Ginsenoside Rb1 inhibits hypoxia-induced epithelial-mesenchymal transition in ovarian cancer cells by regulating microRNA-25

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Abstract. Metastasis frequently occurs in advanced ovarian cancer, which not only leads to substantial mortality but also becomes a major challenge to effective treatment. Epithelial-mesenchymal transition (EMT) is a key mechanism facilitating cancer metastasis. Targeting the EMT process with more efficacious and less toxic agents to prevent metastasis is of significant therapeutic value for ovarian cancer treatment. The anti-EMT function and mechanism of ginsenoside Rb1, a monomer composition extracted from the traditional Chinese herb Panax ginseng or P. notoginseng, was investigated in the present study. Western blotting demonstrated that treatment with ginsenoside Rb1 antagonized hypoxia-induced E-cadherin downregulation and vimentin upregulation in SKOV3 and 3AO human ovarian cancer cells. Wound healing assays and in vitro migration assays indicated that ginsenoside Rb1 weakened hypoxia-enhanced cell migration ability. Furthermore, it was demonstrated that microRNA (miR)-25 is upregulated by hypoxia in ovarian cancer cells, which was attenuated by ginsenoside Rb1 treatment. Additionally, forced expression of miR-25 in ovarian cancer cells was identified to not only trigger EMT, but also block the suppressive effects of ginsenoside Rb1 on hypoxia-induced EMT by negatively targeting the E-cadherin transactivator, EP300. In conclusion, ginsenoside Rb1 may reverse hypoxia-induced EMT by abrogating the suppression of miR-25 on EP300 and E-cadherin, which suggests that ginsenoside Rb1 may be a potential therapeutic candidate for the treatment of ovarian cancer.

Introduction

Ovarian cancer is the most lethal malignancy affecting the female reproductive system, with a 5-year survival rate as low as 30% (1). Cancerous metastasis is the leading cause of mortality in patients with ovarian cancer (2). The difficulty in treating disseminated cases constitutes a major hurdle to improve the prognosis of patients. Identifying agents with non-disclosed, anti-metastatic action from available therapeutics will be a short-cut strategy to combat cancer metastasis.

A growing body of evidence has demonstrated that loss of epithelial features and acquisition of mesenchymal phenotypes promote invasion and distant metastasis of cancer cells, the process is termed epithelial-mesenchymal transition (EMT) (3,4). During EMT, loss of molecules involved in adhesion junctions, tight junctions and desmosomes cause disruption of cell-cell contact and cell polarity in epithelial cells, while acquisition of mesenchymal markers increases cell motility and invasion (5). EMT has been found to be positively implicated in metastatic development of various types of cancer (6). In ovarian cancer, EMT is reported to be associated with peritoneal metastasis, and poor progression-free and overall survival (7). Therefore, identification of agents that block the EMT process may increase the arsenal for treatment of ovarian cancer.

MicroRNA (miR) are a class of small non-coding RNA of ~22 nucleotides in length, that are single-stranded and highly conservative in structure. miR inhibit protein translation or promote mRNA degradation by binding to the 3’-untranslated region of their target genes (8). miR may act as oncogenes or tumor suppressors, depending on the specific function of their targets (9). Research has demonstrated the aberrant expression of miR in human ovarian cancer. For instance, miR-187 is overexpressed in ovarian cancer tissues, which is correlated with the overall survival of ovarian cancer patients (10). miR-149, a member of the miR-200 family, is capable of reversing the EMT process in ovarian cancer cells (11). miR-25, a member of the miR-106-25 cluster, located in intron 13 of the mini-chromosome maintenance complex component 7 on chromosome 7q22.1 (12,13), is upregulated in epithelial ovarian cancer tissues and cell lines (14). Higher level of miR-25 present in tissues of advanced clinical stage and lymph node metastases of ovarian cancer, and patients with upregulated miR-25 tend
to have poorer prognoses (15). However, the specific role of miR-25 in ovarian cancer metastasis remains unclear.

Several monomer compositions from traditional Chinese medicine possess anti-EMT activity. Resveratrol, for example, reverses epidermal growth factor-induced EMT via targeting the extracellular signal-regulated kinase signaling pathway in human breast cancer cell line MCF-7 (16). Ginsenosides are the pharmacologically active components of *Panax ginseng* (17,18). Several effective ingredients of ginsenosides have been identified, such as Rbl, Rg1, Rg3, Rh1 and Rh2, the majority of which have been observed with anticancer activities (18-21). Rbl has been found to have antioxidant, anti-inflammatory (22), anti-senescent and neural protective functions (23). The anti-neoplastic action of Rbl has been reported in the SW480 human colorectal cancer cell line, where it may induce cell apoptosis (24). In the present study, the effect of ginsenoside Rbl on hypoxia-induced EMT in SKOV3 and 3AO ovarian cancer cells was explored, attempting to elucidate the anticancer mechanism of Rbl at the molecular level.

Materials and methods

**Drug and antibodies.** Ginsenoside Rbl with purity of 99% was obtained from Taisly Pharmaceutical Co. (Tianjin, China). Rbl was dissolved in dimethyl sulfoxide at a concentration of 1 mg/ml and stored at -20°C. The stock solution was then added into cell culture medium to generate certain final concentrations. The following primary antibodies were used: β-actin and vimentin (both from Cell Signaling Technology, Inc., Danvers, MA, USA); E-cadherin (Bioworld Technology, Inc., St. Louis Park, MN, USA); and EP300 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany).

**Cell culture and treatment.** Human ovarian cancer cell lines, SKOV3 and 3AO, were obtained from the Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China) and the Shandong Academy of Medical Sciences (Jinan, China), respectively. Cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and maintained in an incubator at 5% CO₂, 37°C and 100% humidity. For hypoxic induction, cells were incubated at 1% O₂ in a HF100 hypoxia chamber (Heat Force, Hong Kong, China) with or without exposure to 160 µg/ml Rbl. Cell morphology was observed and cell images were captured using a phase contrast microscope at a magnification of x100.

**Cell viability assay.** SKOV3 and 3AO ovarian cancer cells were seeded in 96-well plates at a density of 5x10³ cells/well. Rbl was used at final concentrations of 0-320 µg/ml to treat cells at 37°C for 24, 48 and 72 h, respectively. Cell viability was assessed by MTT colorimetric assay, with 20 µl of 5 mg/ml MTT added into each well. This was followed by incubation with cells for 4 h at 37°C and the addition of 150 µl DMSO, as previously described (25). The absorbance was measured at a wavelength of 570 nm by an EnSpire multimode plate reader (PerkinElmer, Inc., Waltham, MA, USA). The survival rate of cells was calculated as: Survival rate (%) = [optical density (OD) 570test - OD570blank]/(OD570control - OD570blank) x 100%.

**miR mimic transfection.** SKOV3 cells were seeded, at a density of 2.5x10⁴ cells/well into 6-well plates 24 h prior to the transfection. A total of 100 nM miR-25 mimic was provided by Guangzhou RiboBio Co., Ltd. (cat. no. miR0000081-1-5; Guangzhou, China) and transfected into cells in each well using the X-tremeGENE siRNA transfection reagent (Roche Molecular Diagnostics, Pleasanton, CA, USA), according to the manufacturer's instructions. A negative control was used in parallel experiments. After 24 or 48 h of transfection, total RNA and protein were collected, respectively.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from treated SKOV3 and 3AO cells and corresponding control cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. miR-25 was quantified by RT-qPCR. cDNA synthesis was conducted with RevertAid first strand cDNA synthesis kit (Thermo Fisher Scientific, Inc.; cat. no. K1622) according to the manufacturer's instructions, as follows: 2 µg RNA was mixed with 1 µl stem-loop reverse transcription primers for miR-25 (cat. no. miRQ0000081-1-1) and U6 (cat. no. MQP-0201; Guangzhou RiboBio Co., Ltd.) in a total volume of 12 µl, and incubated at 65°C for 5 min followed by being chilled on ice for 5 min, then mixed with 4 µl 5x reaction buffer, 2 µl dNTP mixture, 1 µl RNase Inhibitor and 1 µl M-MuLV reverse transcriptase in a final volume of 20 µl. The reactions were performed at 25°C for 5 min, followed by 42°C for 60 min and 70°C for 5 min. The cDNAs were stored at -80°C for subsequent assessments. qPCR was conducted on a CFX-96 real-time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using a SYBR-Green Master Mix (Takara Biotechnology Co., Ltd., Dalian, China). The reactions were incubated at 95°C for 30 sec; followed by 40 cycles of 95°C for 5 sec, 60°C for 30 sec and 72°C for 30 sec. Each measurement was performed in triplicate, and no-template controls were included for each assay. Small nuclear RNA (Rnu6; Guangzhou RiboBio; cat. no. MQP-0201) was used as an internal control. The relative expression of miR-25 was calculated using the 2-ΔΔCq method (26), normalized with the internal control and compared with the level in the negative control cells.

**Western blot analysis.** Total cell protein extracts were collected using mammalian protein extraction reagent (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The samples were quantified using a Quick Start Bradford Protein Assay kit (Bio-Rad Laboratories, Inc.). Proteins were boiled for 5 min prior to separation by 10% SDS-PAGE with 30 µg proteins loaded in each lane, and then transferred onto nitrocellulose membranes (Pall Life Sciences, Port Washington, NY, USA). Membranes were blocked with 5% non-fat milk at room temperature for 2 h. The membranes were incubated with corresponding primary antibodies: β-actin (catalogue no. 3700; Cell Signaling Technology, Danvers, MA, USA); vimentin (cat. no. 5741; Cell Signaling Technology, Inc.); E-cadherin (cat. no. BS1098; Bioworld Technology, Inc., St. Louis Park, MN, USA) and anti-EP300 (cat. no. SAB1400094; Sigma-Aldrich; Merck KGaA) diluted in Tris-buffered saline-Tween-20
As demonstrated in Fig. 2, when exposed to 1% O_2, hypoxic conditions for 24 h, both SKOV3 and 3AO cells experienced typical EMT changes. Morphologically, both cell lines changed from an epithelial appearance of a cobblestone-like cellular shape and tight intracellular connections to a mesenchymal appearance of a spindle-like cellular shape and loose intracellular connections (Fig. 2A). When incubated with 160 µg/ml Rb1 under hypoxic conditions, cell morphology and cell junctions recovered to the epithelial pattern (Fig. 2A). Key EMT markers were further determined by western blot analysis. After hypoxic incubation for 24 h, the expression of E-cadherin was decreased and the expression of vimentin was increased (Fig. 2B). Similar to the morphological observation, co-treatment with Rb1 under hypoxic conditions rescued E-cadherin downregulation and vimentin upregulation (Fig. 2B).

Additionally, cell mobility was detected by wound healing assays. As demonstrated in Fig. 3A and B, the wound healing ability of SKOV3 and 3AO cells was significantly reduced compared with the control group (P<0.05, two-tailed t-test).
was significantly enhanced by 24 h hypoxic incubation compared with the control (P<0.01), which was significantly weakened by Rb1 co-treatment (P<0.01). In vitro migration assays displayed similar results, Rb1 significantly reduced hypoxia-induced cell motility compared with the cells incubated under hypoxic conditions without the addition of Rb1 (P<0.01; Fig. 4). Altogether, these results indicated that Rb1 may inhibit hypoxia-induced EMT in SKOV3 and 3AO cell lines.

Figure 2. Ginsenoside Rb1 blocks the Ho-induced EMT process in SKOV3 and 3AO ovarian cancer cells. (A) Rb1 reversed the morphological EMT changes induced by Ho for 24 h in SKOV3 and 3AO cells. When exposed to 1% O₂ Ho for 24 h, both cell lines changed from a polygonal, cobblestone-like cellular shape with tight intracellular connections to a spindle-like cellular shape with loose intracellular connections. When co-incubated with 160 µg/ml Rb1, the morphology and cell junctions of both SKOV3 and 3AO cells recovered to an epithelial pattern. Scale bar, 100 µm. (B) Rb1 antagonized the Ho-induced expression alterations of EMT markers. Western blot analysis demonstrated Ho 24 h-incubation decreased the expression of E-cadherin and increased the expression of vimentin, which was recovered by Rb1 treatment under the 1% O₂ condition. EMT, epithelial-mesenchymal transition; Ho, hypoxic.

Figure 3. Wound healing assay of SKOV3 and 3AO cells. Scratch healing ability of (A) SKOV3 and (B) 3AO cells was significantly enhanced when cultured under hypoxic conditions for 24 h, which was reversed by co-treatment with Rb1. Percentage of wound healing was calculated and statistically analyzed. **P<0.01 as indicated.
that the expression of miR-25 was significantly increased during hypoxia-induced EMT in both SKOV3 and 3AO cells compared with control cells (P<0.01; Fig. 5A). This increase in expression was significantly reversed by treatment with Rb1 (P<0.01; Fig. 5A). In order to examine the role of miR-25 in the anti-EMT action of Rb1, miR-25 was overexpressed in SKOV3 cells by transfection with miR-25 mimics and detection of its effect on the role of Rb1 in hypoxia-induced EMT. RT-qPCR demonstrated that transfection with miR-25 mimics led to a significant increase (~5-fold) in the miR-25 expression level in SKOV3 cells compared with control cells (P<0.01; Fig. 5B).

As detected by western blotting, exogenous overexpression of miR-25 itself reduced E-cadherin and elevated vimentin (Fig. 5C). miR-25 eliminated the capacity of Rb1 to block hypoxia-induced EMT (Fig. 5C). These results demonstrated that downregulation of miR-25 may be an important mechanism in the suppressive effect of Rb1 on hypoxia-induced EMT in ovarian cancer cells.

Following this, the potential target of miR-25 was identified. As EP300 is the transcriptional activator of E-cadherin (27), the changes in EP300 expression caused by miR-25 were examined. As demonstrated in Fig. 5D, overexpression of miR-25 repressed EP300 protein expression in SKOV3 cells. Furthermore, hypoxia reduced the protein expression level of EP300. Rb1 rescued the EP300 expression level under hypoxia, which was antagonized by exogenous miR-25 overexpression (Fig. 5E). These results suggested that Rb1 blocked the hypoxia-induced EMT process in ovarian cancer cells via downregulation of miR-25, which contributed to the overexpression of E-cadherin transactivator EP300, thus increasing E-cadherin. Therefore, Rb1 may inhibit the hypoxia-induced EMT process in ovarian cancer cells through the miR-25/EP300/E-cadherin pathway (Fig. 6).

Discussion

EMT has been suggested as a predominant driver of poor survival in patients with ovarian carcinoma in a review summarizing gene expression array analyses, in which 108 of 154 genes selected by the authors were EMT-related (28). Efforts have been made to identify agents to block EMT in tumor therapy. EW-7195, a novel small molecule inhibitor specifically targeting TGF-β type I receptor (ALK5) kinase, was demonstrated to decrease phosphorylated Smad2 and the nuclear translocation of Smad2 to inhibit transforming growth factor (TGF)-β1-induced EMT in breast cancer cells (29). Anti-EMT therapeutics, particularly those with reduced cytotoxicity, would likely provide clinical benefits not shared by the commonly used genotoxic chemotherapy drugs that indiscriminately kill both normal and cancer cells, and result in severe side effects and even treatment failure (30). Therefore, identifying pharmacologically active ingredients from natural sources, such as Chinese herbs, to inhibit tumor metastasis could represent an attractive approach to anticancer drug discovery.
Ginsenoside Rb1, the major bioactive component in *P. ginseng*, constitutes 0.47-0.82% of total *P. ginseng* root extract (31). The majority of published research on Rb1 has concentrated on the antioxidant and neovascularization effects of this component (32-35). The present study explored the anti-EMT effect of Rb1 in ovarian cancer. To the best of our knowledge, the present study is the first on the anti-neoplastic bioactivity of Rb1 in ovarian cancer. Being a natural herbal medicine, Rb1 demonstrated no significant toxicity to human ovarian cancer cells. It inhibited EMT and migration of ovarian cancer cells in vitro. These results indicated that Rb1 may be a potential candidate for clinical application against ovarian cancer metastasis. At the same time, the possible underlying mechanism of Rb1’s anti-EMT function was preliminarily explored. The present study demonstrated that Rb1 downregulated the expression of miR-25, leading to the overexpression of EP300 and E-cadherin, which inhibited the hypoxia-induced EMT process in ovarian cancer cells. The same mechanism of miR-25 in inducing EMT was also reported in gastric cancer (14,36) and in renal cell carcinoma (37,38). A study by Smith et al (39) demonstrated that overexpression of miR-25 induced EMT in breast cancer through targeting the tumor-suppressive Smad7 protein, resulting in an increase in TGF-β receptor 1 and downstream activation of TGF signaling. The same mechanism of miR-25 in inducing EMT was also reported in gastric cancer.
cancer (40). A study by Wang et al (15) indicated that high miR-25 expression was significantly associated with advanced clinical stage and lymph node metastases in epithelial ovarian cancer. Notably, patients with a high expression of miR-25 tended to have shorter survival than patients with lower levels (10). Similarly, it was observed in the present study that miR-25 induced EMT in ovarian cancer cells. Rb1 suppressed the EMT process in ovarian cancer cells by downregulating miR-25. A study by Kwok et al (31) also identified that Rb1 may decrease the expression of miR-25 in human dermal fibroblasts, which is consistent with the results of the present. These findings indicated that miR-25 may be a potential target in ovarian cancer metastasis therapy.

EP300 is a transcriptional coactivator and prototype histone acetyltransferase involved in regulating multiple cellular processes, including cell cycle regulation, proliferation, differentiation, apoptosis, DNA damage repair and adhesion, as well as embryonic development (41,42). EP300 has been demonstrated to be a transcriptional activator of E-cadherin and to promote E-cadherin gene expression (43,44). Furthermore, EP300 has been suggested to be the target gene of miR-25 in minimally transformed mammary epithelial cells (MTMEC) (27). In MTMEC cells, by targeting EP300, overexpressed miR-25 led to an EMT-like phenotypic characteristic change, including an increase in cell motility and invasion, as well as the ability to proliferate following treatment with doxorubicin (27). When EP300 was strongly expressed, the association between SNAIL expression and downregulation of E-cadherin was lost in colon carcinoma (45). Research has also demonstrated that loss of EP300 in HCT116 cells resulted in a potentially aggressive phenotype characterized by reduced adhesion and increased migration (46). Furthermore, several critical genes involved in these pathways are differentially expressed, suggesting that EP300 loss promoted EMT (46). Similarly, in the present study, EP300 was repressed during the hypoxia-induced EMT process in ovarian cancer cells, paralleled with E-cadherin downregulation. However, following treatment with Rb1, EP300 expression was recovered. Therefore, EP300 functioned in sustaining epithelial characteristics in cancer cells.

In conclusion, the results of the present study demonstrated that Rb1 could inhibit the hypoxia-induced EMT process in ovarian cancer cells through the miR-25/EP300/E-cadherin pathway. These results provided support for Rb1 acting as a useful drug candidate for ovarian cancer therapy, which warrants further research in the application of ginsenosides in cancer treatment.

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