The milk-derived hexapeptide PGPIPN inhibits the invasion and migration of human ovarian cancer cells by regulating the expression of *MTA1* and *NM23H1* genes

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Abstract. Some bioactive peptides derived from natural resources or synthesized by rational design have been proved to have very good anticancer effect. We studied the inhibition of PGPIPN, a hexapeptide derived from bovine β-casein, on the invasion and metastasis of human ovarian cancer cells *in vitro* and its molecular mechanism. The human ovarian cancer cells studied include the cell line $SKOV_3$ as well as the primary ovarian cancer cells from ovarian tumor tissues of 37 patients at initial debulking surgery, diagnosed as serous ovarian adenocarcinoma. We showed that PGPIPN inhibited the invasion of ovarian cancer cells with Transwell chamber assay, the migration of ovarian cancer cells with cell scratch assay and colony formation of ovarian cancer cells. The expression (mRNAs and proteins) of genes relevant to invasion and metastasis, *MTA1*, and *NM23H1* were analyzed by real-time PCR and western blotting. PGPIPN repressed the expression of *MTA1*, and promoted *NM23H1*. The effects of PGPIPN were dose-dependent. Thus, our study suggests that PGPIPN is a potential therapeutic agent for adjuvant therapy of human malignant ovarian tumors.

Introduction

Ovarian cancer is one of the most common gynecological malignant tumor, although the incidence is lower than that of cervical and uterine cancer, the mortality is the highest (1). The initial debulking surgery is still the main method to cure ovarian cancer, but it often recur after surgery. Although there are some progress in both surgical approach and chemotherapeutic regimen, there is no substantial breakthrough in the treatment of ovarian cancer. Because many ovarian cancer symptoms do not appear in the early stage, it is not easy to be diagnosed. More than 70% of patients are in an advanced stage when diagnosed, for whom the 5-year survival is only 20% (2-4). Currently, cis-diaminodichloroplatinum (DDP) is still chosen as a feasible first-line chemotherapeutic drug for clinical treatment of ovarian cancer, and its antitumor effect has been recorded. However, DDP has significant side effects on patients with renal toxicity, and ototoxicity. Therefore, it is necessary to find an alternative therapeutic drug with high efficiency and low toxicity for ovarian cancer patients.

In recent years, the bioactive peptides derived from milk proteins were found to have anticancer function, especially for gynecological cancer (5-7). Although these bioactive peptides were not as effective as chemotherapy drugs such as DDP in the treatment of cancer, they have no toxicity, or low toxicity to normal cells (untransformed cells). Thus they have a broad prospect and potential for the development as anticancer drugs (8,9).

The hexapeptide (Pro-Gly-Pro-Ile-Pro-Asn, PGPIPN, 63-68 residues of bovine β-casein), also known as immune hexapeptide or immunomodulating peptide, was originally isolated from hydrolysate of bovine β-casein (10-14). Our previous studies showed that PGPIPN significantly decreased the growth of xenografted human ovarian tumor *in vivo*, and induced human ovarian cancer cell apoptosis (9). However, there is no report on whether this peptide could resist the invasion and migration of human ovarian cancer cells or not. We studied the effect and mechanism of PGPIPN in inhibiting the invasion and metastasis of human ovarian cancer cells *in vitro* through both the cell line $SKOV_3$ and primary ovarian cancer cells from fresh primary ovarian tumor tissues of 37 ovarian cancer patients who underwent initial debulking surgery. The findings in this study provide the proof of concept for using PGPIPN as a potential therapeutic

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agent for the treatment of ovarian cancer invasion and metastasis. This study provides the foundation for further *in vivo* experiments (animal model) and clinical trials.

Materials and methods

Reagents. PGPIPN was provided by Shanghai Sangon Biological Engineering Technology (Shanghai, China), and the purity was confirmed by RP-HPLC to be >99.5%. RPMI-1640 culture medium (RPMI: Roswell Park Memorial Institute) was purchased from Gibco BRL Co., Ltd., USA. Fetal bovine serum (FBS) was purchased from Sijiqing Co., Ltd. (Hangzhou, China). DDP was purchased from Qilu Pharmaceutical Co., Ltd. (Jinan, China). Matrigel (artificial reconstituted basement membrane) was purchased from Becton-Dickinson Co. (Franklin Lakes, NJ, USA). Transwell chamber with 8-μm pore was purchased from Coster Company (Santa Fe Springs, CA, USA).

Mouse monoclonal antibodies of MTA1, NM23H and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The horseradish peroxidase conjugated secondary antibody (goat anti-mouse IgG) and Super Signal West Pico Trial kit (ECL chromogenic reagent kit) were purchased from Pierce, USA. BCA kit for protein quantitative assay was purchased from Shanghai Sangon Biological Engineering Technology. The other common biochemical reagents were purchased from Shanghai Biotechnology Co., Ltd.

Cell cultures. Human ovarian cancer cell line $SKOV_3$ (ovarian serous papillary cystadenocarcinoma) was originally purchased from ATCC (American Type Culture Collection, USA) and preserved by the Biochemistry and Molecular Biology Laboratory of Anhui Medical University. Human ovarian cancer cell line $SKOV_3$ was cultured in RPMI-1640 medium with 10% FBS in 5% CO_2 at 37˚C, and then digested with 0.4% trypsin after growing to the logarithm for the next experiment.

For primary ovarian cancer cell culture, fresh primary ovarian tumor tissues, which were assessed and classified as serous ovarian adenocarcinoma (III-IV grade) according to WHO criteria, were obtained from 37 patients with ovarian cancer at initial debulking surgery between January 2014 and March 2015 at the first affiliated hospital of Anhui Medical University. The patients had not received adjuvant therapies such as chemotherapy or radiotherapy before surgery. All the patients signed a written consent documenting donation of their tissue for research purpose according to the Declaration of Helsinki before tissue deposition. The fresh ovarian cancer tissues were washed with PBS 2-3 times to remove the blood, surrounding fats, necrosis tissues and non-tumor tissues, then cut into small pieces ~1 mm^3 and again rinsed with PBS two times. The pieces of tumor tissues were digested with 0.4% trypsin in sterile centrifuge tube at 37˚C for 30 min, shaken once every 5 min. To obtain the single suspension cells, the above digested tissues were filtered with 100 μm cell strainer. After centrifuged at 1,000 rpm for 5 min, the cell pellet was re-suspended in RPMI-1640 medium containing 0.1 mg/l epidermal growth factor (EGF), 0.1 mg/l insulin-like growth factor (IGF) and 0.1 mg/l β-estradiol with 10% FBS, which were subsequently cultured in 5% CO_2 at 37˚C. Once cells reached 70-80% confluence, cell culture medium was drained from the flask, and cells were digested with 0.25% collagenase II until ~1/3 of the cells fell to the bottom of the dish as evaluated using a microscope. Due to their initial shedding, most fibroblasts were eliminated by collagenase digestion. The remaining cells were continually cultured in 5% CO_2 at 37˚C. A portion of these cells were placed on the cell slide and identified by using anti-cytokeratin 7-FITC (FITC: fluorescein isothiocyanate, a green fluorescent dye) for cytokeratin 7 of ovarian cancer cells and Hoechst 33258 (blue fluorescent dye for the nuclei) to assay their purity.

Transwell assay of celI invasion. The Transwell assay was conducted to study cell invasion. The cells in the logarithmic growth were pretreated with PGPIPN at 0 (as control), $1x10^{-5}$, $1x10^{-4}$, $1x10^{-3}$ and $1x10^{-2}$ g/l respectively for 30 min. In addition, DDP at $1x10^{-4}$ g/l was added in the same plate as positive control. Then the above cells at a density of $3x10^5$ cells/ml in sextuplicate were respectively seeded in the upper insert (8-μm pore) using 200 μl RPMI-1640 medium without FBS, which contained the above concentrations of PGPIPN or $1x10^{-4}$ g/l DDP. In addition, the fitted culture dishes contained 500 μl RPMI-1640 medium with 10% FBS. After 30, 36 and 42 h (30 and 36 h for $SKOV_3$ cells; 36 and 42 h for primary ovarian cancer cells) respectively, the chambers were removed from the plates, fixed, and the migrated cells were stained with hematoxylin and eosin. For each well five representative x100 images were quantified. Each experiment was performed in two independent sets. Invasion inhibition rate (%) = (1 - invasive cell number of experimental group/ that of control group) x 100%.

Scratch assay of cell migration. Migration of cells in logarithmic growth was examined using a scratch assay. The cells in sextuplicate were cultured in a 12-well plate at $5x10^4$ cells/well until a confluent monolayer was formed. A 200-μl pipette tip was used to scratch the wells. The wells were sequentially rinsed with PBS and cultured in RPMI-1640 medium with 10% FBS. The wells were treated with 0 (as control), $1x10^{-4}$, $1x10^{-3}$ and $1x10^{-2}$ g/l PGPIPN respectively, and DDP at $1x10^{-4}$ g/l was added in the same plate as positive control. The images of the scratch test were taken by microscopy. The cell migration was measured by Image J software (version 1.48, National Institutes of Health, USA) with contrasting quantification of pixels in the area of scratch at 0, 12 and 24 h. Each experiment was performed in two independent sets. Migration inhibition rate (%) = (1 - experimental group pixels/control group pixels) x 100%.

Colony formation assay. The cells in logarithmic phase were digested by trypsin, resuspended, and counted. The cells in sextuplicate were seeded in 6-well plates at 1,000 cells/well (500 μl), in which RPMI-1640 medium with 10% FBS contained 0 (as control), $1x10^{-5}$, $1x10^{-4}$, $1x10^{-3}$ and $1x10^{-2}$ g/l PGPIPN respectively, and DDP at $1x10^{-4}$ g/l was added in the same plate as positive control. The fresh media were replaced once every 2 days. Cells were cultured in 5% CO_2 at 37˚C for 12 days, the numbers of colonies were counted. A colony containing >30 cells was defined as positive, and counted.

The inhibition rate of colony formation was calculated as follows. Each experiment was performed in two independent sets. Colony inhibition rate (%) = (1 - colony number of experimental group / that of control group) x 100%.

Real-time PCR. An optimized RT-PCR protocol was employed to analyze the mRNA levels of *NM23H1* (metastasis-associated gene-1) and *MTA1* (metastatic tumor antigen 1). β-actin was used as a housekeeping gene. According to primer sequences of *NM23H1*, *MTA1* and *β-actin* genes retrieved from Primer-Bank, primers were designed with Primer 5.0 software, which were synthesized by Shanghai Sangon Biological Engineering Technology. These primer sequences are as follows: *NM23H1* forward, CTTTAGGGATCGTCTTTC AAGG; reverse, TGCCAACTTGTATGCAGAAGTC 399 bp; *MTA1* forward, TGGCAGATAAAGGAGAGATTCG; reverse, TGTCGTAGATGTTCTTGTGGAGA 312 bp; *β-actin* forward, TGACGTGGACATCCGCAAAG; reverse, CTGG AAGGTGGACAGCGAGG 205 bp.

$SKOV_3$ cells or primary ovarian cancer cells were harvested after PGPIPN treatment (sextuplicate) at different doses for 48 h, respectively. The total RNAs in cells were extracted according to the TRIzol kit manufacturer's instructions, and the purity and concentration were determined by ultraviolet spectrophotometry. The reverse transcription reaction conditions were 42˚C, 15 min and 95˚C, 5 min. After the reaction, the reverse-transcribed cDNAs were diluted with RNase-free water to a final volume of 60 μl and preserved at 80˚C. Real-time PCR adopts Takara SYBR Green as real-time PCR Master Mix in ABI7500 fluorescent real-time PCR instrument. The reaction conditions were as follows: 95˚Cx30 sec (1 cycle); 95˚Cx5 sec, 60˚Cx34 sec (40 cycles). The ABI SDS software (Applied Biosystems) was used to determine a critical threshold (C_t), which was defined as the cycle number where the linear phase for each sample crossed the threshold level. The mRNAs of target gene expression were denoted by ΔC_t (ΔC_t = target gene C_t - β-actin C_t value). Finally, the relative mRNA expression of all samples were calculated using the $2^{-\Delta\Delta Ct}$ method (15). All reactions were performed in triplicate, and a mixture lacking a complementary DNA template (NTC) was used as the negative control.

Western blotting. The cells were harvested after PGPIPN treatment (sextuplicate) at different doses for 48 h, respectively. Proteins from the above cells were separated by SDS-PAGE and transferred to PVDF membrane based on the method described by Green and Sambrook (16). After blocking with 5% (w/v) dry skim milk, membranes were incubated with primary antibodies (mouse monoclonal NM23H1, MTA1 and β-actin antibodies, 1:1,000) and then incubated with horseradish peroxidase conjugated secondary antibody (goat anti-mouse IgG, 1:8,000). The proteins were detected with the enhanced chemiluminescence (ECL) system followed by exposure to X-ray film. β-actin was used as a loading control. Digital images were captured by Gel Doc™ gel documentation system (Bio-Rad, USA) and intensities were quantified using Quantity-One software version 4.62 (Bio-Rad).

Statistical analysis. All statistical analyses were performed with SPSS version 16.0. The effect of the treatment on each parameter was analyzed by one-way analysis of variance (ANOVA). Results are shown by mean ± SD, and $P<0.05$ was considered to be statistically significant.

Results

Culture and identification of primary cells. The primary ovarian cancer cells from fresh ovarian tumor tissues, which were assessed and classified as serous ovarian adenocarcinoma (III-IV grade) according to WHO criteria (Fig. 1A), were cultured (Fig. 1B). By immunofluorescence through cytokeratin 7 (CK7), the purities of these primary ovarian cancer cells were identified using anti-cytokeratin 7-FITC and Hoechst 33258 (Fig. 1C and D), their average purity reached 84.5%.

PGPIPN inhibited the invasion and migration of ovarian cancer cells in vitro. The effect of PGPIPN on the invasive ability of ovarian cancer cells was assayed by Transwell. PGPIPN significantly decreased the invasive ability of the $SKOV_3$ cells (Fig. 2A and B) and primary ovarian cancer cells (Fig. 2C and D), in which the maximum inhibition ratios were (28.86±3.01)% in $SKOV_3$ line cells and (27.14±2.14)% in primary ovarian cancer cells. The inhibition effect of PGPIPN showed a dose-dependent manner (Fig. 2B and D). However, the inhibitory effect of the peptide was less than that of clinical anticancer drug-DDP. The invasion inhibition of DDP at $1x10^{-4}$ g/l already exceeded 40% on both ovarian cancer $SKOV_3$ cells and the primary ovarian cancer cells at 30-42 h.

Cell scratch assay was used to detect the effect of PGPIPN on human ovarian cancer cell migration. PGPIPN was found to attenuate cell migrations of both $SKOV_3$ (Fig. 3A and B) and the primary ovarian cancer cells (Fig. 3C and D). PGPIPN significantly attenuated gap closure after 12 and 24 h (Fig. 3). Hence, PGPIPN can significantly inhibit the migration of ovarian cancer cells, which was dose-dependent within the dose range of $1x10^{-4}$-$1x10^{-3}$ g/l (Fig. 3). The effect of the peptide on primary ovarian cancer cells is much better than that of the $SKOV_3$ cells. The inhibitory effects of the peptide to human ovarian cancer cells were similar to that of DDP at 12-24 h.

PGPIPN inhibits colony formation of ovarian cancer $SKOV_3$ cells. We examined the colony formation capacity of $SKOV_3$ cells treated with different concentrations of PGPIPN. The cells were allowed to grow for 12 days to form colonies. PGPIPN can significantly inhibit cell colony formation of ovarian cancer $SKOV_3$ cells (Fig. 4). Our results revealed that PGPIPN could significantly suppress the colony formation capacity of ovarian cancer cells. However, the inhibition effect was slightly less than that of the same concentration of DDP, and the inhibition rate of $1x10^{-4}$ g/l DDP was 39.78±7.8%.

PGPIPN regulates the mRNAs of NM23H1 and MTA1 genes related with invasion and migration of tumor cells. Real-time PCR experiments were performed using *MTA1* and *NM23H1*-specific primers to assess their relative mRNA expression ($2^{-\Delta\Delta Ct}$) in human ovarian cancer cells treated with PGPIPN for 48 h (Fig. 5). The changes of *MTA1* and *NM23H1*

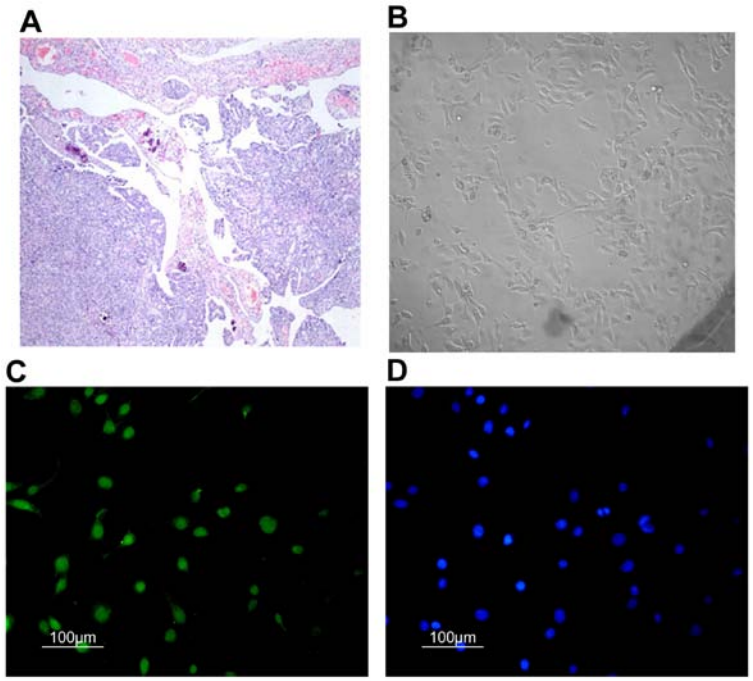

Figure 1. The culture and identification of human primary ovarian cancer cells. (A) Pathological sections of human ovarian cancer tissue (H&E stained, x40), classified as serous ovarian adenocarcinoma (III-IV grade) according to WHO criteria. (B) Representative morphology of human primary ovarian cancer cells in culture (x100). (C) Human primary ovarian cancer cells stained with anti-cytokeratin 7-FITC (x100). (D) Human primary ovarian cancer cells stained with nuclear dye Hoechst 33258 (x100).

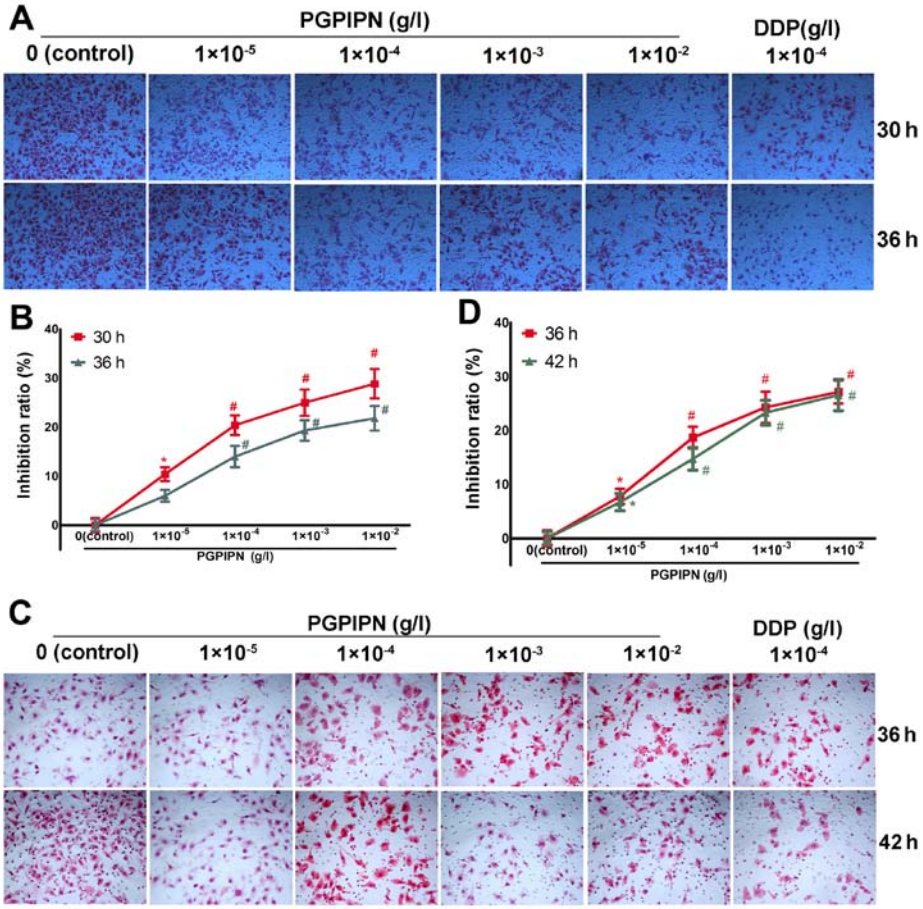

Figure 2. PGPIPN inhibits the invasion of human ovarian cancer cells in Transwell chamber. (A) The $SKOV_3$ cells invading Transwell chambers under the drug treatment (H&E stained, x100). (B) Histogram of inhibition ratio of human ovarian cancer $SKOV_3$ cells treated with PGPIPN at different concentrations. (C) Human primary ovarian cancer cells invading Transwell chambers under drug treatment (H&E stained, x100). (D) Histogram of inhibition ratio of human primary ovarian cancer cells treated with PGPIPN, the data are calculated from 37 primary cancer cell measurements. The data are shown as means ± SD, $^{*}P<0.05$, $^{\#}P<0.01$ compared with control (the vehicle group).

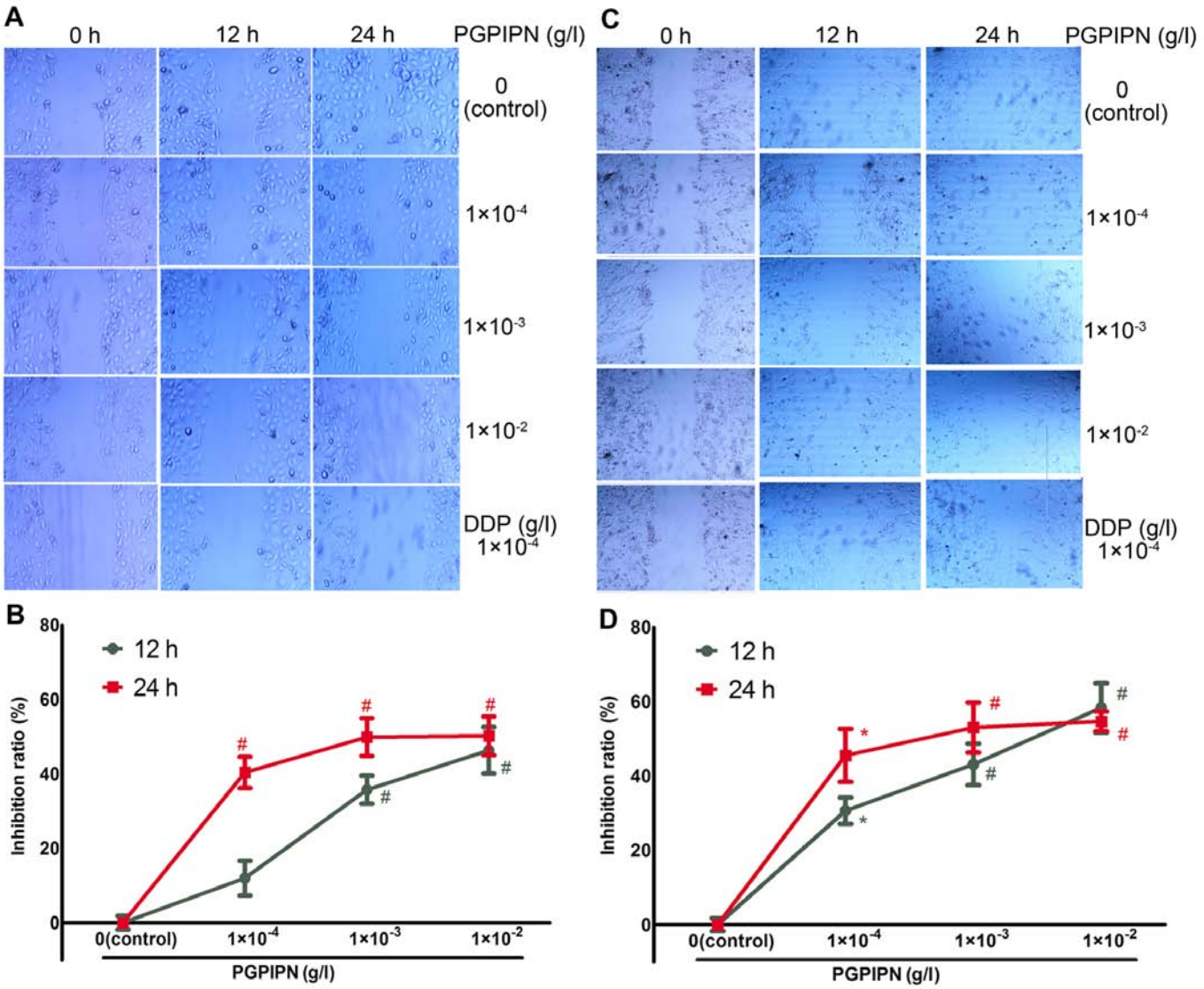

Figure 3. PGPIPN inhibits human ovarian cancer cells migration in cell scratch assay. (A) The $SkOV_3$ cells migrated under drug treatment in the scratch test (H&E stained, x100). (B) Histogram of inhibition ratio of human ovarian cancer $SKOV_3$ cells treated with PGPIPN at different concentrations. (C) Human primary ovarian cancer cells migrated under drug treatment in the scratch test (H&E stained, x100). (D) Histogram of inhibition ratio of human primary ovarian cancer cells treated PGPIPN, the data are calculated from 37 primary cancer cell measurements. The data are shown as means ± SD, $^{*}P<0.05$, $^{\#}P<0.01$ compared with control (the vehicle group).

mRNAs in $SKOV_3$ cells are displayed in Fig. 5A. PGPIPN significantly decreased the mRNA level of *MTA1* gene and increased the mRNA level of *NM23H1* gene in contrast. The effect of PGPIPN on mRNA expression levels of *MTA1* and *NM23H1* genes were dose-dependent.

The PGPIPN also reduced mRNA levels of *MTA1* and *NM23H1* genes in primary ovarian cancer cells similarly to $SKOV_3$ cells (Fig. 5B). Compared with the cell line $SKOV_3$, the effects of PGPIPN on primary ovarian cancer cells were more obvious.

PGPIPN affected levels of MTA1 and NM23H1 proteins related with invasion and migration of tumor cells. Western blotting was used to analyze MTA1 and NM23H1 protein levels of human ovarian cancer cells treated with PGPIPN at different concentrations for 48 h (Fig. 6). NM23H1 in $SKOV_3$ cells was elevated in PGPIPN-treated groups compared to control group, while MTA1 protein gradually decreased with increasing drug concentration (Fig. 6A and B). Notably, PGPIPN-mediated effects on protein levels were significant at the concentration of $\geq 1x10^{-4}$ g/l ($P<0.05$ or $P<0.01$) (Fig. 6B).

PGPIPN also affected NM23H1 and MTA1 protein levels in primary ovarian cancer cells after treatment for 48 h, which was similar to that of $SKOV_3$ cells (Fig. 6C and D).

Discussion

In recent years, studies have shown that many peptides significantly inhibited invasion and metastasis of tumors and were considered potential therapeutic agents for the treatment of malignant tumors, and some were applied in clinical treatment. For example, Chi *et al* (17) reported that CAAT/enhancer binding peptide delta (CEBPD) can reduced and inhibited

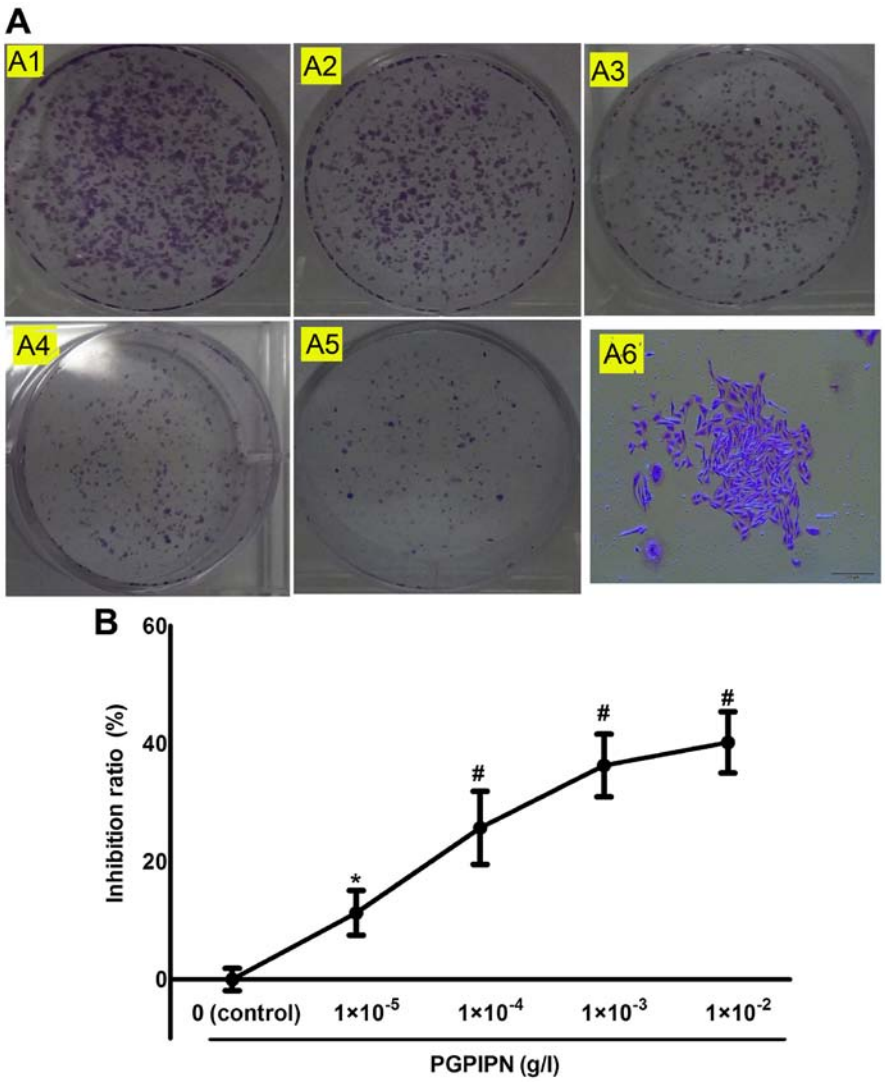

Figure 4. PGPIPN inhibits human ovarian cancer $SKOV_3$ cell colony formation. (A) The colony formation of $SKOV_3$ cells in 6-well plates under drug treatment; A1, A2, A3, A4 and A5 show the colony formation capacity of $SKOV_3$ cell treated with 0 (as control), $1x10^{-5}$, $1x10^{-4}$, $1x10^{-3}$ and $1x10^{-2}$ g/l PGPIPN respectively, and A6 a $SKOV_3$ colony (crystal violet stain, x100). (B) Histogram of inhibition ratio of human ovarian cancer $SKOV_3$ cells treated with PGPIPN at different concentrations. A colony containing >30 cells was defined as positive, and counted. The data are shown as means ± SD, $^*P<0.05$, $^\#P<0.01$ compared with control (the vehicle group).

resistance, invasion and metastasis of malignant tumor cells. Shan *et al* (18) reported the cyclic arginyl-glycyl-aspartic acid (cRGD) peptide had been explored as an αvβ3 integrin receptor-specific targeting moiety for the targeted delivery of nanoparticle-loaded therapeutics. The cRGD peptide has been used as prevention and treatment of breast cancer devopment, invasion and metastasis. In the past 20 years, studies (19,20) have shown that many peptides derived from milk can inhibit tumor metastasis and showed great potential for the treatment of cancer or as adjuvant therapy, some of which have already been applied in clinical treatment. For example, HAMLET (human α-lactalbumin made lethal to tumor cells) and lactoferricin (antibacterial peptide from lactoferrin, Lfcin) have already been applied in clinic as an immunotherapeutic agent for the treatment of cancer (cancer immunotherapies). Lactoferricin, colostrum and other special milk-derived peptides have already been used as nutritional and protective agents for clinical treatment and chemotherapy of cancer (19). Bonuccelli *et al* (21) reported that the milk protein α-casein could effectively inhibit the growth and metastasis of breast cancer tumors by activating the STAT1 signal pathway. The milk-derived peptides have almost no side effects; some short peptides also possess anti-enzymatic hydrolysis and are easily absorbed. Thus, these peptides can be taken orally and also be used as an adjuvant therapy for tumors.

The hexapeptide (PGPIPN) used in this study is derived from the 63-68 amino acid sequence of bovine β-casein. The results of the study showed that PGPIPN could significantly inhibit the invasion and metastasis of both human ovarian cancer $SKOV_3$ cells and the primary ovarian cancer cells from fresh human ovarian cancer tissues *in vitro*, displaying dose-dependency. However, the inhibition effects of PGPIPN were less than that of DDP (a conventional anticancer drug) as the positive control. However, our early studies (9) showed that DDP not only significantly inhibited ovarian cancer cell proliferation, but also had a strong side effect on untransformed normal cells. We showed that PGPIPN had no, or slight side effects on untransformed normal cells by MTT

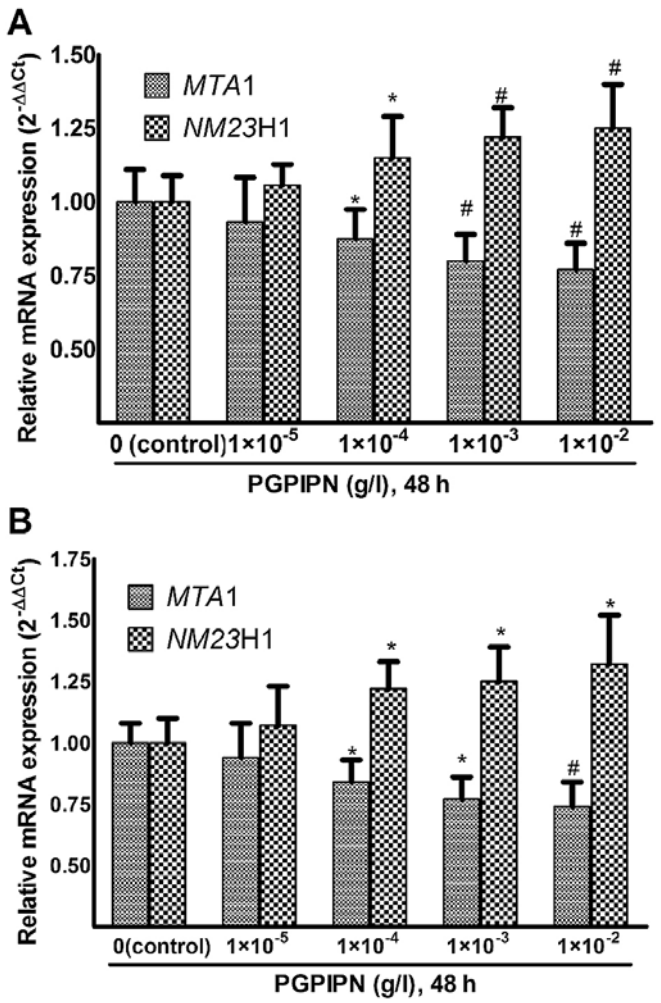

Figure 5. The real-time PCR mRNA levels of *MTA1* and *NM23H1* genes in human ovarian cancer cells treated with PGPIPN. (A) The changes of *MTA1* and *NM23H1* mRNAs in human ovarian cancer $SKOV_3$ cells after PGPIPN treatment at different concentrations for 48 h. (B) The changes of *MTA1* and *NM23H1* mRNAs in human primary ovarian cancer cells after PGPIPN treatment at different concentrations for 48 h. The data in (A) and (B) are shown as means ± SD, the data in (B) shown are from 37 ovarian cancer tissues of 37 patients. $^{*}P<0.05$, $^{\#}P<0.01$ compared with control (the vehicle group), taken β-actin as reference gene.

assay of human normal hepatic cell line LO2, murine embryo fibroblast cells (MEFs) and para-carcinoma tissues of human ovarian cancer.

Tumor metastasis involves many processes, a key step is how to move through biological barriers in the dynamic process of invasion and metastasis of tumor cells (22). Among them, extracellular matrixc (ECM) is a natural barrier for tumor invasion and metastasis, and the degradation of ECM is a key step in tumor metastasis. Tumor cells have the characteristics of migration, secretion of various hydrolytic enzymes, adhesion closely linked with ECM. In addition, the adhesion of tumor cells is related to transmembrane glycoproteins on the surface of the cells. These adhesion molecules can combine with the same or different adhesion molecules and trigger biological effects.

In the study of invasion and metastasis of malignant tumor cells, MTA1 and NM23H1 proteins have attracted much attention. Many studies showed that the levels of MTA1 and NM23H1 proteins were closely related to invasion and metastasis of tumor cells. *MTA1* gene was first isolated as the associated gene of metastasis of breast cancer from rat breast cancer cell line by Pencil *et al* (23) using phage difference hybridization technique in 1993. Toh *et al* (24) determined the nucleotide sequence of the gene and the amino acid sequence of the protein products, named as metastasis-associated 1 (MTA1). MTA1 protein regulated a series of proteins related with invasion and metastasis by signal transduction and gene expression, which can affect the invasion and metastasis of cancer cells. The expression of MTA1 protein was closely related to the staging and the great omentum metastasis of ovarian cancer. The MTA1 protein may have the function of the Src homology 3 (SH3) binding motif participating in regulating signal transduction pathway and cytoskeleton proteins. Moreover, MTA1 may act as a transcription factor (TF) to regulate gene expression. Therefore, MTA1 played an important role in regulating proteins related to invasion and metastasis through affecting signal transduction and gene expression (25,26). The *NM23H1* has been widely investigated as a tumor metastasis suppressor gene. The *NM23H1* gene was found expressed at low levels in many malignant tumors, whose expression was negatively correlated with metastasis of malignant tumors. NM23H1 could inhibit the metastasis of malignant tumors by participating in transmembrane information transfer affecting the related protein synthesis and in polymerizing/depolymerizing microtubules affecting cytoskeletal state. Finally, NM23H1 decreased the activity and the adhesion of the malignant tumor cells (27-29).

We applied real-time PCR and western blotting to determine the changes of expression of *MTA1* and *NM23H1* in ovarian cancer cells. Our results showed that PGPIPN may inhibit the invasion and metastasis of ovarian cancer cells by regulating the expression of *MTA1* and *NM23H1* in ovarian cancer cells and the related signal pathway. PGPIPN as a signal molecule may combine with target molecules of ovarian cancer cells to trigger the signaling pathway affecting the expression of *MTA1* and *NM23H1* genes, thus the invasion and metastasis of tumor cells was reduced. However, the target molecule of PGPIPN in ovarian cancer cells is not yet determined. We investigated how PGPIPN effects the expression of *MTA1* and *NM23H1* genes and the signal pathway. According to our preliminary experiment, this peptide can bind to the cell membrane of ovarian cancer cells (data not shown). Recent studies indicated that bioactive peptides derived from bovine milk proteins are capable of binding and affecting cells. For example, Kreider *et al* reported the milk peptide mixture from normal cow's milk inhibits the tyrosine kinase activity of epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor 2 (VEGFR2) and insulin receptor (IR), respectively (30). Fiedorowicz *et al* reported the bioactive peptides from bovine caseins could bind the μ-opioid receptor on cytomembrane to influence the proliferation and cytokine secretion of human peripheral blood mononuclear cells (PBMCs) (31). To clarify the receptor or ligand of this peptide further studies are required. However, our study confirmed that PGPIPN can significantly inhibit the invasion and metastasis of human ovarian cancer cells *in vitro*, and PGPIPN shows promise as a

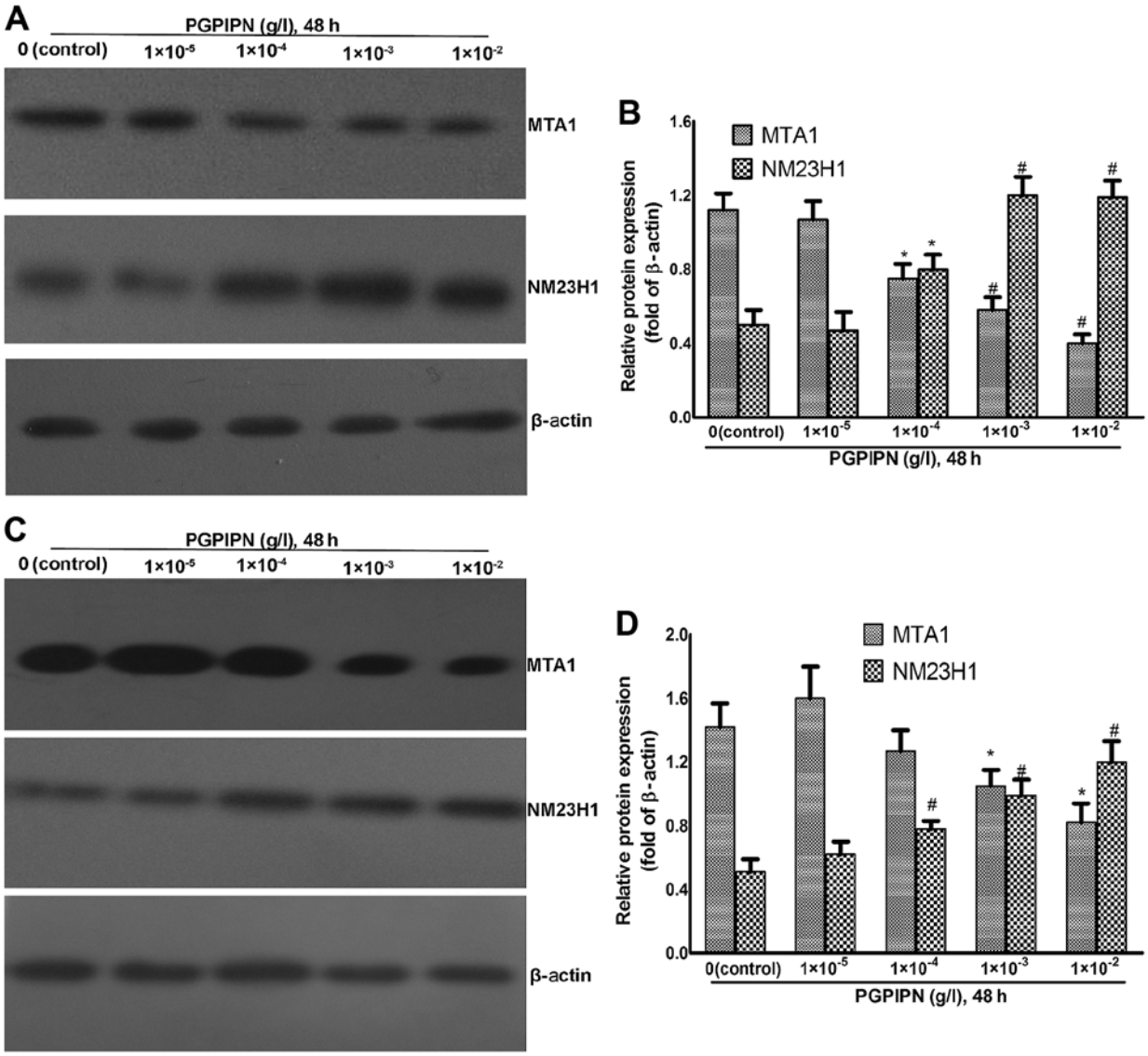

Figure 6. Western blotting levels of MTA1 and NM23H1 in human ovarian cancer cells treated with PGPIPN. (A) Western blot analysis was performed after 48 h PGPIPN treatment in human ovarian cancer $SKOV_3$ cells and antibodies specific to MTA1 and NM23H1 were used to assess protein levels, β-actin was used to show the similar amount of protein loaded in different lanes. (B) The relative intensities of protein bands in (A) were determined using Quantity-One software version 4.62 (Bio-Rad) and normalized using β-actin band intensity. (C) After PGPIPN treatment at different concentrations for 48 h, MTA1 and NM23H1 were detected in human primary ovarian cancer cells, β-actin was used to show the similar amount of protein loaded in different lanes. (D) The relative intensities of protein bands in (C) were determined using Quantity-One software version 4.62 and normalized using β-actin band intensity. The data in (B) and (D) are shown as means ± SD, the data in (D) are from 37 ovarian cancer tissues of 37 patients. $^{*}P<0.05$, $^{\#}P<0.01$ compared with control (the vehicle group).

new therapeutic drug for adjuvant therapy of human ovarian cancer.

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