Ampelopsin reduces the migration and invasion of ovarian cancer cells via inhibition of epithelial-to-mesenchymal transition

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Abstract. Ampelopsin has displayed anticancer activity in several types of cancers. However, no evidence has been reported for the direct effect of ampelopsin on ovarian cancer cell migration and invasion, and the underling mechanisms have not yet been clearly established. The aim of the present study was to investigate the influence of ampelopsin on the migration and invasion of ovarian cancer. Proliferation and viability of the ovarian cancer cells were detected by MTT assay. Migration and invasion of the cells were detected, respectively, by scratch wound healing assay and Transwell assay. The expression levels of epithelial-to-mesenchymal transition (EMT) markers were detected at the protein level after stimulation with ampelopsin. Then, the expression levels of NF-κB and p-IκBα were detected with western blot analysis. Meanwhile, an inhibitor of NF-κB was used to investigate the effect of ampelopsin. Finally, the expression of Snail was also detected. Proliferation, migration and invasion of the A2780 cells were all inhibited following the application of ampelopsin. Ampelopsin upregulated E-cadherin and downregulated N-cadherin and vimentin in a concentration- and time-dependent manner. Ampelopsin also exerted its ability to suppress the nuclear translocation of the NF-κB pathway. Administration of the inhibitor BAY11-7082 confirmed the roles of NF-κB in the expression of EMT markers and its transcription factor. These results demonstrated that ampelopsin inhibited EMT and reduced the invasion of ovarian cancer cells via the NF-κB/Snail pathway.

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Introduction

Ovarian cancer is one of the most prevalent cancers and has the highest mortality rate among women with gynecological malignancies (1). Without an effective method of early detection and due to the highly invasive property of ovarian cancer cells, the majority of patients suffer distant metastasis at the time of diagnosis. It has been shown that approximately 70-80% of patients with ovarian cancer of stage III and stage IV die within 5 years of diagnosis, even when undergoing aggressive cytoreductive surgery and combination chemotherapy (2). Therefore, it is urgent to elucidate the molecular mechanisms associated with ovarian cancer metastasis and to identify new therapeutic approaches, in order to achieve better treatment outcome.

In recent years, accumulating evidence has demonstrated that epithelial-to-mesenchymal transition (EMT), which is a morphologic conversion process that was first described in the context of embryogenesis, is associated with the acquisition of mesenchymal phenotypes and malignant characteristics in ovarian cancer cells, representing mechanisms of escaping from apoptosis and migrating through the extracellular environment (3-5). Loss of the epithelial molecule E-cadherin and gain of mesenchymal markers N-cadherin and vimentin have been considered as the most important hallmarks of EMT (5). Among the stimuli that trigger EMT, Snail family members, including Snail, Slug, Twist, Zeb1 and SIP1, have been found to play an important role in promoting EMT (4).

Ampelopsin [(2R,3R)-3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)-2,3-dihydrochromen-4-one] (Fig. 1A), also called dihydromyricetin, is a type of flavonoid and is isolated from the stems and leaves of *Ampelopsis grossedentata*. Numerous pharmacological activities of ampelopsin have been reported, such as anti-inflammatory (6), antioxidant, and antimicrobial activity (7). In recent years, ampelopsin has been described to possess anticancer activity in various types of cancers. Ampelopsin was found to inhibit the growth and invasion of breast cancer cells *in vitro* (8), and to inhibit the growth of prostate cancer *in vivo* (9). Ampelopsin also showed activity for inhibiting vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), suppressing angiogenesis in hepatocellular carcinoma (10). However, no evidence

has been reported for the direct effect of ampelopsin on ovarian cancer cell invasion and the mechanisms of this effect.

The present study was designed to investigate the effects of ampelopsin on ovarian cancer cell migration and invasion, as well as its influence on EMT.

Materials and methods

Reagents. Ampelopsin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit monoclonal to E-cadherin and vimentin antibodies were from Abcam (Cambridge, UK). Rabbit polyclonal to N-cadherin, Snail and GAPDH were also from Abcam. Rabbit anti-mouse antibodies for NF-κB (p65) and IκBα were both purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). BAY11-7082, a selective inhibitor of NF-κB, was purchased from Sigma-Aldrich.

Cell culture. The A2780 cell line (human ovarian cancer cell line) was obtained from the American Type Culture Collection (Manassas, VA, USA), and was cultured in RPMI-1640 (HyClone Laboratories, Inc., Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone) in an atmosphere containing 5% CO_2.

Cell viability and proliferation assay. Cell viability and proliferation activity were assessed with the MTT colorimetric assay. A2780 cells were seeded into 96-well plates (Corning Inc., Corning, NY, USA) at a concentration of 5,000 cells/well. After stimulation with ampelopsin of various concentrations (0, 5, 10, 25, and 50 μM) for various time-points (0, 1, 3, 6, 12, 24, and 48 h), MTT (5 μg/ml, 20 μl) was added into each well for a 4-h incubation. Then, 80 μl of dimethyl sulfoxide (DMSO) (Sigma-Aldrich) was added for another 15 min to fully solubilize the formazan (the metabolic product of MTT). Finally, the liberated purple product was detected using a microplate luminometer at 490 nm.

Scratch wound healing assay. A scratch wound healing assay was used to evaluate the migratory ability of the A2780 cells. A2780 cells ($1x10^6$/well, Corning Inc.) were cultured in 6-well plates. Straight scratches of the same width were made in the monolayer of A2780 cells with a pipette tip. After incubation with ampelopsin of 25 μM for 24 h, images were captured to measure the wound healing under a microscope.

Transwell assay. The effect of ampelopsin on the invasive ability of the A2780 cells was detected with modified Boyden chambers with 8-μm pore filter inserts (Corning Inc.). The A2780 cells were cultured in 24-well plates, and the upper chamber contained cells in RPMI-1640 plus 1% FBS, while the lower chamber contained RPMI-1640 plus 10% FBS. Cells ($1x10^5$/well) were re-suspended in the upper chamber at 37˚C in 5% CO_2. After a 24-h-incubation, the cells on the lower surface were fixed with methanol for 30 min and stained with hexamethylpararosaniline, while the cells remaining on the upper surface were wiped away.

Western blot analysis. After stimulation, the A2780 cells were collected and lysed. The extracted protein concentration was measured using BCA protein assay kit (Beyotime Biotechnology, China). Proteins of equal amounts were separated via 10% SDS-polyacrylamide gel, and transferred onto nitrocellulose (NC) membranes (Millipore, Billerica, MA, USA). Blots were blocked and incubated with the primary antibodies followed by incubation with the secondary antibodies. Finally, the blots were visualized with electrochemiluminescence (ECL) detection system (Millipore).

Statistical analysis. All data in the present study were evaluated with predictive analytics software (PASW) statistics 18.0 (SPSS Inc., Chicago, IL, USA). The normally distributed data were analyzed by one-way ANOVA and the non-parametric variables were analyzed by the Mann-Whitney U test. Statistical significance was confirmed as $P<0.05$.

Results

Ampelopsin inhibits the proliferation of ovarian cancer cells. To clarify the specific role of ampelopsin in ovarian cancer cell proliferation, various concentrations (0, 5, 10, 25, and 50 μM) of ampelopsin were added into the cultured A2780 cells. As shown in Fig. 1B, after a 24-h incubation at 50 μM, ampelopsin significantly inhibited the cell viability as detected by MTT assay. However, at concentrations below 50 μM (5, 10, and 25 μM), the inhibition was not significant. Subsequently, 25 and 50 μM of ampelopsin were selected to stimulate the cells for different times (0, 1, 3, 6, 12, 24 and 48 h). As shown in Fig. 1C, ampelopsin of 50 μM significantly inhibited the proliferation after a 24-h stimulation, while ampelopsin of 25 μM did not inhibit the proliferation after a 48-h stimulation. As a result, we chose 25 μM of ampelopsin for the subsequent migration and invasion experiments so that the influence of proliferation was excluded.

Ampelopsin inhibits the migration and invasion of ovarian cancer cells. A wound healing assay was used to assess the migration of the ovarian cancer cells, while a Transwell assay was applied to evaluate the invasion of the ovarian cancer cells. A2780 ovarian cancer cells were treated with ampelopsin at the concentration of 25 μM for 24 h. As shown in Fig. 2A, the results of the wound healing assay demonstrated that healing over the scratch was significantly reduced after treatment with ampelopsin. As shown in Fig. 2B, the results of the Transwell assay demonstrated that the number of invading cells migrating from the upper to the lower surface was also significantly reduced after treatment with ampelopsin.

Effects of ampelopsin on expression of EMT markers in the ovarian cancer cells. EMT is thought to play an important role in the process of cancer cell migration and invasion. We thus assessed the effect of ampelopsin on EMT marker expression in the A2780 ovarian cancer cells by western blot analysis. Various concentrations of ampelopsin (5, 10, 25, and 50 μM) were respectively added to the cells, and the cancer cells were cultured for another 12 h. As shown in Fig. 3A, ampelopsin treatment significantly increased the expression of epithelial marker E-cadherin and decreased the expression of mesenchymal markers N-cadherin and vimentin in the A2780 cells. The results above suggest that ampelopsin may alter the expression of EMT markers in a concentration-dependent manner and

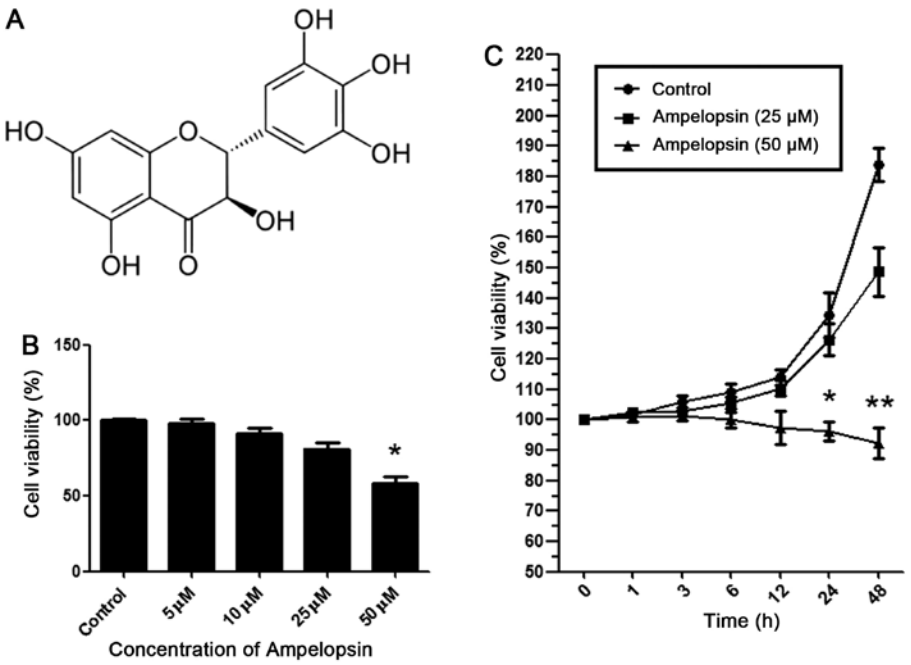

Figure 1. Ampelopsin inhibits the proliferation of A2780 ovarian cancer cells. (A) Chemical structure of ampelopsin. (B) After the A2780 cells were stimulated at 24 h with ampelopsin of different concentration gradients (0, 5, 10, 25, and 50 μM), MTT assay was used to detect the viability of the A2780 cells. (C) After A2780 cells were stimulated with 0, 25 and 50 μM ampelopsin for different time periods (0, 1, 3, 6, 12, 24 and 48 h), MTT assay was used to detect the viability of the A2780 cells. *P<0.05; **P<0.01 vs. the control group. Data shown are means ± SEM from 3 independent experiments in duplicate.

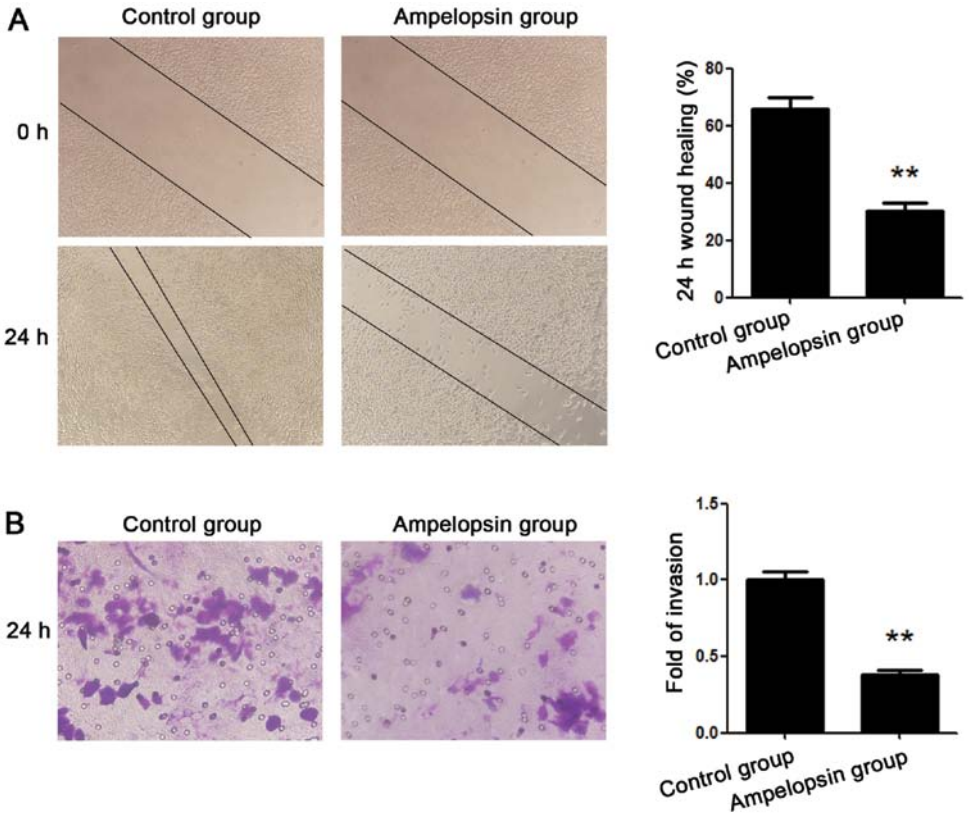

Figure 2. Ampelopsin inhibits the migration and invasion of A2780 ovarian cancer cells. (A) A2780 cells were treated with ampelopsin at the concentration of 25 μM for 24 h. The effect of ampelopsin on the migration of A2780 cells was assessed by a wound healing assay. (B) A2780 cells were treated with ampelopsin at the concentrations of 25 μM for 24 h. The effect of ampelopsin on invasion of A2780 ovarian cancer cells was assessed by a Transwell assay. **P<0.01 vs. the control group. Data shown are means ± SEM from 3 independent experiments in duplicate.

indicate that the inhibitory effect of ampelopsin on A2780 cell invasion and migration may be associated with EMT.

NF-κB pathway is involved in the anti-metastatic mechanism of ampelopsin. As a transcription factor, NF-κB shows a

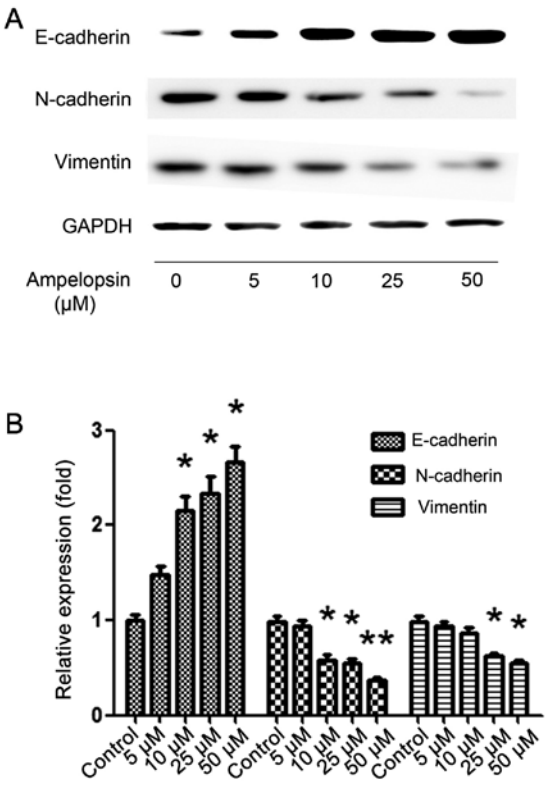

Figure 3. Ampelopsin increases the expression of E-cadherin and decreases the expression of N-cadherin and vimentin. (A) A2780 ovarian cancer cells were treated with ampelopsin at various concentrations (0, 5, 10, 25, and 50 μM) for 12 h and western blot analysis was utilized to assess the changes of E-cadherin, N-cadherin and vimentin protein expression. (B) Statistical analysis of the western blotting results. *P<0.05 and **P<0.01 vs. the control group. Data shown are means ± SEM from 3 independent experiments in duplicate.

significantly increased expression in ovarian cancer, and plays an important role in the aggressiveness of tumors (11,12). NF-κB is tightly masked by its inhibitor protein IκB and thereby sequestered in the cytoplasm (13). Various stimulation signals may phosphorylate IκBα and trigger a ubiquitination-mediated degradation of IκBα (13), which allows phosphorylation of p65 and translocation of NF-κB from the cytoplasm to the nucleus where it binds with the promoter of its target genes. A previous study of pancreatic carcinoma cells demonstrated that blockage of NF-κB signaling may render cells resistant to TGF-β-induced EMT and then suppress migration and invasion (14). Thus, in the present study, we aimed to ascertain whether ampelopsin exerts its anti-metastatic effects and alters the expression of EMT markers via the NF-κB pathway.

In order to determine the effects of ampelopsin on NF-κB, we first stimulated the cells with different concentrations of ampelopsin (5, 10, 25 and 50 μM) for 12 h. Then cytosol and nuclear proteins were extracted, respectively, followed by the detection of the expression of p65 and IκBα. As shown in Fig. 4A and B, ampelopsin significantly increased the expression of p65 in the cytosol but decreased its expression in the nucleus in a concentration-dependent manner. Meanwhile, the phosphorylation of IκBα in the cytosol was decreased after ampelopsin treatment. Subsequently, to further determine the effect of NF-κB on the expression of EMT markers, we blocked the NF-κB pathway by its inhibitor BAY11-7082 (20 μM), which was chosen to pretreat the cells for 2 h before ampelopsin. As shown in Fig. 4C, BAY11-7082 significantly reversed ampelopsin-induced E-cadherin expression and N-cadherin and vimentin expression. The results above indicate that NF-κB activation is critical for EMT and ampelopsin exerted its effect on cancer cell migration and invasion, as well as EMT, through suppressing the NF-κB pathway.

Ampelopsin induces Snail upregulation via NF-κB activation. As a complex multistep process occurring during tumor progression, EMT is governed by a variety of regulatory networks, in particular, the Snail family (15). Among the family members, Snail was the first described transcriptional repressor of E-cadherin and is the most extensively studied transcription factor. Thus, the effects of ampelopsin on the expression of Snail and the relationship between NF-κB and Snail were investigated. We choose the concentration gradient of ampelopsin (5, 10, 25, and 50 μM) to stimulate the ovarian cancer cells for 24 h. As shown in Fig. 5A, ampelopsin obviously suppresses the expression of Snail along with the increase in concentration, reaching a peak activity at the concentration of 50 μM. Then, the cells were also pretreated with BAY11-7082 (20 μM) for 2 h. As shown in Fig. 5B, pretreatment with the NF-κB inhibitor BAY11-7082 significantly abrogated the inhibitory effect of ampelopsin. These results indicate that NF-κB is a key regulator of Snail, and ampelopsin suppresses the expression of Snail through blocking the NF-κB pathway at least in part.

Discussion

Metastasis is considered to be a primary cause of mortality among most ovarian cancer patients. Thus, understanding the molecular mechanisms of metastasis and searching for effective approaches to inhibit metastasis are the most important issues in cancer research. As a type of flavonoid extracted from *Ampelopsis grossedentata*, ampelopsin exhibits multiple functions in inflammation and oxidation. In recent years, more and more evidence suggests that ampelopsin has the ability to inhibit cell proliferation, migration and invasion in breast and prostate cancer. However, although the previous research (8-10) shed light on the action of ampelopsin's anticarcinogenesis, direct evidence involving ovarian cancer and the detailed molecular mechanisms have not been clearly elucidated.

As shown in a previous study (9), ampelopsin exhibits potent activity in inhibiting the proliferation of cancer cells by inducing apoptosis and downregulating Bcl-2. Thus, in the present study, we firstly detected the effect of ampelopsin on the viability of cultured cancer cells, and the results revealed that ampelopsin of 50 μM significantly suppressed ovarian cancer cell proliferation after 24 and 48 h of incubation, while there was no difference at 12 h. As a result, in the following experiments involving EMT markers, we selected 12 h as the stimulation time-point and 25 μM as the stimulation concentration, so that the influence of proliferation was excluded. Subsequently, the wound healing and Transwell assays were respectively applied, and the results demonstrated that migration and invasion of ovarian cancer cells were both suppressed after incubation with ampelopsin, which indicated the anti-metastatic activity of ampelopsin.

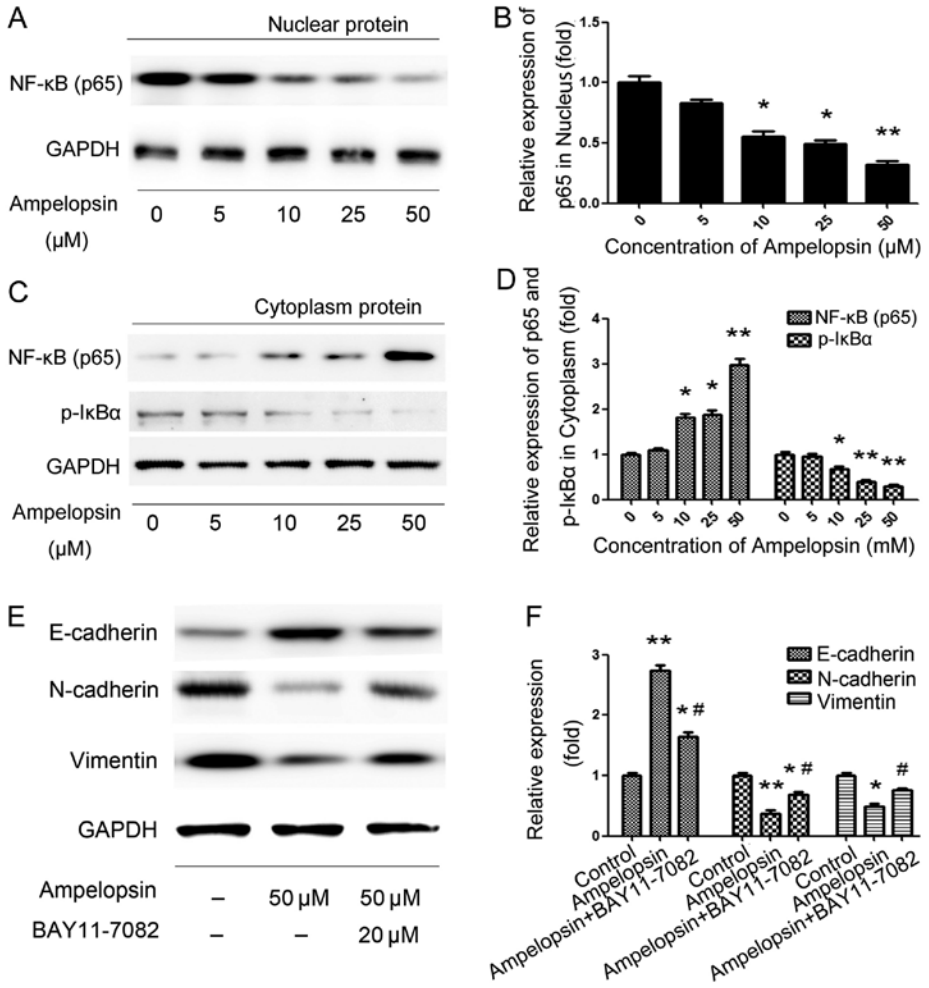

Figure 4. NF-κB mediates the regulation of epithelial-to-mesenchymal transition (EMT). (A and B) Treatment with ampelopsin for 12 h concentration-dependently decreased the expression of p65 in the nucleus. (C and D) Treatment with ampelopsin for 12 h concentration-dependently increased the expression of p65 in the cytosol, but decreased the phosphorylation of IκBα in the cytosol. (E and F) Western blot analysis showed that BAY11-7082 (20 μM) significantly reversed the effects of ampelopsin on E-cadherin, N-cadherin and vimentin protein expression. *P<0.05 and **P<0.01 vs. the control group; $^{\#}$P<0.05 vs. the ampelopsin group. Data shown are means ± SEM from 3 independent experiments in duplicate.

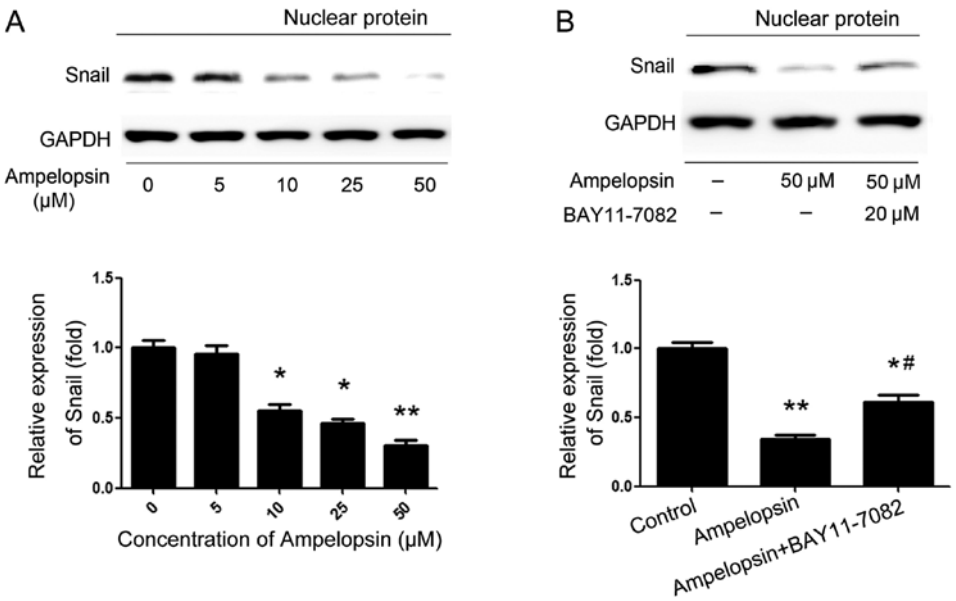

Figure 5. NF-κB mediates the regulation of Snail. (A) Treatment with ampelopsin for 12 h concentration-dependently decreased the expression of Snail in the nucleus. (B) Western blot analysis showed that BAY11-7082 (20 μM) significantly reversed the effects of ampelopsin on Snail protein expression. *P<0.05 and **P<0.01 vs. the control group; $^{\#}$P<0.05 vs. the ampelopsin group. Data shown are means ± SEM from 3 independent experiments in duplicate.

EMT, a developmental reprogramming process through which polarized, immotile epithelial cells undergo transdifferentiation into motile mesenchymal cells, is characterized by loss of epithelial markers, such as E-cadherin, and in turn, acquisition of mesenchymal markers, such as N-cadherin and vimentin. Previous studies have demonstrated that, during ovarian cancer progression, EMT plays an important role in inducing matrix metalloproteinase production and in promoting dissemination of tumor cells, thereby increasing cell invasion and contributing to the poor outcome of ovarian cancer patients (16-18). In addition, evidence suggests that EMT has been found to give rise to resistance to chemotherapeutic drugs in ovarian cancer (19,20). Therefore, in the present study, we aimed to ascertain the specific roles of ampelopsin in the metastasis of ovarian cancer cells, as well as the underlying relationship between ampelopsin and EMT. The results demonstrated that incubation with ampelopsin for 12 h concentration-dependently promoted the expression of epithelial marker E-cadherin, and inhibited mesenchymal markers N-cadherin and vimentin, which indicated that EMT was promoted by ampelopsin in ovarian cancer cells.

NF-κB, a pleiotropic transcription factor, plays important roles in pathological processes associated with cancer development, such as proliferation, migration, invasion, angiogenesis, drug resistance and inflammation (21-23). It was also found in previous studies that the induction of EMT was closely associated with the activation of NF-κB. Cichon and Radisky found that ROS-induced EMT in mammary epithelial cells was mediated by NF-κB (24), while Liu and colleagues found that triptolide reversed hypoxia-induced EMT in pancreatic cancer by NF-κB downregulation (25). In addition, it was also reported in several other studies that EMT was induced by many factors via the NF-κB signaling pathway in hypopharyngeal cancer (26), tongue squamous cell carcinoma (27), and prostate cancer (28). In the present study, our results demonstrated that the expression of p65 in the cytosol was increased while its expression in the nucleus was decreased, which indicated that the inhibition of NF-κB nuclear translocation mediated the most important biological effects of NF-κB. The phosphorylation of IκBα in the cytosol, which is the major inhibitor of NF-κB, was detected simultaneously, and its expression was decreased after ampelopsin treatment. Subsequently, the application of BAY11-7082 significantly reversed ampelopsin's effects on E-cadherin, N-cadherin and vimentin expression, which further proved the important role of NF-κB in the induction of EMT by ampelopsin.

Finally, we detected the effect of ampelopsin and NF-κB on transcription factor Snail. Snail, a zinc finger protein, is the most important member of the Snail superfamily. Previous studies have confirmed that it mediates EMT through downregulation of epithelial marker E-cadherin and upregulation of mesenchymal markers N-cadherin and vimentin, through binding with several boxes in the promotor region (29). Snail is overexpressed in several types of cancers, especially in ovarian cancer, and has also been associated with tumor progression (30). Notably, Snail was also found to confer migration and invasion properties to cancer cells and promote carcinoma metastasis. The localization, expression and activity of Snail may be regulated by various factors, and the NF-κB signaling pathway is the most important and hackneyed mediator (31,32). The results also revealed that ampelopsin concentration-dependently increased the expression of Snail in the nucleus, while BAY11-7082 significantly reversed the effects, further indicating that NF-κB was upstream of Snail.

In summary, the present study firstly demonstrated that ampelopsin inhibited EMT and reduced the invasion of ovarian cancer cells. Moreover, the effect of ampelopsin was mediated by the NF-κB/Snail signaling pathway. According to the results of the present study, the invasive ability of ovarian cancer cells may be restrained by ampelopsin by inhibiting the NF-κB/Snail signaling pathway and EMT. Further *in vivo* studies should be performed.

Acknowledgements

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