 2 μM) for 48 h. Our results showed that PA could effectively inhibit the in vitro growth of HO-8910 cells in dose-dependent manners in 72 h, suppressed migration and invasion of HO-8910 cells in concentration-dependent manners at 24 h, caused the increased accumulation of G1 phase cells, and caused down-regulation of β-catenin and COX-2 and up-regulation of E-cadherin expression level. Taken together, it could conclude that PA might inhibit proliferation and invasion of ovarian carcinoma cell through decreasing β-catenin and COX-2 expression and increasing E-cadherin exprssion.

Keywords: HO-8910 cells, pachymic acid, proliferation, invasion

Introduction

Ovarian cancer is one of the most common gynecological malignancy that with high mortality and pose a serious threat to women health [1, 2]. The high mortality mainly attributes to metastases throughout the peritoneal cavity in 70% of women with ovarian cancer [3, 4]. Clinically, there are only 20% of ovarian cancer patients diagnosed limited to the ovaries (stage I) and approximately 90% of these patients can be cured by currently available therapy [4, 5]. Unfortunately, the majority of ovarian cancer patients are found in advanced stage (III and IV) [1]. And at this stage, ovarian cancer cells have been metastasized to other organization, which makes this disease difficult to treat [6]. Over these years, although chemotherapy has improved the overall survival of advanced stage ovarian cancer patients, the overall survival remains poor [7]. Therefore, there is an exceedingly critical need to search for useful drugs or therapeutic methods against ovarian cancer disease.

Studies have reported that E-cadherin, β-catenin and COX-2 play a key role in ovarian cancer carcinogenesis [1, 8, 9]. In particularly, E-cadherin mediated cell-cell adhesion is important for the progress of ovarian cancer metastases and loss of E-cadherin can promote the ovarian cancer metastases [8, 10]. As the core molecule of adherens junctions, the extracellular tail of E-cadherin mediates cell-cell adhesion and its cytoplasmic part interacts with the other adheresion components such as β-catenin [11, 12]. β-catenin has two different functions including cadherin-mediated adhesion at the plasma membrane by complexed with the cytoplasmic tail of E-cadherin, and as a main nuclear signal in the Wnt/β-catenin pathway [13, 14]. β-catenin activates the target gene expression by Wnt signalling and promotes cell growth such as proliferation and metastases [1, 15]. In addition, COX-2 has been demonstrated to be as a target gene of the Wnt pathway and promote ovarian cancer cell growth [9].

Pachymic acid (PA) is a lanostane-type triterpenoid from poria cocos, which has been reported to possess anticancer, anti-inflammatory, anti-reject and anti-oxidant activity in various cancer and inflammatory models [16-23]. According to publishing data, PA has been shown to be effective in preventing breast cancer cell invasion in vitro by targeting NF-κB signalling and inhibiting lung cancer cell growth by suppressing proliferation and inducing apoptosis in A549 cells [20, 21]. In addition, PA is found to suppress the growth of human prostate cancer cells by inhibiting phospholipase A_2 [16]. These previous research results showed that PA is a potential anti-cancer agent.

At present, the potential of PA against ovarian cancer is poorly known. Hence, our study is to explore the effect of PA on proliferation, cell cycle, and invasion of human ovarian carcinoma cell lines HO-8910 cell. Our finds will provide a new promising chemotherapy for treating ovarian cancer.

Materials and methods

Cell lines and reagents

Human ovarian cancer cell line HO-8910 was obtained from the Shanghai cell bank of Chinese academy of sciences (Shanghai, China). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Biochem (Shanghai, China); Matrigel were purchased from BD Biosciences (San Jose, CA); Transwell well culture chambers were purchased from Corning (New York, USA); Propidium iodide was purchased from Beyotime (Jiangsu, China) Antibodies against GAPDH and E-cadherin were obtained from Santa Cruz Bio-technology (Santa Cruz, CA). β-catenin and COX-2 were purchased from Cell Signaling Technology (Beverly, MA) and Abcam (Cambrige, MA), respectively; goat-anti-rabbit/rat horseradish-peroxidase-conjugated secondary antibodies were purchased from Beyotime (Jiangsu, China). In addition, pachymic acid was isolated from P. cocos and characterized previously as previous described methods [19, 21].

Cell culture

HO-8910 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and antibiotics (100 U/ml penicillin G and 100 mg/ml of streptomycin) at 37°C in a 95% humidified atmosphere and 5% CO_2.

Cell viability assay by cck-8

HO-8910 cells were seeded in the 96-well plates (4×103 cells/well) and cultured for 24 h at 37°C. After that, cells were treated by PA with desired concentrations for 24 h and then cell counting kit-8 (cck-8) was used to determine the viability of HO-8910 cells following the manufacturer's protocol.

Cell invasion assay

Transwell well culture chambers with Matrigel were used to determine the effect of PA on HO-8910 cell invasion. Briefly, 0.75 ml RPMI-1640 with 10% FBS was placed in the lower chamber. 0.5 ml HO-8910 cells (1×105 cells/well) were seeded in the upper chamber and treated with indicated drug at 37°C in 5% CO_2. After 48 h, the invaded cells through Matrigel-coated PVPF filter were fixed with 4% formaldehyde for 10 min and stained with 0.5% crystal violet solution for 30 min, and counted by using the optical microscope (Olympus, Japan).

Cell cycle assay by flow cytometry

Flow cytometry was used to determine the effect of PA on the cell cycle distribution of the HO-8910 cells. HO-8910 cells were cultured with or without PA for 48 h and then were treated with 0.25% trypsin to prepare a single cell suspension in phosphate buffered saline with 10% FBS. These cells were fixed in 70% precooled ethanol and 1mg/ml RNase A was used to remove RNA. Finally, cells were stained by propidium iodide and measured by flow cytometry (FACSCalibur, BD Biosciences).

Western blotting

After PA treatment for 48 h, HO-8910 cells were collected and total proteins were extracted. The protein content was quantified by the BCA Protein Assay Reagent (Sangon Biotech, Shanghai, China). Total protein (20 μg) was loaded to sodium dodecyl sulfate-polyacrylamide electrophoresis gels (SDS-PAGE) and then transferred to nitrocellulose filter membrane, which was incubated with anti-E cadherin, β-catenin, COX-2 and GAPDH antibodies at 4°C for 12 h. After that, the membranes

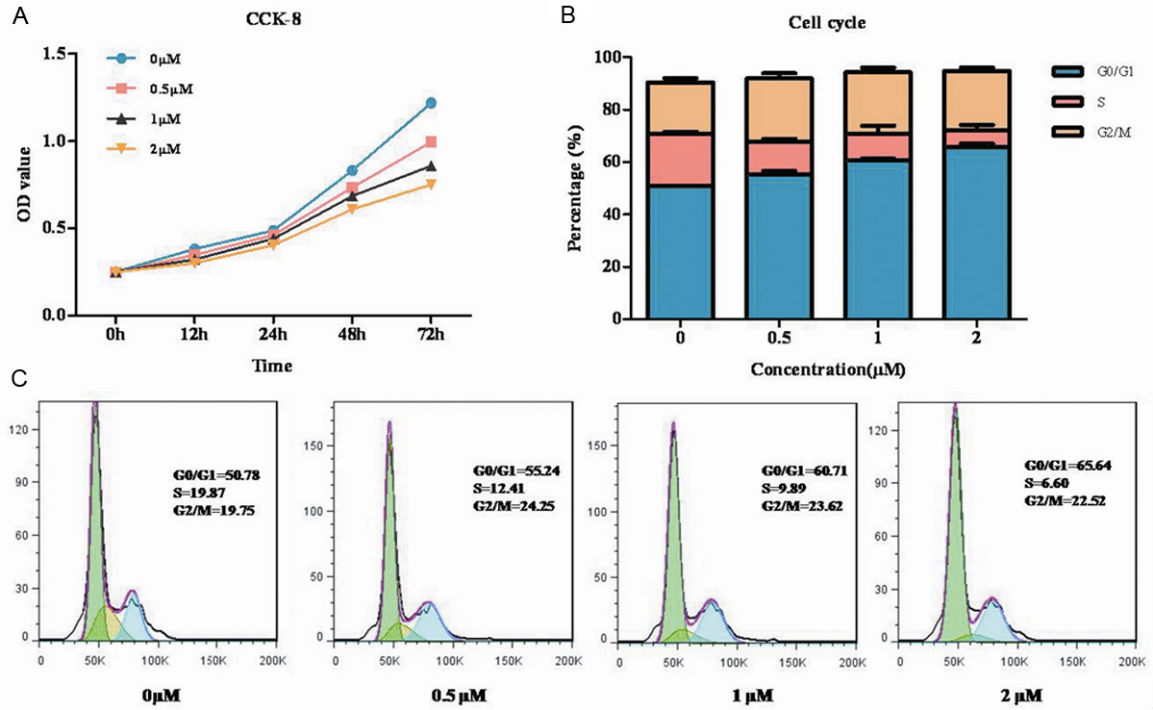

Figure 1. Effects of PA on HO-8910 cell proliferation and cell cycle. A. After treatment with or without different concentrations of PA, cell viability was determined at 24 h, 48 h or 72 h (n=3). The effect of PA on cell viability was quantified as relative percentage of cell viability to that untreated control cells set at 100%. B, C. After 24 h treatment with or without different concentrations of PA, HO-8910 cell cycle distribution was measured by flow cytometry. Results are expressed as means ± SD of three independent experiments; SD denoted by error bars.

were incubated with corresponding horseradish-peroxidase-conjugated secondary antibodies and the immunoreactive band was visualized with ECL-detecting reagents (Beyotime, Jiangsu, China).

Statistical analysis

Data were expressed as mean ± standard deviation and $P<0.05$ was considered statistically significant. One way analysis of variance (ANOVA) was used to compare the means between two groups. Data analysis was performed using GraphPad Prism 5.0 software package.

Results

PA blocked ovarian carcinoma cells proliferation in vitro

The effect of PA on HO-8910 cell viability was detected by the CCK-8 assay. As shown in **Figure 1A**, the proliferation of HO-8910 cells treated with PA (0.5, 1, 2 μM) were significantly inhibited at 24, 48, and 72 h ($P<0.01$, $P<0.01$, $P<0.01$, respectively), in dose-dependent manner, compared to the untreated HO-8910 cells. Therefore, this data indicated that PA could suppress HO-8910 cells growth.

The effect of PA on HO-8910 cell cycle

The cell cycle distribution was detected using propidium iodide staining in HO-8910 cells. According to the results of flow cytometry, compared with the untreated cells, treated HO-8910 cells exhibited a significantly increased accumulation of cells in the G1 phase after PA exposure for 24 h (**Figure 1B**, **1C**). Additionally, the HO8910-PA cells had decreased percentages of cells in S phases. Therefore, our results revealed that PA induced the HO8910-PA cells in G1 phase.

PA inhibits ovarian carcinoma cells invasion in vitro

We determined the inhibitory effect of PA on HO-8910 cell invasion by Transwell well culture chambers with Matrigel. As shown in **Figure 2**, PA at 0.5, 1, 2 μM significantly inhibited the

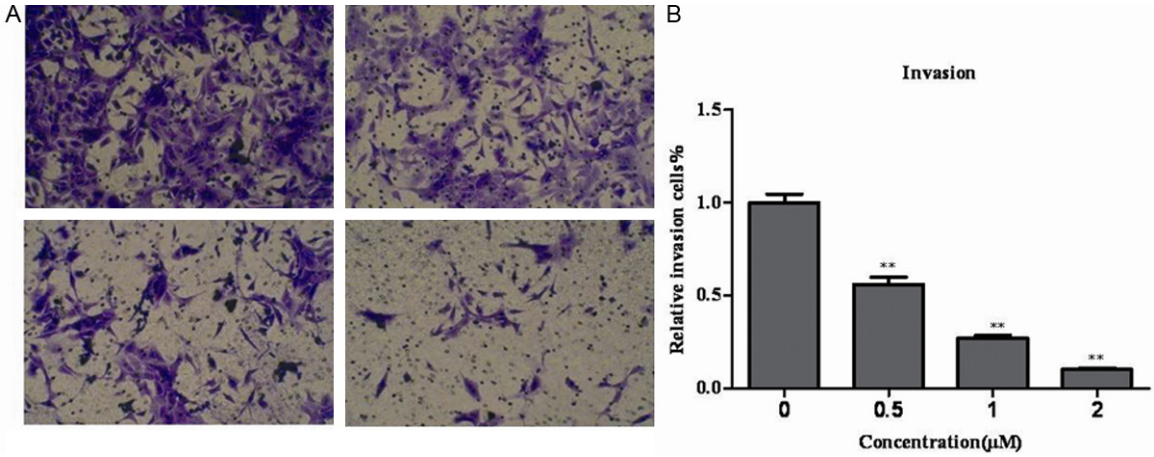

Figure 2. PA inhibited HO-8910 cells invasion *in vitro*. Cells that had invaded were stained and effects of PA on cell invasion were presented as percent cell invasion compared with untreated cells set at 100%. Results are presented as means ± SD of three independent experiments; SD denoted by error bars. $^{**}P<0.01$, compared to the untreated PA HO-8910 cells.

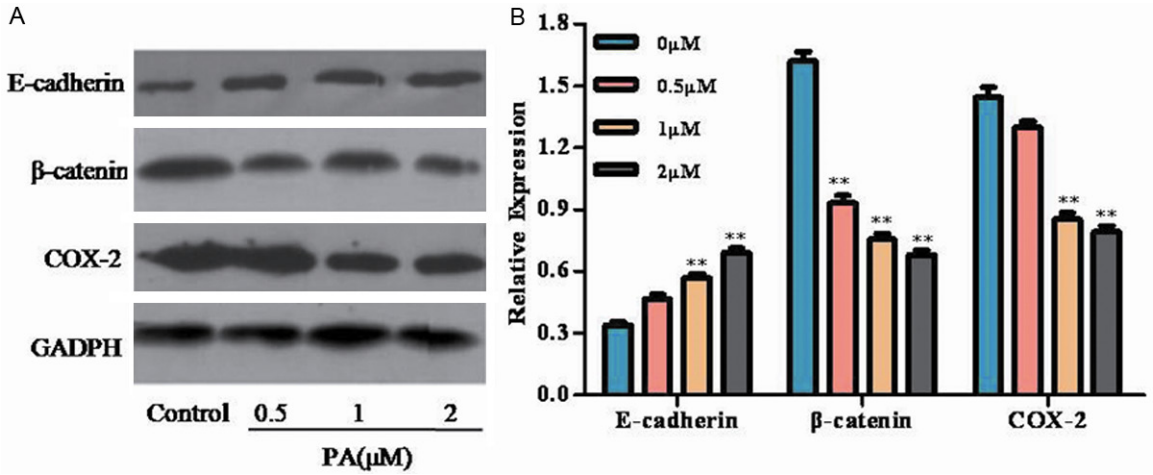

Figure 3. Effects of PA on the expression of E-cadherin, β-catenin and COX-2 in HO-8910 cells. After treatment with or without different concentrations of PA, corresponding proteins were determined at 48 h.

invasiveness of HO-8910 cells after 24 h exposure, in dose-dependent manner, respectively.

PA affected the protein expression of E-cadherin, β-catenin and COX-2

Further, to explore the mechanism of how PA blocks HO-8910 cell proliferation and invasion, we detected the effect of PA on the protein expression of E-cadherin, β-catenin and COX-2 by western blotting. As shown in **Figure 3**, after PA treatment for 48 h, the E-cadherin protein expression level was increased in the HO-8910 cells while the β-catenin and COX-2 expression levels were down-regulated. Thus, these data in our study revealed that the anti-proliferative mechanisms of PA in HO-8910 cells might be related to cell cycle distribution, and E-cadherin/β-catenin signaling pathway.

Discussion

In this study, our results were the first report that PA could suppress human ovarian carcinoma cell growth in vitro. In particular, PA exhibited the inhibition of HO-8910 cell proliferation and invasion in dose-dependent manner. Moreover, we found that these combined effects may be associated with influencing cell cycle distribution and E-cadherin/β-catenin signaling pathway.

The metastatic dissemination of tumor cells is a principle cause of death in patients diagnosed with ovarian cancer. Because of invasion and spread of cancer cells to other organs, the therapy for ovarian cancer is very difficult. With tumor progression, tumor cells show marked changes in cell-cell and cell-matrix adhesion. Cell-cell adhesion appears to be important in tumor cell invasion and metastasis. Experiments have demonstrated that E-cadherin/β-catenin-mediated cell adhesion system, as an "invasion suppressor system", plays a crucial role in the metastasis process [12]. And COX-2 has been reported to promote the development of ovarian cancer [24].

E-cadherin plays a key role in cell-cell adhesion, proliferation, migration, apoptosis and tissue integrity [8, 25, 26]. It is reported that E-cadherin mediated cell-cell adhesion is critical for the establishment of ovarian cancer metastases [15]. In vivo and in vitro, loss or impaired function of E-cadherin has been demonstrated to increase the tumor formation and invasion [10, 12]. In addition to as a cell-cell adhesion molecular, E-cadherin is a negative regulator of the canonical Wnt signalling pathway and β-catenin appears to be the main mediator. Interestingly, in our present study, the expression level of E-cadherin protein was increased by treatment with PA in HO-8910 cells.

E-cadherin functions by activating downstream signals and β-catenin appears to be the main mediator [27]. The β-catenin has different functions in different intracellular localizations in epithelial cells. In normal epithelial cells, β-catenin is mainly associated with E-cadherin mediated cell-cell adhesion [13]. After loss of E-cadherin expression, β-catenin localizes to the nucleus and promotes transcription of proliferation and survival genes by activating Wnt signaling [13, 28]. Previous data have demonstrated that β-catenin is a central mediator in the canonical Wnt pathway while E-cadherin acts as a negative mediator of this pathway [29]. In our present data, after treatment with different concentrations of PA for 48 h, the β-catenin protein level was also decreased.

COX-2 is one of the Wnt target genes. In published data, positive COX-2 expression was detected in 56% of all the ovarian carcinoma specimens and the COX-2 expression was increased in the ovarian cancer [9, 24]. COX-2 has been demonstrated to possess promoting anti-apoptotic proteins expression, up-regulating growth factor expression and increasing matrix metalloproteinase expression to promote the carcinoma cells growth [30]. Thus, COX-2 is also an important therapeutic target for ovarian cancer. Importantly, we found that PA can suppress COX-2 protein expression of HO-8910 cells in vitro. This finding is consistent with the previous data that PA could effectively reduce IL-1β-induced COX-2 activation of A549 cells in vitro at nontoxic levels [20].

In conclusion, our present data indicated that PA could inhibit the proliferation, cell cycle and invasion of HO-8910 cells in vitro. And the mechanism of these combined effects may be involved in modulating E-cadherin/β-catenin signaling pathway. It is possible that PA acts as a potential agent against ovarian cancer. For this purpose, in vivo effects and further molecular mechanisms studies are need to be demonstrated during ovarian carcinogenesis.

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Disclosure of conflict of interest

None.

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