Emodin exerts antitumor effects in ovarian cancer cell lines by preventing the development of cancer stem cells via epithelial mesenchymal transition

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Abstract. Ovarian cancer has the worst prognosis among all types of gynecological malignancies and patients are often diagnosed at an advanced stage with distant metastasis. In the present study, it was found that emodin, a small molecular chemical drug derived from natural plants, has antitumor effects on ovarian cancer cells. Emodin induced cytotoxicity and inhibited proliferation in the ovarian cancer cell lines, SK-OV-3, A2780 and PA-1. In addition, emodin inhibited the migration and invasion abilities of the ovarian cancer cells by inhibiting epithelial-mesenchymal transition (EMT), which was evidenced by the downregulation of N-cadherin and vimentin, and the upregulation of E-cadherin protein expression levels. When a subcutaneous xenograft SK-OV-3 tumor mouse model was used, emodin notably reduced the tumor growth rate and inhibited tumor cell proliferation. Furthermore, mechanical analysis revealed that emodin markedly inhibited EMT and reduced the stemness of tumor cells, which was evidenced by the decrease in the protein expression of CD133 and Oct4. Pulmonary metastasis of the ovarian cancer cells was significantly suppressed in the tumor mouse model by the administration of emodin. In addition, flow cytometry analysis indicated that emodin significantly reduced the proportion of ovarian cancer stem-like cells in metastatic lung tissues. In conclusion, emodin, a potent inhibitor of EMT, could serve as a potential candidate for ovarian cancer therapy.

Introduction

Ovarian cancer is a malignant carcinoma that is usually diagnosed at a late stage, by which time metastasis can be observed at distant sites, including the lungs, liver and lymph (1,2). Ovarian cancer usually develops from the ovarian surface epithelium or from serous intra-epithelial carcinoma (3). With the advancement of medical technology, debulking surgery and cis-platinum-based chemotherapy are the major therapeutics for ovarian cancer. However, the outcome of debulking surgery is dependent on surgical skill and the genetic features of the tumor (4,5). Debulking surgery combined with cis-platinum based chemotherapy can lead to remission in patients with ovarian cancer; however, the majority of patients suffer from cancer resistance, metastasis and relapse (6,7). Following relapse, the outcome of chemotherapy declines significantly, with rapid disease progression and drug resistance. Furthermore, genetic alterations in cancer cells, which are widely investigated for drug resistance and cancer metastasis, result in drug inactivation, enhanced DNA repair mechanisms and changes in the intracellular pathways (8). However, cancer metastasis and relapse remain important obstacles for ovarian cancer therapy, and novel and efficient therapeutics for ovarian cancer are urgently required.

Epithelial-mesenchymal transition (EMT) plays key roles in cancer proliferation and distant metastasis (9,10). Various signaling pathways have been associated with extracellular cues, such as TGF-β and play important roles in reprogramming gene expression during EMT (11,12). Furthermore, cancer stem cells (CSCs), which are characterized by the markers, CD133 (also known as prominin-1) and octamer binding transcription factor 4 (OCT4), exhibit a CD44+/CD24−/low phenotype (13) and self-renewal ability. They also constitute a minor proportion of neoplastic cells in the tumor microenvironment, which are usually regarded as the tumor-initiating cells (TICs) (14,15). The proportion of TICs in tumors is an important source of metastatic lesions in breast cancer. Current evidence has revealed that cells, which undergo EMT, exhibit stem cell-resembling characteristics (16,17). For example, Mani et al (16) demonstrated that stem-like cells isolated from either mouse or human mammary glands or mammary carcinomas express EMT markers, illustrating an association
between EMT and the gain of epithelial stem cell properties. Wang et al (18) revealed that fusobacterium nucleatum produced CSC characteristics by activating IL-6/STAT3 and eliciting EMT-resembling activation. Furthermore, EMT induced cancer cell mesenchymal characteristics and promoted cancer cells to gain stemness (19,20). In addition, EMT induces invasion and dissemination of tumor cells, and also assists CSCs to invade to distant organs, leading to cancer distant metastasis (21,22). Numerous studies have demonstrated that poor outcomes of ovarian cancer therapies, i.e., metastasis and relapse, are usually due to a small proportion of CSCs that have escaped from the primary cancer lesion (23,24). Thus, CSC-target therapy is a promising strategy to conquer ovarian cancer metastasis and relapse.

Traditional Chinese Medicine has important roles and has shown good therapeutic efficiency in the prevention of various diseases. In recent years, numerous bioactive small molecular drugs extracted from herbal medicine have been widely investigated in diseases, such as cancer (25-28). Emodin, which can be isolated from several Chinese herbs, including Rheum palmatum L and Poly gonum cuspidatum, has been reported to possess numerous bioactivities in regulating resistance to oxidation and blood glucose levels, in addition to having anti-bacterial properties (29-31). The use of emodin in Traditional Chinese Medicine is ‘attack stagnation, clear heat and dampness, purge fire, cool blood, remove blood stasis and detoxification’ (32). In addition, according to recent studies, emodin significantly inhibited colon cancer, prostate cancer, breast cancer and skin cancer cells by triggering cancer cell apoptosis (29,33,34). However, the anti-proliferation and anti-metastasis effect of emodin on ovarian cancer has been rarely studied. Therefore, the antitumor effect of emodin in vitro and in vivo was investigated in the present study. In addition, the corresponding mechanism was also analyzed to provide evidence for the therapeutic application of emodin in ovarian cancer.

Materials and methods

Cell lines and cell culture. The human ovarian cancer cell lines, SK-OV-3 and A2780 were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.), and PA-1 were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.), containing 10% (FBS) (Gibco; Thermo Fisher Scientific, Inc.), were purchased from American Type Culture Collection. All the cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂. Emodin was purchased from Sigma-Aldrich (Merck KGaA), dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C for further use. The antibodies against N-cadherin (cat. no. 13116; 1:1,000), E-cadherin (cat. no. 14472, dilution, 1:1,000), vimentin (cat. no. 5741; 1:1,000), CD133 (cat. no. 64326; 1:1,000), OCT4 (cat. no. 2840; 1:1,000) and β-actin (cat. no. 3700; 1:1,000) were purchased from Cell Signaling Technology, Inc.

Cell proliferation assay. Briefly, suspensions of the SK-OV-3, A2780 and PA-1 cell lines (2,000-5,000 cells/100 µl) were added to 96-well microplates. Approximately 12 h later, the cells were treated with different concentrations (0, 2.5, 5, 10, 20, 40 and 80 µM) of emodin for 24, 48 or 72 h. Then, the cell culture medium in each well was replaced with 20 µl MTT solution (5 mg/ml) and the samples were incubated at 37°C for a further 2-4 h. Subsequently, 150 µl DMSO was added to each well to dissolve the formazan crystal produced by the living cells. Finally, the optical density of each well was measured using a Spectra MAX M5 microplate spectrophotometer (Molecular Devices, LLC) at 570 nm. At least three independent experiments were performed.

Colony formation assay. The clonogenic ability of the ovarian cancer cell lines was assessed using the colony formation assay following treatment with emodin. Briefly, the ovarian cancer cell lines (400-600 cells/well) were seeded in 6-well plates and cultured for a further 12 h. The cells were treated to various doses of emodin (0, 5, 10, 20 and 40 µM) and cultured for a further 12 days. The cell culture medium was replaced with fresh medium containing corresponding concentrations of emodin every 3 days. Subsequently, the culture medium was discarded, and the cell colonies were washed three times with PBS, fixed with methanol for 15 min at 25°C and stained with 0.5% crystal violet for 20 min at 25°C. Finally, the number of cell colonies (>30 cells) was counted manually and images were captured using an inverted microscope (Axiovert 200; Zeiss AG).

Wound healing assay. The ovarian cancer cell lines were seeded in 6-well plates and cultured for 12 h. When the monolayer of the cells reached 80% confluence, the wound was created using a sterile 200 µl micropipette tip. Then, the cells were washed with fresh medium and cultured in medium (with 0.5% FBS) and different concentrations of emodin (0, 10, 20 and 40 µM) for 48 h. Next, the cell culture medium was discarded, the cells were washed three times with PBS, then images were captured using a light microscope (Axiovert 200; Zeiss AG). The migration rates of the treated cells were quantified using the following equation: Migration rate=(1-W1/W0)/(1-W3/W1), where W0 represents the width of the wound at 0 h; W1 represents the width of the wound at 48 h in the control group and W3 represents the width of the wound at 48 h in emodin treatment group. The migration rate of the control cells was regarded as 100%.

Transwell and Matrigel assays. For the analysis of the migratory effect of emodin, 1×10⁶ SK-OV-3, A2780 and PA-1 cells, suspended in 100 µl serum-free medium, were added to the upper chamber, while 600 µl complete medium, containing 10% FBS, was added to the bottom chamber. Different concentrations of emodin (0, 10, 20 and 40 µM) were added to medium in the upper chambers. After migration for ~48 h at 25°C, non-migratory cells remaining in the upper chamber were discarded using a cotton swab and the migrated cells located on the bottom of membrane were washed, fixed with methanol for 15 min at 25°C, then stained with 0.5% crystal violet for 20 min at 25°C. Images of the migrated cells were captured using a light microscope and five random fields of view were counted to compare the migration rate.

A Matrigel assay was performed to determine the invasion ability of the cells treated with emodin. Briefly, the upper surface of a 24-well Transwell plate (MilliporeSigma) was pre-coated with ~70 µl Matrigel (BD Biosciences) diluted...
weight of the mice was measured and recorded every 3 days. After treatment with the indicated concentrations of emodin (0, 10, 20 and 40 µM) for 48 h, the SK-OV-3, A2780 and PA-1 cell lines were harvested and lysed with RIPA buffer (Beyotime Institute of Biotechnology) to obtain the total protein. Concentration of total protein was determined by BCA method. An equal amount of protein (~30 μg), from the differently treated samples were separated using SDS-PAGE (10%), then transferred onto PVDF membranes (Amersham Bioscience; Cytiva). The membranes were blocked with skimmed milk (5% in TBST buffer (TWEEN-20, 0.5%) for 1 h at 37°C and incubated with the primary antibodies overnight at 4°C, then incubated with the corresponding secondary antibodies (horseradish peroxidase-conjugated goat Anti-Rabbit IgG H&L; cat. No.: ab205718, dilution: 1:10,000, Abcam) overnight at 4°C. The sections were washed with PBS 3 or 4 times, incubated with the biotinylated secondary antibody (Abcam, ab64256, dilution: 1:1,000) at 37°C for 1 h, then treated with streptavidin horseradish peroxidase at 37°C for 15 min at 25°C, then stained with 0.5% crystal violet or 20 min at 25°C. The cells were analyzed using flow cytometry (FACS Canto II, BD Biosciences) and the data were analyzed using Calcuta II, BD Biosciences) and the data were analyzed using
Emodin inhibits ovarian cancer cell migration and invasion by affecting EMT. A wound healing assay was performed using the SK-OV-3, A2780 and PA-1 cell lines to investigate the in vitro anti-migration ability of emodin in ovarian cancer cells. As displayed in Fig. 2A and B, emodin significantly inhibited the wound healing rate of the SK-OV-3 and A2780 cell lines in a dose-dependent manner, suggesting an anti-migratory effect of emodin in ovarian cancer cells. In addition, Transwell and Matrigel assays were performed to further investigate the anti-migratory and anti-invasive abilities of emodin in ovarian cancer cells. The results demonstrated that the migration abilities of the SK-OV-3, A2780 and PA-1 cell lines were significantly suppressed in the presence of emodin compared with that in the control group (Fig. 2C and D). As indicated in Fig. 2E and F, treatment with different concentrations of emodin distinctly inhibited the invasive abilities of the SK-OV-3, A2780 and PA-1 cell lines. Furthermore, as there
is an association between cancer cell migration and invasion, and EMT (35), it was investigated whether emodin inhibited the migration and invasion abilities of the ovarian cancer cells via EMT. As demonstrated in Fig. 2G-I, the protein expression levels of N-cadherin and vimentin were decreased in the SK-OV-3, A2780 and PA-1 cell lines following treatment with emodin, whereas the protein expression levels of E-cadherin were increased, indicating EMT was affected in the ovarian cancer cell lines. Taken together, these results suggest that emodin suppressed the migration and invasion abilities of the ovarian cancer cells by affecting EMT.

Emodin significantly inhibits tumor growth in a xenograft model of human ovarian cancer. To investigate whether the antitumor activity of emodin in vivo was consistent with its anti-proliferation effect in vitro, SK-OV-3 tumor-bearing mice were treated with different doses of emodin (20 and 40 mg/kg) for 18 days following injection of the cells.
As shown in Fig. 3A, tumor growth was significantly reduced following treatment with 20 and 40 mg/kg emodin compared with that in the control group. Mice were sacrificed at the study endpoint, and tumors from each group were isolated. The tumor size and average weight in the 20 and 40 mg/kg treatment groups were notably smaller compared with that in the control group (Fig. 3B and C). Furthermore, no adverse effects, such as toxic death, skin ulceration and body weight loss were observed, during the treatment of the SK-OV-3 tumor-bearing mice with emodin. The body weight of the mice was not significantly different between the three treatment groups (Fig. 3D). In addition, after pathological evaluation of the major organs (heart, liver, spleen, the lungs and the kidneys), no notable pathological changes were found in the emodin treatment groups, suggesting that emodin is safe to use in vivo (Fig. 3E).

The antitumor effect of emodin was also analyzed using IHC from the tumor sections of each group. As displayed in Fig. 4A and B, increased expression of cleaved-caspase 3 and decreased number of Ki-67 positive cells was observed in the emodin treatment groups. Furthermore, compared with that in the control group, CD133, a key marker of ovarian CSCs (36), was significantly downregulated, suggesting a reduction in the development of ovarian CSCs following treatment with emodin.

Subsequently, western blot analysis was used to verify the anti-CSC effect of emodin. As demonstrated in Fig. 4C and D, the protein expression level of E-cadherin was increased following treatment with emodin, whereas the expression level of N-cadherin was decreased, suggesting that emodin inhibited EMT. In addition, the stemness of the tumors in the emodin treatment groups was significantly suppressed, as evidenced by the decrease in protein expression level of OCT4, which has been validated as a master regulator in the maintenance of the cancer stem-like phenotype (37).

Emodin reduces pulmonary metastasis of ovarian cancer cells by killing cancer stem cells. A pulmonary metastasis mouse model was established using SK-OV-3 cells to evaluate the anti-metastasis properties of emodin. Following treatment with emodin for 18 days, the mice were sacrificed, the weight of the lung tissues was measured, and the number of metastatic lung nodules were counted. As indicated in Fig. 5A and B, compared with that in the control group, the number of metastatic nodules on the lung was markedly reduced in the emodin treatment groups. In addition, the weight of the lungs in the emodin treatment groups was significantly inhibited compared with that in the control group.

It is reported that poor survival and distant metastasis in patients with ovarian cancer are caused by the renewal of CSCs, and increased CSCs have been associated with tumor recurrence or relapse, distant metastasis and chemoresistance (38,39). As aforementioned, the protein expression of the CSC markers, CD133 and OCT4 was significantly decreased in the emodin treatment groups. Various studies have indicated that the CD44+/CD24− population may represent stem cell-like properties of ovarian cancer cells (40-42). To further evaluate whether emodin inhibited lung metastasis of ovarian cancer cells by destruction of the ovarian CSCs, flow cytometry was used to measure the proportion of ovarian CSCs (CD44+/CD24−) in metastatic lung tissues following emodin treatment for 18 days. As shown in Fig. 5D and E,
the percentage of CD44+/CD24− cells was 32.65±2.95% in the control group, while treatment with 20 and 40 mg/kg emodin significantly reduced the proportion of CD44+/CD24− cells to 27.82±3.21 and 17.89±2.76%, respectively (P<0.001).
indicating that emodin reduces pulmonary metastasis of ovarian cancer cells by killing cancer stem cells.

Discussion

Ovarian cancer has the worst prognosis among all types of gynecological malignancies and patients are often only diagnosed at an advanced stage (1). Even though chemotherapy combined with surgery can result in remission in ovarian cancer patients, resistance and distant metastasis remain an obstacle for ovarian cancer therapy (43). Once the patient has relapsed, the outcome of chemotherapy declines significantly, with rapid tumor progression and drug resistance (44). Therefore, the discovery of novel potential drug candidates to prevent tumor metastasis is urgently required.

CSCs have a key role in tumor initiation, invasion, metastasis and therapeutic resistance, as well as in local recurrence following curative resection (45). Therefore, the elimination of CSCs in patients with ovarian cancer is considered to represent an effective strategy for the treatment of this highly refractory malignancy. Furthermore, EMT is an important component of cancer proliferation and distant metastasis (46). The crosstalk between CSC and EMT has been shown to increase cancer cell mesenchymal characteristics on the CSCs and promote cancer cells to gain stemness (19,20). However, there are currently limited agents that preferentially inhibit ovarian CSCs by regulating EMT.

Emodin, which is derived from natural plants, has been reported to exhibit therapeutic effects in several diseases such as hyperlipidemia, anti-viral and anti-liver fibrosis (47-49). Various in vitro studies have demonstrated its effectiveness on the promotion of apoptosis or the inhibition of proliferation in lung, breast and cervical cancer cells (50-52). In the present study, it was found that emodin could reduce the viability of ovarian cancer cells at low concentrations. The anti-proliferation activity of emodin against ovarian cancer cells was verified with MTT and colony formation assays.

Emodin was also found to exhibit an inhibitory effect on migration and invasion in ovarian cancer cells by inhibiting EMT, as evidenced by the decrease in the protein expression level of N-cadherin and vimentin, and the increase in the protein expression level of E-cadherin following treatment with emodin. It has previously been reported that emodin inhibited pancreatic cancer EMT and invasion by increase the expression level of microRNA-1271 (53). It has been demonstrated that emodin inhibited colon cancer cell invasion and migration by suppressing EMT via the WNT/β-catenin pathway (54). Emodin could also suppress the proliferation and invasion of colorectal cancer cells by inhibiting VEGFR2 protein expression (55). Therefore, the antitumor effects of emodin were analyzed using a subcutaneous xenograft SK-OV-3 tumor mouse model. The results of the animal experiments suggested that the tumor growth rate and tumor weight were significantly inhibited by the administration of emodin (40 mg/kg), with an inhibitory rate of ~45%. The effects of emodin (20 mg/kg) on tumor growth rate and tumor weight were lower than that of 40 mg/kg. Mechanistic analysis demonstrated that emodin reduced the proliferative ability of the tumors and decreased EMT. In addition, decreased protein expression levels of CD133 and Oct4 were observed in the tumor tissues following treatment with emodin, which indicates that emodin could inhibit the stemness of ovarian cancer cells. Various studies have demonstrated that inhibition of EMT results in impairment of stemness during the reprogramming of somatic cells (56,57). Liu et al (51) reported that emodin reduced breast cancer lung metastasis by suppressing macrophage-induced breast cancer cell EMT and cancer stem cell formation. It has also been reported that fusobacterium nucleatum produced cancer stem cell characteristics by activating IL-6/STAT3 and eliciting EMT-resembling activation (18). However, there is not enough evidence to demonstrate that downregulation of CD133 and OCT4 could directly lead to impairment of stemness of tumor cells. CD133 and OCT4 are only markers of tumor stem cells and emodin was only found to markedly inhibit EMT activity and impair the stemness of tumor cells from the decrease in the expression level of CD133 and Oct4. In future experiments, CD133 and Oct4 expression will be knocked down or overexpressed using transfection with small inhibiting RNA/overexpression plasmid to further investigate the function of CD133 and Oct4 in the formation of cancer stem cells.

Ovarian cancer, which starts as a local tumor lesion can metastasize to distant organs, including the lymph nodes, the lungs and the breasts (58). The metastatic process of cancer cells from the primary tumor to the distant site is complex, and requires migration from local lesions to blood vessels (59). As emodin reduced the migratory and invasion ability of the ovarian cancer cell lines, the anti-metastasis effect of emodin was investigated using a pulmonary metastasis tumor mouse model. The results showed that administration of emodin, at 20 and 40 mg/kg, significantly inhibited lung metastasis of ovarian cancer, which was consistent with the in vitro experiments. Furthermore, poor outcomes of ovarian cancer therapies are usually caused by a small proportion of CSCs, that have escaped from the primary cancer lesion (60). Thus, elimination of CSCs in the tumor microenvironment could be a promising strategy to conquer ovarian cancer metastasis. In the present study, it was demonstrated that emodin could reduce the proportion of ovarian CSC in metastatic lung tissues, suggesting that emodin suppressed pulmonary metastasis of ovarian cancer.

In summary, the present study provided important information regarding the antitumor activities of emodin in ovarian cancer. Emodin exhibited antitumor and anti-metastasis effects on ovarian cancer by inhibiting EMT and ovarian CSC formation. The results suggest that emodin could serve as a potential drug for treating ovarian cancer and metastasis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.
Authors’ contributions

HML and HYC conceived and designed the study, HML acquired and analyzed the data. HML, HMC and JY interpreted the data and wrote the manuscript. HML and HYC confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All the animal experiments in this study were performed according to the National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of Gannan Medical University (Jiangxi, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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