Vitexin attenuates epithelial ovarian cancer cell viability and motility *in vitro* and carcinogenesis *in vivo* via p38 and ERK1/2 pathways related VEGFA

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**Background:** Epithelial ovarian cancer (EOC) is the most common type of ovarian tumor, however, effective treatment does not currently exist for this condition. This study evaluated the role of vitexin in mitigating EOC both *in vitro* and *in vivo*.

**Method:** SKOV-3 cells were used for *in vitro* experimentation. Xenotransplantation mouse models were set up by subcutaneously injecting mice with SKOV-3 cells. CCK8 was used to screen the optimal dose *in vitro*. Cell proliferation, invasion, number of microtubule nodules and apoptosis were respectively detected by colony formation assay, transwell assay, microtubule formation assay and flow cytometry. TUNEL and immunohistochemistry were used to detect tissues apoptosis and VEGF content. Western blot assay was used to detect the expression of Ki67, caspase-3, VEGFA, VEGFR2, ERK1/2 and p38.

**Results:** *In vitro* experiment, compared with the control group, 10 μL of vitexin significantly reduced Ki67 levels and enhanced tumor cell apoptosis rate. Additionally, the colony forming rate, invasive cells per field, and number of nodes/HPF in vitexin treated group decreased dramatically. The result of western blot showed that levels of p-p38/p38 and p-ERK1/2/ERK1/2 also noticeably decreased.

**Conclusions:** Vitexin decreased the proliferation and invasion of SKOV-3 cells and noticeably reduced tumor growth. These findings suggest that vitexin could be a promising therapy for EOC.

**Keywords:** Epithelial ovarian cancer (EOC); vitexin; vascular endothelial growth factor (VEGF); SKOV-3 cells; attenuate; clone formation

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**Introduction**

Epithelial ovarian cancer (EOC) is a serious type of ovarian tumor that occurs worldwide and affects 3–12/100,000 women per year. EOC is characterized by high mortality rates and there is no effective routine treatment for the advanced stages of this disease (1-3). The leading cause of relapse and death in EOC patients is chemoresistance. Novel and adjuvant therapies have therefore become the new direction of research for EOC treatment.

Blood vessels are the impetus for the rapid growth and metastasis of cancer cells. Neovascularization therefore plays a critical supporting role in the occurrence and
development of tumors, and a variety of signaling molecules are involved in its regulation (4,5). Vascular endothelial growth factor (VEGF) is the primary factor that induces endothelial cell proliferation and is closely associated with tumor tissue angiogenesis. VEGFA is a member of the VEGF family, which exert their biological effect when bound to their receptors. The primary VEGF receptors include Flt-4, VEGFR2, and Flt-1 (6,7). VEGFR, VEGF, and their downstream signaling pathways are markers of tumor angiogenesis, and VEGFA/VEGFR2 signal transduction plays a dominant role in vascular endothelial cell formation (8,9). VEGFA/VEGFR2 signal transduction inhibition can therefore suppress tumor cell growth by blocking angiogenesis (10,11).

Lignans, a class of complex polyphenolic antioxidants found in plants, were once considered one of the most promising dietary agents for cancer prevention (12,13). One type of lignan compound is vitexin (chemical structure: Figure 1A), which is isolated from the seed of Vitex negundo. Zhou et al. reported that vitexin exerts extensive antitumor activity in cancer xenograft models, such as inducing caspase activation and promoting apoptosis (14). Numerous evidence demonstrates that vitexin exerts antitumor effects on multiple human cancers, including glioblastoma (15), hepatocellular carcinoma (16), and leukemia (17). It remains unknown whether vitexin possesses antitumor effects on EOC.

In this study, we investigated the suppression effect of vitexin on EOC cell viability and motility in vitro and carcinogenesis in vivo.

We present the following article in accordance with the ARRIVE reporting checklist (available at http://dx.doi.org/10.21037/atm-20-5586).

**Method**

**Cell culture**

The ovarian cancer cell line SKOV-3 was purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). Cells were cultivated in complete medium (Gibco, Rockville, MD, USA) with T75 cell culture flasks (Corning, New York, NY, USA) according to the suppliers’ instructions.

**Animal model**

Athymic nude mice (female) were obtained from laboratories of The First Affiliated Hospital of Xinxiang Medical University. The mice were housed in climate-controlled conditions and fed with free access to chow and water. The environment of the animals was a 12-hour light/dark cycle with a constant temperature of 22±1 °C and humidity of 55%±5%. Tumorigenic assays were performed as described in previous studies (18). SKOV-3 cells were transduced with lentiviral shRNA and treated with puromycin (1.5 mg/mL) for 7 days to select the transduced cells. Transduced cells with a density of 5×10^6 cells/well were injected subcutaneously into the right thighs of mice to set up the model. Upon tumor formation, the mice were injected intraperitoneally with vitexin (20, 40, or 80 mg/kg) and solvent twice weekly for 4 weeks. The dosage of Vitexin was based on the previous study (19). Tumor sizes were measured weekly, and the mice were sacrificed after 4 weeks.

All animal experiments were carried out in accordance...
with the NIH Guide for the Care and Use of Laboratory Animals 2018 and were approved by The First Affiliated Hospital of Xinxiang Medical University, Weihui, Henan.

**Cell viability assay**

Two hundred μL SKOV-3 cells were plated in 96-well plates at a density of 5×10^5 cells/well. Upon 24-hour culture, the cells were treated with different concentrations of vitexin (0, 0.1, 0.5, 1, 2.5, 5, 10, 20, 50, 100, 200, 300, and 400 μL) for 4 hours in a 5% CO_2 incubator at 37 °C. CCK-8 dye was then added to the medium and the cells were incubated at 37 °C for 4 hours. Finally, the absorbance in five parallel wells was measured.

**Colony formation assay**

SKOV-3 cells at a density of 5×10^5 cells/well were seeded in triplicate in 6-well plates overnight. After 10 days of culture, visible cells were washed with phosphate-buffered saline (PBS), fixed with methanol, and then stained with Giemsa. Colonies of N50 cells were counted (20).

**Flow cytometry**

The cell apoptosis rate was measured by flow cytometry; this process is described in previous literature (21). Briefly, after treatment, cells were washed with PBS and incubated with 10 μL Annexin V-FITC (fluorescein isothiocyanate) and 5 μL propidium iodine (PI) for 15 min at room temperature in the dark. Finally, cells were analyzed using a FACSCalibur Flow Cytometer (BD Biosciences).

**Western blot analysis**

Cells were harvested after 48 hours of vitexin pretreatment. Western blot analyses were carried out according to processes described in previous literature (22). The primary antibodies used in this study were as follows: VEGF (ab32152, 1:250), MMP-9 (ab76003, 1:1,000), E-cadherin (ab76055, 1:1,000), PCNA (ab29, 1:1,000), MMP-14 (ab51074, 1:5,000), caspase-3 (ab13847, 1:500), VEGFA (ab52917, 1:10,000), ERK1/2 (b17942, 1:1,000), Ki67 (ab15580, 1:500), p-ERK1/2 (ab214362, 1:1,000), p38 (ab31828, 1:1,000), and p-p38 (ab4822, 1:1,000). The samples were analyzed using an Odyssey Infrared Imaging System (LI-COR Inc., Lincoln, NE, USA).

**Cell apoptosis assay**

Hoechst 33258 staining was used to detecte apoptosis. In brief, 3×10^5 cells per well were washed with PBS twice. Then, 1 mL of Hoechst 33258 reagent (Beyotime, Nantong, China) was added to each well, and the cells were incubated at 37 °C for 30 min in the dark. Then, the Hoechst 33258 reagent was removed, and cells were washed with PBS for 3 times (5 min × 3 times). Morphological changes of apoptotic cells were observed under an inverted fluorescence microscope, and images were captured.

**Transwell assay**

Cells were left either untreated or treated with vitexin for 48 hours. Cell suspensions of 200 mL/chamber in serum-free medium were then seeded into the upper chambers of transwell inserts. Complete medium was added to the lower chambers. After a 36-hour incubation period, cells were fixed with methanol and stained with Giemsa. Cells on the upper surface of the membrane were then cleared, allowing cells on the lower surface to be examined by microscope (Leica, Germany). The average number of invasive cells was recorded.

**Microtubule formation assay**

Cells were left either untreated or treated with vitexin for 48 hours. Serum-free RPMI medium and matrigel were then added and mixed in a ratio of 1:1 at 4 °C. The mixture was added to the 24-well plates in an amount of 300 μL/well and incubated at 37 °C under 5% CO_2 conditions for 30 minutes. The microtubule structures were observed every 4 hours. The images were analyzed with Microvision Saisam software.

**Transferase dUTP nick end labeling (TUNEL) assay**

The prepared paraffin sections were dewaxed, rehydrated, and washed. Then, the sections were incubated with TUNEL reaction mixture (Roche Diagnostics, Indianapolis, IN, USA) according to the instructions of the manufacturer. Apoptotic cells were detected by fluorescence microscopy (Olympus, Japan).

**Immunohistochemistry**

Prior to cutting tumor tissue into 3 μm-thick sections, the
tissue was fixed with 4% formalin and paraffin embedding. The sections were then routinely deparaffinized in xylene and rehydrated in an alcohol gradient. Sections were sealed with 5% normal goat serum for 1 hour and co-incubated with primary antibodies Ki67 (ab15580, 1:500) and VEGF (ab32152, 1:250) overnight at 4℃. Sections were then washed three times in PBS and incubated with secondary antibody (ZSGB-BIO, Beijing, China) for 30 minutes at room temperature. Sections were then analyzed using an Olympus BX-UCB light-field microscope according to the manufacturer’s instructions.

Statistical analysis
Analyses were performed using SPSS 21.0 (SPSS, Inc., Chicago, IL, USA). ANOVA and Student’s t-tests were performed to assess statistical significance, as appropriate. Data are presented as mean ± SEM.

Results
Effect of vitexin treatment on SKOV-3 cell viability
Vitexin solutions of varying concentrations (0, 0.1, 0.5, 1, 2.5, 5, 10, 20, 50, 100, 200, 300, and 400 μL) were used to treat SKOV-3 cells and determine the optimal therapeutic dose. Upon 24-hour treatment, compared with the control group, vitexin concentrations of 0.1–20 μL caused minimal toxicity to SKOV-3 cells, while concentrations above 20 μL caused significant toxicity (Figure 1B). Three dose concentrations (5, 10, and 20 μL) were selected for use in subsequent experiments in this study.

Vitexin treatment impaired SKOV-3 cell proliferation and promoted SKOV-3 cell apoptosis
As shown in Figure 2A, compared with no vitexin treatment, 5, 10, and 20 μL vitexin treatment significantly suppressed the colony forming rate of SKOV-3 cells. Compared with the control group, treatment of 10 μL Vitexin enhanced tumor cell apoptosis rate (2.6%±0.6% versus 14.6%±6.0%, *P<0.05, Figure 2B). Figure 2C showed that Ki67 expressed highly in SKOV-3 cells, which is consistent with previous report (23). Of note, compared with the control group, 10 μL of vitexin significantly reduced Ki67 levels (0.08%±0.02% versus 0.02%±0.01%, *P<0.05) and accelerated caspase-3 activation. These results suggest that vitexin treatment can greatly impair SKOV-3 cell proliferation and promote SKOV-3 cell apoptosis.

Vitexin treatment reduced SKOV-3 cell invasive ability
Typical pictures of transwell assay was showed in Figure 3A. From Figure 3B, 10 and 20 μL vitexin treatment could significantly decrease the number of invasive cells per field, which revealed that the invasive ability of SKOV-3 cells were suppressed by vitexin treatment. Typical pictures of microtubule formation assay was showed in Figure 3C. From Figure 3D, 10 and 20 μL vitexin treatment could significantly decrease the formation of microtubule, which indicated that the microtubule formation ability of SKOV-3 cells were suppressed by vitexin treatment. In order to make the results more reliable, we used Western blot to detect the relative content of proteins VEGF, MMP-9 and E-cadherin, which related to the microtubule formation and invasion of cancer cells. The results was showed in Figure 3E,F.

Vitexin treatment attenuated the viability and motility of SKOV-3 cells via VEGFA-dependent ERK1/2 and p38
As shown in Figure 4A, vitexin treatment greatly downregulated the expression of VEGFA and VEGFR2 simultaneously, which in turn downregulated the phosphorylation of ERK1/2 and p38. The relative protein levels of VEGFA, VEGFR2, p-ERK1/2/ERK1/2, and p-p38/p38 decreased significantly (Figure 4A). To further determine the effect of Vitexin treatment on ERK1/2 and p38 pathways, skatole, the activator of ERK1/2 and p38 pathways, was introduced. From Figure 4B,C,D,E,F, skatole treatment partially attenuated the inhibitory effect of Vitexin on the proliferation and invasion of SKOV-3 cells, and counteracted the promoting effect of Vitexin on the apoptosis of SKOV-3 cells. These results suggest that vitexin treatment attenuated the viability and motility of SKOV-3 cells through VEGFA-dependent ERK1/2 and p38.

Vitexin treatment suppressed tumor formation in vivo
Vitexin notably inhibited tumor growth (Figure 5A). Tumor
Figure 2 Vitexin treatment inhibited proliferation and promoted apoptosis of SKOV-3 cells. (A) Proliferation was detected by clonal formation assay (magnification ×200). (B) Cell apoptosis rate was detected by flow cytometry [propidium iodide (PI) staining]. (C) Relative protein levels of Ki67 and caspase-3 were detected by western blot assay (*, P<0.05 vs. control).

Discussion

Chemotherapy is currently the primary treatment for cancer. This treatment, however, produces strong and unpleasant side effects. Natural products, especially compounds that originate in plants, have recently attracted the attention of researchers due to their potential antitumor properties, and Chinese medicine is now being researched as adjuvant treatment for cancer and other malignant diseases. Numerous studies have shown that Chinese medicine can effectively decrease the side effects and toxicity of chemotherapy, as well as enhance disease resistance and promote apoptosis (24,25). Vitexin, which is isolated from the seed of *Vitex negundo*, has potential antitumor activity against many human cancers. For example, it can exert cytotoxic effects by inducing apoptosis mediated by the activation of caspases (26). Vitexin has also been shown to inhibit the growth of esophageal cancer cells.

weight decreased greatly with increased vitexin dose (Figure 5B). As shown in Figure 5C, vitexin treatment clearly attenuated tissue apoptosis. Figure 5D,E show that vitexin treatment reduced the percentage of apoptotic cells and downregulated VEGF expression in tumor tissues. These results suggest that vitexin inhibited tumor formation in vivo.
and induce their apoptosis (27). In this study, we found that low doses of vitexin (≤20 μL) had minimal toxicity on SKOV-3 cells. Vitexin treatment impaired the proliferation of SKOV-3 cells and promoted their apoptosis in vitro, as well as attenuating cell invasion and microtubule formation. Interestingly, vitexin treatment greatly downregulated the expression of VEGFA and VEGFR2 simultaneously. Additionally, vitexin treatment markedly inhibited ERK1/2 and p38 phosphorylation. Vitexin treatment also suppressed tumor formation in vivo.

A typical feature of tumor cells is clonal proliferation. Ki67 is an important indicator of cancer cell proliferation and is a powerful prognostic marker for several types of cancer (28). As apoptosis is a necessary cell death program, activation of this process is an effective anticancer pathway (29). Caspase-3 is an important part of the apoptotic process. In the present study, Ki67 expression was significantly decreased in SKOV-3 cells treated with vitexin, while the cleavage of caspase-3 and caspase-9 were simultaneously enhanced. Bhardwaj et al. reported that vitexin produces good pharmacological effects by inducing the apoptosis of rectal cancer cells (30). Consistent with our results, in this study vitexin treatment induced the cleavage of caspase-9 and caspase-3. This suggests that vitexin may inhibit SKOV-3 cell proliferation and promote SKOV-3 cell apoptosis.

Figure 3 Vitexin treatment inhibited the invasive ability of SKOV-3 cells. (A) SKOV-3 cell invasion was detected by transwell assay (magnification ×200). (B) Invasive cells per field. (C) Microtubule formation by SKOV-3 cells (magnification ×200). (D) Number of nodes/HPF in the microtubule formation experiment. (E) Relative protein levels of VEGF, MMP-9 and E-cadherin were detected by western blot assay. (F) Relative protein level of VEGF, MMP-9 and E-cadherin. (*, P<0.05 vs. control).
Tumor metastasis is the leading cause of cancer-related death and infiltration plays a critical role in this process. Infiltration involves tumor cells losing their intercellular junctions, followed by cell degradation, remodeling, adhering to the surrounding extracellular matrix (ECM), and finally migrating to distant locations. In our study, transwell assays and microtubule formation assays showed that vitexin played a positive role in preventing tumor cell invasion and microtubule formation, respectively. Consistent with our results, Choi et al. found that vitexin suppressed the rate of invasion and migration of PC12 cells (31). This suggests that vitexin can suppress the
invasive ability of SKOV-3 cells.

Tumor growth is closely related with oxygen and blood supply provided by new blood vessels (32). This process is mediated in many tumors by VEGFA, which is a highly specific pro-vascular endothelial growth factor that accelerates increased vascular permeability, extracellular matrix degeneration, vascular endothelial cell migration, proliferation, and angiogenesis. In the present study, vitexin treatment downregulated VEGFA and VEGFR2 expression and inhibited ERK1/2 and p38 phosphorylation. Scarpa et al. found that vitexin-2-0-xylocides (XXYs) and avenanthramides (AVNs) downregulated the expression of VEGFA, which resulted in the inhibition of CaCo-2 and HepG2 cell proliferation (33). Our findings are also in
agreement with Rosa et al. These researchers reported that vitexin reduced the expression of p-p38 and p-ERK1/2 in LPS-elicited RAW 264.7 cells (34). Curtarello et al. exposed ovarian cancer cells to anti-VEGF therapy and achieved good results (35). Together, these results indicated that vitexin influenced SKOV-3 cells by affecting VEGFA and VEGFR2 expression, and the related pathways may be ERK1/2 and p38.

To further verify the mitigation effect of vitexin on EOC, we established a xenotransplantation mouse model for experimentation. Our results showed that vitexin treatment significantly inhibited both tumor growth and the percentage of VEGF positive cells while significantly promoting tissue apoptosis.

In conclusion, we found that vitexin treatment inhibited SKOV-3 cell invasion and promoted SKOV-3 cell apoptosis. Vitexin treatment downregulated the expression of VEGFA and VEGFR2, and inhibited the phosphorylation of ERK1/2 and p38. Vitexin also significantly inhibited tumor growth and promoted tumor tissue apoptosis in vivo.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All animal experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals 2018 and were approved by The First Affiliated Hospital of Xinxiang Medical University, Weihui, Henan (No. HNXX-20191103).

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