A novel uPAg-KPI fusion protein inhibits the growth and invasion of human ovarian cancer cells *in vitro*

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Abstract. Urokinase-type plasminogen activator (uPA) acts by breaking down the basement membrane and is involved in cell proliferation, migration and invasion. These actions are mediated by binding to the uPA receptor (uPAR) via its growth factor domain (GFD). The present study evaluated the effects of uPAg-KPI, a fusion protein of uPA-GFD and a kunitz protease inhibitor (KPI) domain that is present in the amyloid β-protein precursor. Using SKOV-3 cells, an ovarian cancer cell line, we examined cell viability, migration, invasion and also protein expression. Furthermore, we examined wound healing, and migration and invasion using a Transwell assay. Our data showed that uPAg-KPI treatment reduced the viability of ovarian cancer SKOV-3 cells in both a concentration and time-dependent manner by arresting tumor cells at G1/G0 phase of the cell cycle. The IC_{50} of uPAg-KPI was 0.5 μg/μl after 48 h treatment. At this concentration, uPAg-KPI also inhibited tumor cell colony formation, wound closure, as well as cell migration and invasion capacity. At the protein level, western blot analysis demonstrated that uPAg-KPI exerted no significant effect on the expression of total extracellular signal-regulated kinase (ERK)1/ERK2 and AKT, whereas it suppressed levels of phosphorylated ERK1/ERK2 and AKT. Thus, we suggest that this novel uPAg-KPI fusion protein reduced cell viability, colony formation, wound healing and the invasive ability of human ovarian cancer SKOV-3 cells *in vitro* by regulating ERK and AKT signaling. Further studies using other cell lines will confirm these findings.

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Introduction

Ovarian cancer is a significant health problem worldwide, accounting for more than 200,000 new cases of cancer and 125,000 cancer-related deaths each year. Furthermore, ovarian cancer is ranked as the sixth most common cancer and seventh most common cause of mortality for women (1). Despite recent advancements in the prevention, detection and treatment of ovarian cancer, it remains the cause of the highest number of gynecological-related mortalities in Western countries. This is thought to be due to late diagnosis, thereby reducing treatment options (stages III-IV) (2). As a result of this situation, novel treatments and earlier diagnostic tools are urgently required in order to effectively target ovarian cancer.

In ovarian cancer, similar to other cancers, tumor metastasis has been shown to be responsible for a significant number of mortalities, and tumor cell invasion is dependent on finely regulated extracellular proteolytic activity, which allows tumor cells to invade the extracellular matrix (3). Urokinase-type plasminogen activator (uPA) breaks down the basement membrane upon binding to the uPA receptor (uPAR) through the respective growth factor domain (GFD) (4). Thus, the activated uPA/uPAR/plasmin proteolytic cascade plays a key role in tumor invasion and the dissemination of various malignancies (5,6). In addition to its recognized function in extracellular matrix degradation, uPA signaling has been implicated in tumor cell proliferation, migration and the invasion of local tissues (4). However, it has been hypothesized that downregulation of uPA and uPAR, blockade of uPA activity using an antibody or small molecule inhibitor, or interfering with uPA binding to its receptor could suppress the promotion of cell mobility and growth. This approach is considered to be a novel method in treating tumors. Indeed, certain of these potential approaches have demonstrated potent anticancer effects and are currently being further evaluated in clinical trials (7-10) (http://www.wilex.de/portfolio/mesupron/phase-i-mit-wx-uk1/).

In cases of ovarian cancer, the expression of uPA and uPAR has been associated with epithelial ovarian cancer progression (11,12). Previously, it has been demonstrated that high levels of expression of uPA are associated with residual tumor progression in ovarian cancer patients, suggesting that targeting uPA signaling helps clinicians to stratify patients (13). Thus, in the present study, we constructed and expressed a fusion protein,

uPAg-kunitz protease inhibitor (KPI). This involved fusing uPA GFD with KPI, a kunitz protease inhibitor domain that is found in amyloid beta-protein precursor (APP). Incorporation of the KPI-amyloid protein precursor allowed for inhibition of serine protease hydrolysis (14). Subsequently, we assessed uPAg-KPI antitumor activity in ovarian cancer cells *in vitro*. Our previous study expressed uPAg-KPI in *Pichia pastoris*, and used ion exchange chromatography to purify the target protein. Renaturalization was carried out using ammonium sulfate, and biological analysis demonstrated that this fusion protein inhibited serine protease activity (15). The present study provides insightful information in relation to the effect of uPAg-KPI regulation on ovarian cancer cell viability, migration and invasion *in vitro*.

Materials and methods

Chemicals and reagents. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were both purchased from Sigma (St. Louis, MO, USA), and the BrdU Flow kit and Biocoat Matrigel™ invasion inserts were both purchased from BD Biosciences (San Jose, CA, USA). The Transwell chamber was from Corning (Corning, NY, USA), and Iscove's modified Dulbecco's medium (IMDM) and fetal bovine serum (FBS) were both from HyClone (Logan, UT, USA). Crystal violet, Trypan blue, penicillin-streptomycin and trypsin-EDTA were all purchased from Solarbio (Beijing, China), and the primary antibodies used for western blot analysis of p-extracellular signal-regulated kinase (ERK; 4695), p-ERK (9101), AKT (9272) and p-AKT (4058) were all purchased from Cell Signaling Technology (Danvers, MA, USA). uPAg-KPI was constructed and prepared in the College of Pharmacy, Biological Engineering Laboratory (Jilin University, Changchun, China), it was expressed in *Pichia pastoris*, by renaturation and ion exchange chromatography; biological analysis demonstrated that the fusion protein inhibited serine protease activity, as previously described (15).

Human ovarian adenocarcinoma cell line and culture. The human ovarian adenocarcinoma cell line SKOV-3 was obtained from Biochemical Teaching and Research Section, Basic Medical Institute, Jilin University (Changchun, China) and cultured in IMDM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator with 5% CO_2 and 95% air at 37˚C.

Cell viability MTT assay. SKOV-3 cells were seeded and grown on a 96-well plate with a flat bottom at a density of 5,000 cells/well in complete medium supplemented with 10% FBS for 24 h. Following incubation, the wells were treated with 0, 0.05, 0.1, 0.5 and 1.0 μg/μl of uPAg-KPI, respectively for up to 72 h. At the end of each experiment, 20 μl MTT solution (5 mg/ml) was added and cells were further incubated for 4 h at 37˚C. Following this, cell medium was replaced with 100 μl/well DMSO and optical density (A490 nm) was read on an automated microplate reader (BioTek, Winooski, VT, USA). The data were calculated as the means ± SD.

Flow cytometric cell cycle distribution assay. To analyze cell cycle distribution, cells were cultured overnight in serum-free IMDM overnight and in IMDM-containing 10% FBS for treatment with 0.5 μg/μl concentration of uPAg-KPI for 48 h. BrdU was then added (10 μl/ml) to cell cultures for an additional 0.5 h. Cells were then collected by trypsinization, fixed in a Cytofix/Cytoperm buffer and Cytoperm-plus buffer in the dark, and then washed in a Perm/Wash buffer, and digested with RNase (all from BD Biosciences) for 1 h at 37˚C. The resulting solution was resuspended in 1 ml of Perm/Wash buffer and stained with 0.01 mg/ml of propidium iodide in the dark for 30 min at room temperature. Cells were analyzed using a flow cytometer (BD Accuri C6; BD Biosciences).

Colony formation assay. Cells at a density of 200 cells/well were added to 6-well plates and cultured in IMDM containing 10% FBS in triplicate for 12 h and then treated with uPAg-KPI (0.5 μg/μl) at 37˚C for 14 days. Following treatment, cells were washed twice with phosphate-buffered saline (PBS; Solarbio) and fixed with 4% paraformaldehyde for 30 min and then stained with 0.1% crystal violet hydrate solution for 20 min. The number of colonies with ≥50 cells was counted under an inverted microscope (Nikon, Tokyo, Japan) and calculated as: plate clone formation efficiency = number of colonies/number of cells inoculated x100.

In vitro 'wound' closure assay. SKOV-3 cells were plated in 6-well plates and treated with 0.5 μg/μl uPAg-KPI until cells reached confluence. A 'wound' was then created by scratching the surface of the well with a pipette tip. Following injury, cells were further cultured in serum-free IMDM with or without uPAg-KPI (0.5 μg/μl) for 24 and 48 h. Cells were then photographed using a phase-contrast microscope (x100) and the wound closure was measured as previously described (16).

Tumor cell migration and invasion assay. In the present study, SKOV-3 cells were treated in the presence or absence of 0.5 μg/μl uPAg-KPI for two days, and tumor cell migration and invasion capacity were measured using the Transwell system (Corning) with or without Matrigel precoating (BD Biocoat™ Matrigel Invasion chamber with an 8-μm filter), respectively. In brief, cells were trypsinized and resuspended in serum-free IMDM and placed in the upper chamber of a Transwell plate at a density of $2x10^4$ cells/well, containing 0.5 μg/μl of uPAg-KPI or control loading solution (PBS). The bottom chambers contained IMDM and 20% FBS. Cells were incubated for 48 h in a humidified atmosphere with 95% air and 5% CO_2 at 37˚C. Non-migrated or invasive cells in the upper chamber were removed by wiping the upper side of the membrane with a cotton swab. Migrated or invading cells on the reverse side of the membrane were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet for 15 min and counted under a light microscope at a magnification of x200.

Protein extraction and western blot analysis. SKOV-3 cells were plated onto a 10-cm tissue culture dish and grown for 24 h. uPAg-KPI (0.5 μg/μl) was then added to each dish and PBS (the solvent) was used as a vehicle control. Cells were incubated at 37˚C for 0, 12, 24 and 48 h and harvested and resuspended in ice-cold PBS (pH 7.4) containing 2 mM EDTA and 0.1% Triton X-100. Collected cells were sonicated and centrifuged at 12,000 rpm for 20 min at 4˚C in order to remove
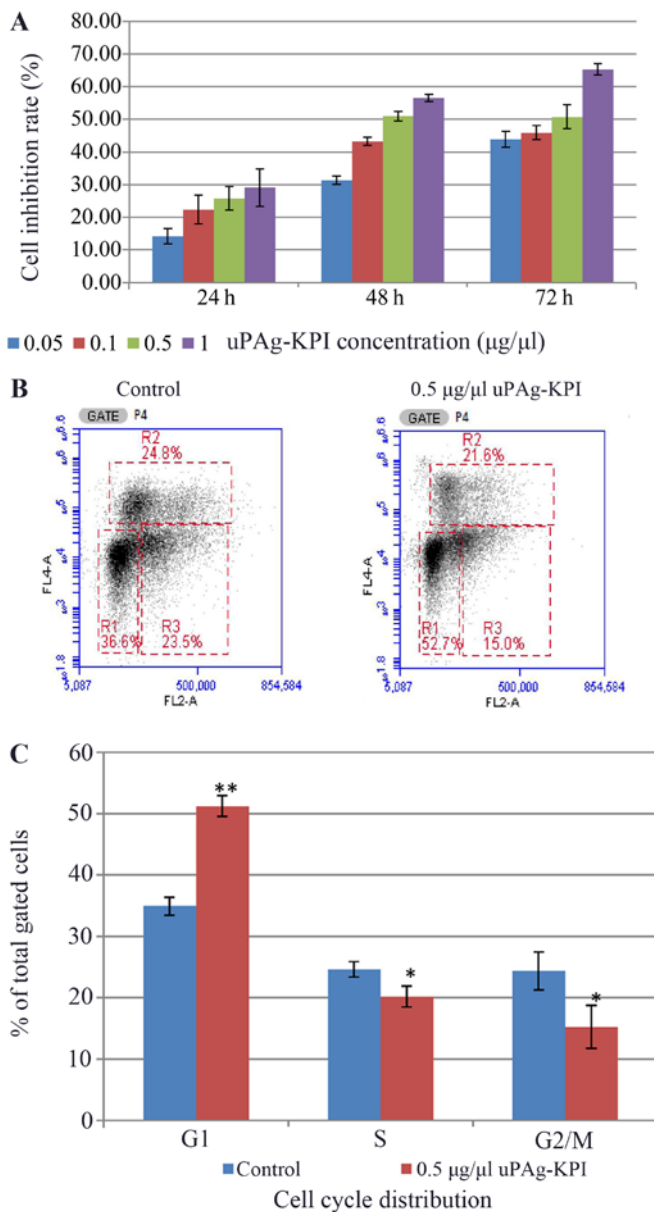
Figure 1. Effect of urokinase-type plasminogen activator (uPA)g-kunitz protease inhibitor (KPI) on inhibition of SKOV-3 cell viability. (A) Cell viability MTT assay. SKOV-3 cells were cultured with different concentrations of uPAg-KPI for up to 72 h and then subjected to MTT assay. The data are represented as the means ± SD of three independent experiments. (B) Flow cytometric cell cycle analysis. Cells were grown and treated with or without uPAg-KPI (0.5 μg/μl) for 48 h and then subjected to flow cytometric cell cycle analysis. (C) Summarized data of (B) cell cycle distribution. The experiment was repeated three times. $^*P<0.05$ and $^{**}P<0.01$.
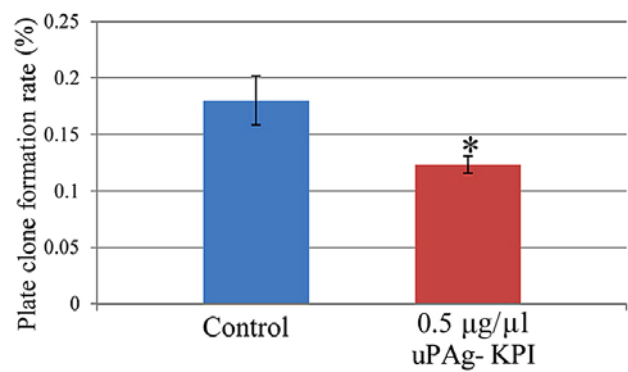

Figure 2. Effect of urokinase-type plasminogen activator (uPA)g-kunitz protease inhibitor (KPI) on the inhibition of SKOV-3 cell plate colony formation. Cells were grown and treated with 0.5 μg/μl uPAg-KPI for 14 days and the colony formation rate of the control and treatment groups was counted after staining with crystal violet hydrate solution. The data are summarized and presented as the means ± SD of three independent experiments. $^*P<0.05$.

Statistical analysis. In the present study, all data are represented as the means ± SD of three independent experiments. Statistical difference was evaluated using the Student's t-test, and a P-value <0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS, version 19 software (SPSS, Chicago, IL, USA) to compare the experimental versus control groups.

Results

Effects of uPAg-KPI on inhibition of SKOV-3 cell viability. In order to assess the effect of uPAg-KPI on the inhibition of ovarian cancer cell growth, we treated cells with various concentrations of uPAg-KPI for up to 72 h and performed the cell viability MTT assay. Our data demonstrated that uPAg-KPI markedly reduced cell viability in a dose- and time-dependent manner in SKOV-3 cells (Fig. 1A). The IC_{50} value of uPAg-KPI was 0.5 μg/μl in SKOV-3 cells following 48 h treatment. We thus selected 0.5 μg/μl uPAg-KPI for 48 h treatment for subsequent experiments. A reduction in cell viability by uPAg-KPI was also confirmed by Trypan blue dye exclusion (data not shown).

Following the aforementioned experiments, flow cytometric analysis (cell cycle distribution assay) demonstrated that cells treated with uPAg-KPI (0.5 μg/μl) for 48 h accumulated at the G1 phase of cell cycle (52.7 vs. 36.6% of control) (Fig. 1B and C). Moreover, the number of cells in the S and G2/M phases declined (21.6 vs. 24.8% and 15.0 vs. 23.5% of control, respectively). These results demonstrated that uPAg-KPI inhibited SKOV-3 cell proliferation (Fig. 1B and C).

Effects of uPAg-KPI on the inhibition of tumor cell colony formation. Considering uPAg-KPI has the propensity to affect cell proliferation *in vitro*, a plate colony formation assay was performed. The efficiency of plate colony formation indicated that there was a significant difference between cells treated with 0.5 μg/μl of uPAg-KPI compared to untreated control cells. The number of colonies after treatment was notably fewer compared to the controls (Fig. 2). The result, to an extent, further confirmed that treatment with uPAg-KPI reduced the proliferation of ovarian cancer cells.

cell debris. The supernatant was collected for determination of total protein concentration using a Pierce™ protein BCA assay kit (Thermo Fisher Scientific, Waltham, MA, USA) with bovine serum albumin (BSA) as a standard. Briefly, samples were electrophoresed and then transferred to polyvinylidene difluoride membrane using a semidry transfer system. Non-specific binding sites were blocked for 1 h in Tris-buffered saline, pH 7.6, containing 5% dried skimmed milk. Blots were incubated with ERK1/2, p-ERK1/2, AKT and p-AKT antibodies overnight at 4˚C, then diluted with secondary antibody for 1 h. After several washes, reactive bands were visualized using an ECL chemiluminescence detection kit (Thermo Fisher Scientific) (17).
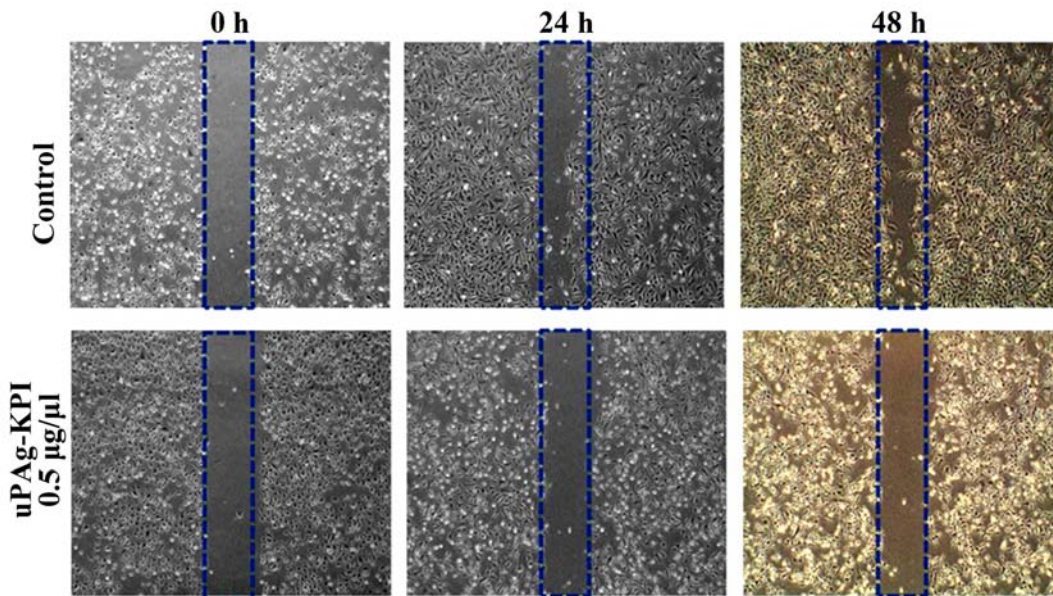
Figure 3. Effect of urokinase-type plasminogen activator (uPA)g-kunitz protease inhibitor (KPI) on the inhibition of SKOV-3 cell wound closure. Cells were grown and treated with 0.5 μg/μl uPAg-KPI for 24 or 48 h. When cells reached a confluent monolayer, a wound was created using a pipette tip, cell debris was rinsed away using ice-cold PBS and the remaining cells were cultured for 24 or 48 h. The relative wound closure was photographed and measured using a phase-contrast microscope.

Effects of uPAg-KPI on the inhibition of SKOV-3 cell migration and invasive capacity. We analyzed the effects of uPAg-KPI on the migration and invasive capacity of SKOV-3 cells using the previously mentioned wound healing assay. As shown in Fig. 3, relative wound closure was decreased following treatment with uPAg-KPI (0.5 μg/μl) for 24 or 48 h compared to the controls.

Following this, we performed Transwell tumor cell migration and invasion assays and demonstrated that uPAg-KPI had a significant inhibitory effect on cell migration and invasion at the concentration 0.5 μg/μl (Fig. 4).

Effects of uPAg-KPI on the regulation of ERK1/2 and AKT activity in SKOV-3 cells. After establishing the ability of uPAg-KPI to inhibit cell migration, we assessed the underlying molecular mechanisms of uPAg-KPI. Using western blot analysis, we demonstrated that treatment with uPAg-KPI suppressed p-ERK and p-AKT activity. However, no marked effects on the total levels of ERK1/2 and AKT proteins were noted (Fig. 5).

Discussion

Ovarian cancer is a highly metastatic type of cancer, and identification of the underlying molecular signaling pathways will help effectively control disease progression (18). Generally, degradation of the extracellular matrix is considered to be the first step of tumor invasion or metastasis. Activation of the uPA/uPAR/plasmin proteolytic signaling network plays a key role in extracellular matrix degradation. Previous studies using natural and synthetic uPA inhibitors have demonstrated abrogated tumor cell growth, invasion and metastasis due to inhibition of uPA serine protease activities (19,20) and the ability of certain uPA antagonists (21-23) to block binding to uPAR on the tumor cell surface.

In our previous study, we constructed uPAg-KPI, which contained uPA GFD conjugated with a kunitz protease

inhibitor (KPI) domain that is present in the amyloid beta-protein precursor (APP). Our previous data demonstrated that this fusion protein inhibited serine protease activity *in vitro* (15). Thus, in the present study, we assessed the effects of this fusion protein uPAg-KPI on the regulation of ovarian cancer cell phenotypes and protein expression. We found that uPAg-KPI treatment reduced the viability of ovarian cancer cells in a concentration and time-dependent manner and arrested tumor cells at the G1/G0 phase of the cell cycle. The IC$_{50}$ of uPAg-KPI was 0.5 μg/μl after 48 h treatment, and treatment of ovarian cancer cells with this concentration inhibited tumor cell colony formation, wound healing, and tumor cell migration and invasive capacity. Molecularly, uPAg-KPI suppressed the activity of ERK1/2 and AKT proteins. Thus, we demonstrated that this novel uPAg-KPI fusion protein exerted antitumor activity in ovarian cancer SKOV-3 cells *in vitro* by regulation of ERK and AKT signaling.

uPA was originally isolated from human urine and is present in the bloodstream and the extracellular matrix (24). The primary physiological substrate of uPA is plasminogen, and activation of plasmin triggers a proteolytic cascade to promote thrombolysis or extracellular matrix degradation. Altered expression or altered activity of uPA is linked to a variety of vascular diseases and cancers (25,26). Extracellular matrix degradation, following plasminogen activation has been shown to induce tumor cell tissue invasion and metastasis, whereas inhibition of uPA activity or expression has been used as an anticancer agent (27,28). Indeed, Mesupron®, a small molecule serine protease inhibitor developed by WILEX, has been used in clinical trials (http://www.wilex.de/portfolio/mesupron/phase-i-ii-mit-mesupron/). Studies have suggested that the drug appears to be safe when combined with chemotherapy in cases of breast cancer (http://www.wilex.de/portfolio/mesupron/phase-i-ii-mit-mesupron/).

In the present study, we found that the fusion protein uPAg-KPI not only demonstrated the ability to inhibit tumor
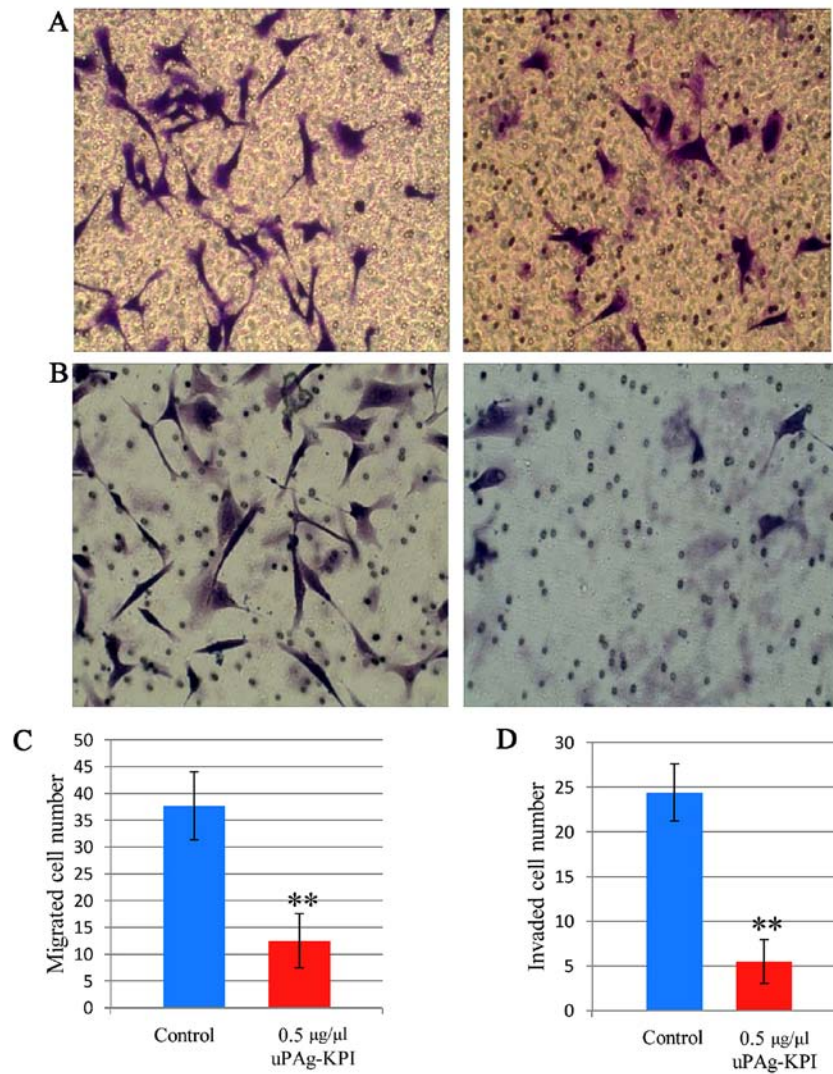
Figure 4. Effect of urokinase-type plasminogen activator (uPA)g-kunitz protease inhibitor (KPI) on inhibition of SKOV-3 cell migration and invasion. (A and C) Cell migration assay. Cells at a density of 2×10^4 cells/100 μl were plated in the upper chamber in the absence or presence of uPAg-KPI (0.5 μg/μl), and the bottom chambers were filled with regular IMDM/10% FBS. Cells were cultured for 48 h. At the end of the experiment, cells which had migrated into the bottom filter were counted under a phase-contrast microscope (x200) after staining with 1% crystal violet. **P<0.01. (B and D) Transwell tumor cell invasion assay. Transwell filters were pre-coated with Matrigel and the rest of the procedures were the same as for the Transwell tumor cell migration assay.
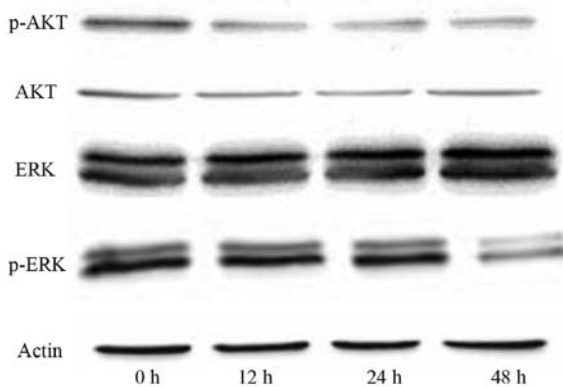

Figure 5. Effect of urokinase-type plasminogen activator (uPA)g-kunitz protease inhibitor (KPI) protein on the regulation of AKT, p-AKT, extracellular signal-regulated kinase (ERK)1/2, and p-ERK1/2 protein expression in SKOV-3 cells, as measured by western blot analysis. SKOV-3 cells were grown and treated with 0.5 μg/μl uPAg-KPI for 0, 12, 24 and 48 h and then subjected to western blot analysis to measure the AKT, p-AKT, ERK1/2 and p-ERK1/2 protein levels. The experiments were repeated three times with similar results.

cell growth, but also inhibited tumor cell invasion and metastasis. It is envisioned that futire *in vivo* studies will assess the effectiveness of this fusion protein uPAg-KPI in animals before clinical trials. However, the uPA signal transduction pathway is complex, and there is a plethora of combining pathways. For example, previous studies have shown that the uPA/uPAR signaling cascade may be at the intersection of multiple tumor invasion and metastasis-related signaling molecules or pathways (29-32). In addition to activating extracellular matrix degradation, the uPA/uPAR system also activates Src, Raf, FAK, ERK or MAPK signaling pathways, which play an important role in tumor progression (33-35). With respect to the induction of tumor cell proliferation, previous studies have shown that uPA induced a cascade of several cell proliferation signaling pathways, such as the signal transducer and activator of transcription (Stat3) pathway, ERK1/2 pathway and the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway (36-39). In order to investigate the possible mechanisms by which uPAg-KPI induced cell growth arrest and

inhibition of tumor cell invasion, the present study detected the level of ERK, p-ERK, AKT and p-AKT proteins and found that uPAg-KPI suppressed the expression of phosphorylated ERK1/ERK2 and AKT. These two pathways have previously been shown to regulate cell growth and invasion (40,41). Thus, the data obtained from the present study suggest that uPAg-KPI binds to membrane-anchored uPAR and restrains plasminogen activation on the tumor cell surface. This blocks the ERK and AKT signaling pathways and thus significantly decreases tumor growth and invasion. However, further investigation is required in order to elucidate how exactly uPAg-KPI suppresses phosphorylation and the activity of ERK1/ERK2 and AKT proteins.

Acknowledgements

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