Expression and activity analysis of a new fusion protein targeting ovarian cancer cells

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Abstract. The aim of the present study was to develop a new therapeutic drug to improve the prognosis of ovarian cancer patients. Human urokinase-type plasminogen activator $(uPA)_{17-34}$-kunitz-type protease inhibitor (KPI) eukaryotic expression vector was constructed and recombinant human uPA_{17-34}-KPI (rhuPA$_{17-34}$-KPI) in *P. pastoris* was expressed. In the present study, the DNA sequences that encode uPA 17-34 amino acids were created according to the native amino acids sequence and inserted into the KPI-pPICZαC vector, which was constructed. Then, uPA_{17-34}-KPI-pPICZαC was transformed into *P. pastoris* X-33, and rhuPA$_{17-34}$-KPI was expressed by induction of methanol. The bioactivities of a recombinant fusion protein were detected with trypsin inhibition analysis, and the inhibitory effects on the growth of ovarian cancer cells were identified using the TUNEL assay, *in vitro* wound-healing assay and Matrigel model analysis. The results of the DNA sequence analysis of the recombinant vector uPA_{17-34}-KPI-pPICZα demonstrated that the DNA-encoding human uPA 17-34 amino acids, 285-288 amino acids of amyloid precursor protein (APP) and 1-57 amino acids of KPI were correctly inserted into the pPICZαC vector. Following induction by methonal, the fusion protein with a molecular weight of 8.8 kDa was observed using SDS-PAGE and western blot analysis. RhuPA$_{17-34}$-KPI was expressed in *P. pastoris* with a yield of 50 mg/l in a 50-ml tube. The recombinant fusion protein was able to inhibit the activity of trypsin, inhibit growth and induce apoptosis of SKOV3 cells, and inhibit the invasion and metastasis of ovarian cancer cells. By considering uPA_{17-34} amino acid specific binding uPAR as the targeted part of fusion protein and utilizing the serine protease inhibitor activity of KPI, it was found that the recombinant fusion protein uPA_{17-34}-KPI inhibited the invasion and metastasis of ovarian tumors, and may therefore be regarded as effective in targeted treatment.

Introduction

Ovarian cancer, which is a heterogeneous and rapidly progressive disease of low prevalence, is a gynecological malignant tumor with a high mortality rate (1). Due to the resistance of chemotherapy and tumor recurrence, the mortality rate among women diagnosed with metastatic diseases caused by ovarian cancer remains high, with only 27% of patients surviving >5 years post-diagnosis (2). Therefore, the development of new therapeutic drugs is important to improve the prognosis of ovarian cancer patients.

The human urokinase plasminogen activator (uPA) gene is located at the long arm of chromosome 10, is 6.5 kb in length and consists of 11 exons, and can be transcribed into mature mRNA of 2.4 kb in length. The uPA is an extracellular serine protease that binds to its receptor, urokinase plasminogen activator receptor (uPAR and CD87), and converts plasminogen into plasmin at the cell-matrix interface (3,4). uPA and uPAR are involved in the control of extracellular matrix turnover, cell migration, invasion and cell signaling leading to a variety of different responses under physiological and pathological conditions (5,6). Previous findings indicate that during the period of cancer cell invasion and metastasis, proteolytic enzymes participate in the degradation of extracellular matrix components (7,8). The specific uPA/uPAR binding results in the enzymatic activity of uPA being concentrated on the cell surface, not only reinforcing the activation of plasminogen, and consequently accelerating pathological processes such as extracellular stromatin degradation and cellular invasion, but also participating in the generation of new blood vessels closely associated with tumor metastasis (9,10). uPA and

uPAR are overexpressed in human ovarian cancer cells (11,12), and the binding of uPA with uPAR on the surface of ovarian cancer cell membranes is considered as the key to promoting metastasis of ovarian cancer by uPA (8,9).

uPA comprises a carboxyl-terminal serine protease domain and an amino-terminal fragment (ATF) that contains all of the determinants required for binding to its receptor. The 17-34 amino acids of ATF consist of an amino acid fragment that is crucial to uPA/uPAR binding (13,14). Therefore, we aimed to create a type of fusion protein that contains uPA 17-34 amino acids and activated protein. Through targeted binding between the shortest conjugation fragment of uPA 17-34 amino acids and uPAR on ovarian cancer tissues, the fusion protein we designed was able to enter the targeted cells accurately.

The major isoforms of human amyloid precursor protein (APP), APP695 and APP751, differ due to the presence of a kunitz-type protease inhibitor (KPI) domain in the extracellular region (15). KPI is a type of stable serine protease inhibitor that inhibits the activity of serine protease and its decomposition on proteins, blocks the invasion and metastasis processes of tumor cells and recovers the cell barrier.

We constructed the pPICZαC-uPA$_{17-34}$-KPI expression vector which contained the nucleotide sequences encoding human uPA 17-34 and 285-345 amino acids of APP, which contained the 57 amino acid KPI domain (residues 289-345) (16). The *P. pastoris* expression system was then used to express the secreted recombinant human uPA$_{17-34}$-KPI (rhuPA$_{17-34}$-KPI) fusion protein. By considering uPA$_{17-34}$ amino acid specific binding uPAR as the targeted part of fusion protein, and utilizing serine protease inhibitor activity of KPI, the recombinant fusion protein, uPA$_{17-34}$-KPI, was found to inhibit the invasion and metastasis of ovarian tumors and serve as an effective targeted treatment.

Materials and methods

Strains, vectors and reagents. The DNA sequence that encodes human uPA$_{17-34}$ amino acids was produced by Sangon Biotech Co., Ltd. (Shanghai, China). The *P. pastoris* X-33, H-DMEM culture medium and zeocin antibiotic were purchased from Invitrogen-Life Technologies (Carlsbad, CA, USA). The restrictive enzyme, T4 DNA ligase, *Taq* DNA polymerase and plasmid preparation kit were purchased from Takara (Biotechnology Co., Ltd., Dalian, China). SP Sepharose XL and Source™ 30 RPC reversed phase hydrophobic chromatography were purchased from Phamarcia (Uppsala, Sweden). YPD, BMGY, and BMMY media were prepared as a formula (Invitrogen-Life Technologies). The SKOV3 human ovarian cancer cell line was kept in the School of Pharmaceutical Sciences of Jilin University, and KPI-pPICZαC was constructed in our laboratory.

Construction of the uPA$_{17-34}$-KPI-pPICZαC expression vector. The DNA that encodes uPA 17-34 amino acids was produced according to its native amino acids sequence (Sangon Biotech Co.,Ltd). To improve the yield of recombinant protein, synonymous codons were replaced by yeast-biased codons. The DNA sequence used was: 5'-TC GAG AAG AGA GGT ACT TGT GTT TCT AAC AAG TAC TTC TCT AAC ATT CAC TGG TGT AAC TGT CCA GAG GTT GTT AGA GAG GTT TGC

A-3'; complementary strand, 5'-AAC CTC TCT AAC AAC CTC TGG ACA GTT ACA CCA GTG AAT GTT AGA GAA GTA CTT GTT AGA AAC ACA AGT ACC TCT CTT C-3'.

Annealing was performed as follows: 6 µl 0.5 mmol NaCl and 25 µmol single-strand DNA were mixed. The compound was then boiled for 3 min at 80˚C and cooled down to room temperature gradually. After being annealed, the complementary strands contained cohesive ends of *Xho*I and *Pst*I, while the *Kex*2 site was followed by the *Xho*I site.

KPI-pPICZαC was constructed and kept in our laboratory. In addition to the 57 amino acids KPI domain, the recombinant plasmid contained DNA that encodes an additional four amino acid residues that correspond to amino acids 285-288 of APP (16). The DNA sequence of 61 amino acids was inserted into pPICZαC between *Xho*I and *Xba*I. The KPI-pPICZαC plasmid was digested with *Xho*I and *Pst*I and then the DNA sequence that encodes uPA 17-34 amino acids was inserted into the corresponding sites to construct the uPA$_{17-34}$-KPI-pPICZαC expression plasmid.

The recombinant plasmid was transformed into competent cells of *Escherichia coli* XL-Blue, and the recombinant colonies were selected by zeocin (25 µg/ml) resistance. The positive clones were incubated in LB liquid medium for 12 h and the plasmids were extracted using a plasmid preparation kit. The nucleotide sequences of the inserted DNA and flanking sequence were verified by sequencing with the Genome Lab DTCS-Quick Start kit and CEQ 2000 DNA analysis system (Beckman Coulter Inc., Brea, CA, USA).

Transformation and induced expression of rhuPA$_{17-34}$-KPI. The recombinant plasmid with correct sequence was linearized with *Sac*I and introduced into the competent cells of the *P. pastoris* X-33 strain by electroporation using a micropulser (Bio-Rad, Hercules, CA, USA) according to the pPICZα vector manual. After electroporation, the mixture was spread on a YPD culture plate containing zeocin (100 mg/l) and cultured at 30˚C for 3 days. The blank plasmid of pPICZαC was also transformed as a negative control.

After the transformants with zeocin resistance became visible, some monoclonal transformants were randomly selected and cultured in 10 ml BMGY culture medium at 30˚C with agitation at 225 rpm for 24 h. The culture media (0.5 ml) were sampled, and centrifuged at 10,000 rpm for 5 min at 4˚C and the cell pellets were used for genomic DNA analysis. The yeast genomic DNA was extracted using the boiling-freezing-boiling method and PCR identification was carried out to select the *P. pastoris* X-33 strain which was successfully. transformed into recombinant plasmid

The remaining culture medium was centrifuged and cell pellets were resuspended in 10 ml BMMY medium to induce expression for 7 days. The culture medium (0.5 ml) was sampled daily and centrifuged at 10,000 rpm for 5 min at 4˚C. The supernatant was used for recombinant protein detection. Methanol was added every 24 h to a final concentration of 0.5% (v/v) to induce the expression of the target protein. SDS-PAGE and trypsin inhibition analyses were performed to select high-level expression clone cells.

Optimized expression of rhuPA$_{17-34}$-KPI in P. pastoris. To ascertain the optimized pH value and induction time in

Table I. Protocol of trypsin inhibition activity analysis of rhuPA$_{17-34}$-KPI.

Materials	Tube 0 (μl)	Tube 1 (μl)	Sample tube (μl)
Tris-HCl buffer (pH 8.0)	650	650	650
Trypsin solution (20 U/ml)	25	25	25
RhuPA$_{17-34}$-KPI culture supernatant	60	60	60
30% HAc	450	0	0
	37°C 20 min		
DL-BAPNA (200 mmol/l)	10	10	10
	37°C 10 min		
30% HAc	0	450	450

KPI, kunitz-type protease inhibitor.

order to achieve a high-level expression of rhuPA$_{17-34}$-KPI, a high-level expression cell colony was cultured with different pH values (3.5-7.5 with 0.5 intervals) using culture medium. The procedure followed was the same as mentioned above, and the pH values were adjusted daily with disodium hydrogen phosphate and citric acid. At the desired time points, 0.5 ml cell aliquots were removed and replaced with equal amounts of fresh medium. The supernatant samples were used for trypsin inhibition analysis. To optimize the induction time, the supernatant samples from 0 to 7 days under the same pH value were detected by trypsin inhibition analysis.

SDS-PAGE and western blot analyses of rhuPA$_{17-34}$-KPI. SDS-PAGE analysis was performed using 18% separation gel. For western blot analysis, proteins in the gel were transferred to a nitrocellulose membrane. The membrane was blocked with 5% BSA for 1 h and then incubated with the murine anti-human urokinase monoclonal antibody (American Diagnostica Inc., Stamford, CA, USA) for 12 h. After being washed, the membrane was incubated with the goat anti-mouse HRP-conjugated IgG (Dingguo, China), diluted to 1:250. The bound antibody was detected using 3,3'-diamino-benzidine (DAB).

Trypsin inhibition activity analysis of rhuPA$_{17-34}$-KPI. The trypsin inhibition experiment was used to determine the activity and quantity of rhuPA$_{17-34}$-KPI. As shown in Table I, 650 μl Tris-HCl (50 mmol/l, pH 8.0) buffer, 60 μl culture supernatant and 25 μl trypsin (20 U/ml) were mixed in 1.5-ml tubes and incubated at 37°C for 20 min. Then, 10 μl N-benzoyl-DL-arginine-4-nitroanilide hydrochloride (DL-BAPNA; 200 mM/l) was added. After being incubated at 37°C for 10 min, the reaction was stopped by adding 450 μl 30% (v/v) acetic acid. The absorbance of each tube was measured at 410 nm. Each sample was measured three times and the mean value was used for analysis. The percentage of trypsin inhibition activity (TIA) was calculated based on the formula:

$$\text{TIA (\%)} = \frac{A1 - As}{A1 - Ao} \times 100\%$$

where A_0, A_1, A_{410} were tube 0 and 1; and As, A_{410} the sample tube (tube S).

Purification of rhuPA$_{17-34}$-KPI. After being induced by methanol for 5 days, the supernatant of the fermentation broth was collected by centrifugation. After being diluted four times with 20 mM NaAc-HAc (pH 3.0) buffer, the pH of the supernatant was adjusted to 3.0 with 1 M acetate acid. The supernatant was loaded onto the cation exchange chromatography (SP Sepharose XL) which was equilibrated with 20 mM NaAc-HAc (pH 3.0) buffer. The column was washed extensively with the same buffer. The bound protein was eluted with a linear gradient of NaCl (0.1-1.0 M) and protein elution was monitored by measuring the absorbance at 280 nm and identified by trypsin inhibition analysis. The column effluent containing rhuPA$_{17-34}$-KPI was loaded onto a reverse phase column (Source 30) which was equilibrated with 0.1% trifluoroacetic acid (TFA) for further purification. RhuPA$_{17-34}$-KPI was eluted using 50% methanol containing 0.1% TFA and was concentrated by vacuum distillation and freeze drying to remove the methanol.

N-terminal amino acid sequence analysis. To determine the N-terminal sequence, the purified rhuPA$_{17-34}$-KPI was electrophoresed on 18% SDS-PAGE gel and electroblotted on a PVDF membrane. After being blotted, the PVDF membrane was stained with Coomassie Brilliant Blue R250, and the rhuPA$_{17-34}$-KPI band was extracted and determined by automated Edman degradation performed on a model PPSQ-21A protein sequencer (Shimadzu, Japan).

Inhibitory effects of rhuPA$_{17-34}$-KPI on the growth of ovarian cancer cells. The growth and proliferation of SKOV3 ovarian carcinoma cells was observed using a Cell Counting Kit-8 (CCK-8) assay. Briefly, the SKOV3 human ovarian cancer cell line was maintained in H-DMEM with 10% fetal bovine serum (FBS) and 100 U/ml of penicillin/streptomycin, at 37°C in a humidified atmosphere containing 5% CO_2. The cells (2×10^4) were seeded in 96-well plates containing complete medium and incubated for 24 h followed by different doses of rhuPA$_{17-34}$-KPI. SKOV3 cells were treated with H-DMEM (as control) and 0.075, 0.15, 0.3, 0.6, 1.2 and 2.4 mg/ml rhuPA$_{17-34}$-KPI, respectively, for 48 h. Subsequently, 10 μl of the CCK-8 solution was added to each well and the cells were incubated for 2 h. The absorbance was detected at 450 nm using a microplate reader (Bio-Rad Instruments, Hercules, CA, USA). The percentage of inhibition rate of rhuPA$_{17-34}$-KPI (I) was calculated using the formula:

$$\text{I (\%)} = \frac{Ac - As}{Ac} \times 100\%$$

where Ac, A_{450} was the control group; and As, A_{450} the different doses of rhuPA$_{17-34}$-KPI.

TUNEL assay for apoptosis. Apoptosis was determined using a TUNEL assay. SKOV3 cells at a density of 1×10^5 cells/well were seeded in 1.3 mm coverslips in 24-well plates and incubated

for 24 h followed by different doses of rhuPA$_{17-34}$-KPI for 24 h. SKOV3 cells were treated with H-DMEM (as control), 1.2 and 2.4 mg/ml rhuPA$_{17-34}$-KPI, respectively, and the cells were fixed with freshly prepared paraformaldehyde [4% in PBS (pH 7.4)], rinsed with PBS and incubated in permeabilization solution. TUNEL staining was performed with an In Situ Cell Death Detection kit (Bioss, China) according to the manufacturer's instructions. The TUNEL reaction mixture containing reaction buffer, terminal deoxynucleotidyl transferase and bromodeoxyuridine triphosphate was added onto the slides and incubated for 2 h at 37°C, followed by washing and incubation with an HRP-labeled anti-bromodeoxyuridine monoclonal antibody for 30 min at room temperature. The presence of antigen was then visualized with diaminobenzidine. Slides were subsequently counterstained with hematoxylin and imaged under bright-field microscopy. The number of TUNEL-positive cells was counted in six different fields and representative fields were photographed. The percentages of apoptotic cells were calculated from the ratio of apoptotic cells to total cells counted.

Inhibitory effects of rhuPA$_{17-34}$-KPI on the cell migration of ovarian cancer cells. The effects of rhuPA$_{17-34}$-KPI on the migration of SKOV3 cells were examined using the wound-healing assay (17). SKOV3 cells were cultured in 24-well plates and allowed to reach 90% confluence. The cells were wounded with a 200-*μ*l pipette tip and washed with PBS. After incubation with rhuPA$_{17-34}$-KPI at a concentration of 1.2 or 2.4 mg/ml for 24 h, the migration of SKOV3 cells into the wound area was visualized and photographed.

Ovarian cancer cell invasion assay. The effects of rhuPA$_{17-34}$-KPI on the inhibition of invasion of SKOV3 cells were detected in a two-compartment Boyden chamber (Transwell) as previously described (18). Briefly, SKOV3 cells were treated with rhuPA$_{17-34}$-KPI at a concentration of 1.2 or 2.4 mg/ml for 24 h. The cells were collected and resuspended with serum-free H-DMEM. Cells ($2x10^4$) were seeded in the upper compartment, and H-DMEM with 20% FBS was placed in the lower compartment of the Boyden chamber. After incubation at 37°C for 6 h, the cells on the lower side were fixed, stained and observed. The invasion ability of tumor cells was determined by the mean value of cells migrating through the membrane.

Statistical analysis. Data are presented as means ± SEM. Statistical analyses were performed using the Student's t-test. P<0.05 was considered to indicate a statistically significant result.

Results

Identification of recombinant plasmid uPA$_{17-34}$-KPI-pPICZα. Since there is a *Pst*I site in the DNA sequence that encodes KPI 6-8 amino acids, we created a DNA sequence that encodes uPA 17-34 amino acids and a few amino acids corresponding to the N-terminal amino acid sequence of KPI. After the uPA sequence was inserted into the KPI-pPICZαC plasmid (digested with *Xho*I and *Pst*I), the recombinant plasmid uPA$_{17-34}$-KPI-pPICZα was performed using DNA sequence analysis. The results of the DNA sequence analysis of the
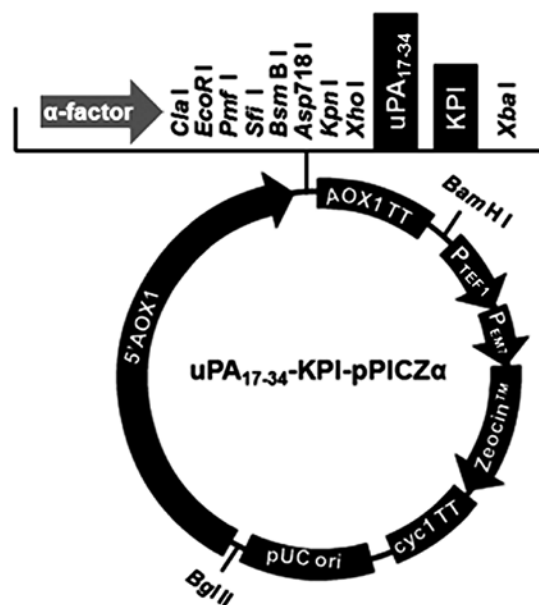

Figure 1. The schematic drawing of uPA$_{17-34}$-KPI-pPICZα UPA17-34-KPI which contains the DNA sequences encoding human uPA 17-34 and 285-345 amino acids of APP was inserted into the pPICZαC vector between the *Xho*I and *Xba*I sites. KPI, kunitz-type protease inhibitor; APP, amyloid precursor protein.

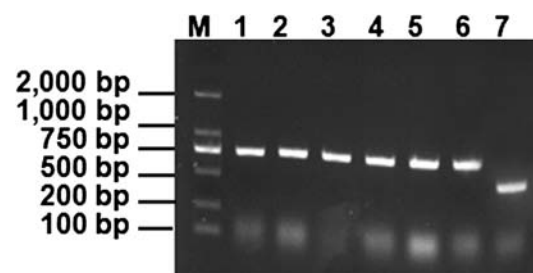Figure 2. Agarose gel electrophoresis analysis of PCR product of the yeast genomic DNA Lane M, DL 2000 marker. Lanes 1-6, PCR products of yeast genomic DNA transformed with uPA$_{17-34}$-KPI-pPICZα. Lane 7, PCR product of yeast genomic DNA transformed with pPICZαC blank plasmid. KPI, kunitz-type protease inhibitor.

recombinant expression vector uPA$_{17-34}$-KPI-pPICZα (data not shown) demonstrated that the DNA sequences encoding human uPA 17-34 and 285-288 amino acids of APP, and 1-57 amino acids of KPI were correctly inserted into the pPICZαC vector and the amino acid sequence was identical to that logged in GenBank. The schematic drawing of uPA$_{17-34}$-KPI-pPICZα presents the structure of recombinant plasmid (Fig. 1).

Screening of positively transformed yeast strain with PCR. After being cultured at 30°C for 3 days, a number of transformants with zeocin resistance became visible on the YPD agar plates containing 0.1 g/l zeocin. 5'AOX and 3'AOX were used as primers to perform PCR analysis of genomic DNA. PCR products were used to carry out 1.0% agarose gel electrophoresis analysis. There were ~750 bp amplification bands for the samples that were transformed with uPA$_{17-34}$-KPI-pPICZα, but ~400 bp for the control sample which was transformed with the pPICZαC blank plasmid (Fig. 2). The results showed that, the DNA sequence encoding the fusion protein was integrated

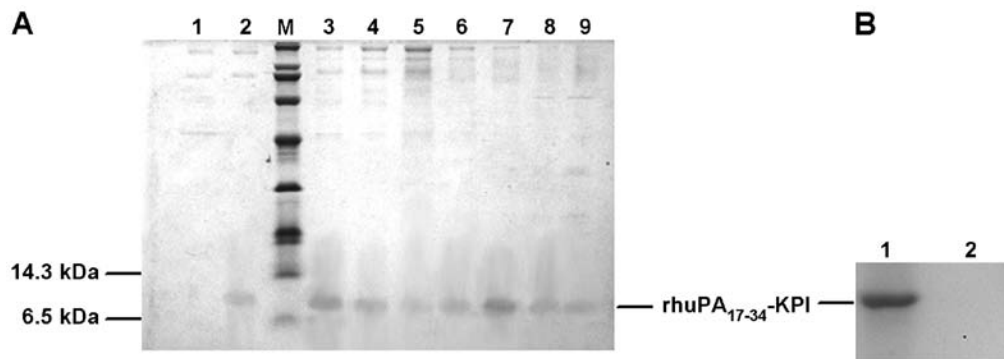Figure 3. SDS-PAGE and western blot analyses of rhuPA$_{17-34}$-KPI. (A) SDS-PAGE analysis of rhuPA$_{17-34}$-KPI. SDS-PAGE was performed on 18% gel and stained with Coomassie brilliant blue. Lane M, protein molecular weight marker (broad). Lane 1, supernatant from the negative stain transformed with pPICZα blank plasmid. Lanes 2-9, supernatant from different rhuPA$_{17-34}$-KPI transformants after induction of methanol for 72 h. (B) Western blot analysis of rhuPA$_{17-34}$-KPI. Lane 1, purified rhuPA$_{17-34}$-KPI. Lane 2, supernatant from the negative stain transformed with pPICZαC blank plasmid as a negative control. KPI, kunitz-type protease inhibitor.
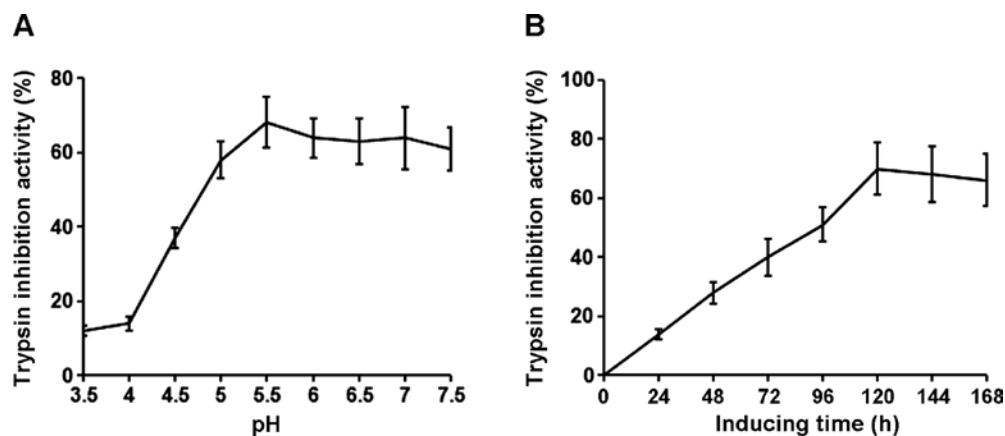

Figure 4. Optimized expression of recombinant human uPA$_{17-34}$-KPI in *P. pastoris*. (A) Optimization of the pH value. (B) Optimization of the methanol inducing time-points. Supernatants collected at each evaluated condition were processed by trypsin inhibition activity analysis.

into >90% of clones which were transformed with the recombinant expression vector, uPA$_{17-34}$-KPI-pPICZα.

Expression of rhuPA$_{17-34}$-KPI. The positive transformants were induced to express recombinant protein by methanol for 7 days. On the third day after induction with methanol the supernatants were identified using SDS-PAGE analysis and the transformant presenting with the highest expression level of rhuPA$_{17-34}$-KPI was utilized to optimize the expression of the fusion protein in *P. pastoris*. The results indicated that rhuPA$_{17-34}$-KPI was expressed following the induction of methanol. However, the recombinant protein expression in the transformant-containing blank plasmids of pPICZαC was negative. Based on the amino acids sequence, the calculated molecular weight of rhuPA$_{17-34}$-KPI was 8.8 kDa, which was consistent with the result of the SDS-PAGE measurement (Fig. 3A).

Identification of rhuPA$_{17-34}$-KPI. The primary purified recombinant protein was identified by western blot analysis. The results demonstrated that the recombinant protein bound to the anti-human urokinase monoclonal antibody. No band was observed in lane 2, which was the supernatant of transformant containing blank plasmids of pPICZαC (Fig. 3B).

N-terminal amino acid sequence analysis of the purified recombinant protein offered the first 15 amino acids as G T C V S N K Y F S N I H W C. The N-terminal sequence of rhuPA$_{17-34}$-KPI was identical to that of human uPA$_{17-34}$, confirming the successful expression and purification of rhuPA$_{17-34}$-KPI.

Optimized expression of rhuPA$_{17-34}$-KPI. Trypsin inhibition activity analysis was performed to determine the optimal expression conditions of rhuPA$_{17-34}$-KPI. After a series of experiments, the optimal expression conditions of rhuPA$_{17-34}$-KPI were obtained as follows: the optimal pH was 5.5 (Fig. 4A), and the optimal induction time-points were identified on the 5th day for the strain (Fig. 4B) at 30˚C, following daily methanol addition at a concentration of 0.5% (v/v). Under these conditions, the yield of rhuPA$_{17-34}$-KPI was 50 mg/l.

Anticancer effects of rhuPA$_{17-34}$-KPI. The anticancer activity of rhuPA$_{17-34}$-KPI was detected by its antiproliferation effects on SKOV3 cells. We observed the cell morphology of SKOV3 cells following treatment with rhuPA$_{17-34}$-KPI at different doses. In the control group, the cells grew well with adherence and the cells were fusiform or diamond shaped. However, following treatment with rhuPA$_{17-34}$-KPI, the cells
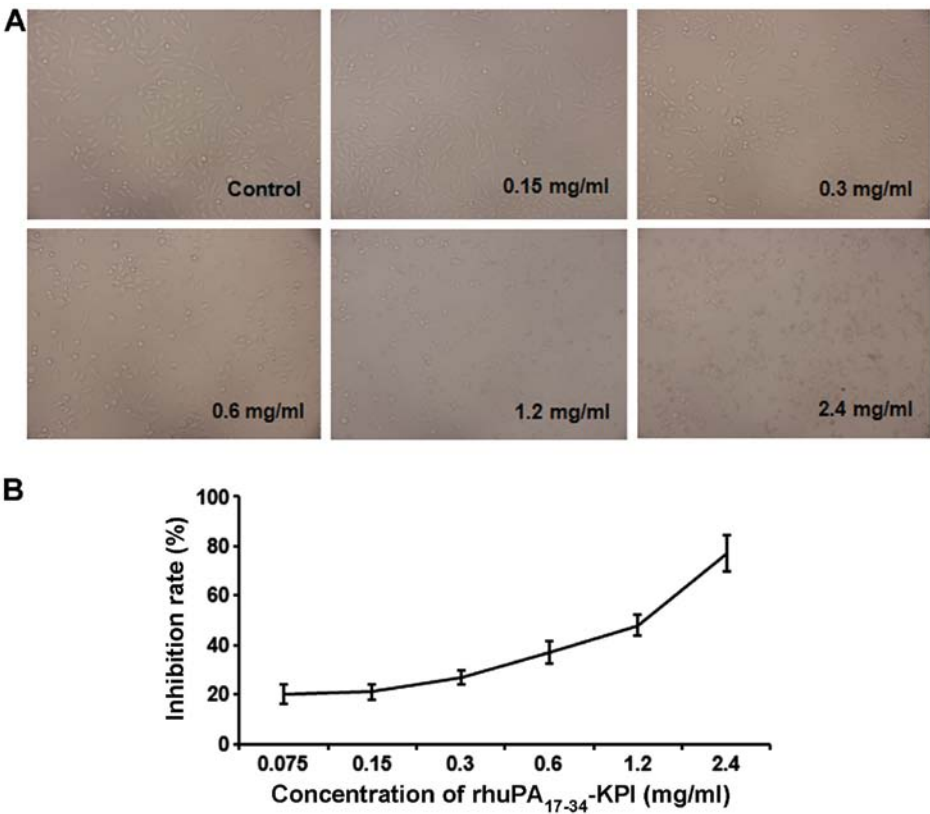
Figure 5. Inhibition effects of rhuPA$_{17-34}$-KPI on the growth of ovarian cancer cells. (A) Morphology of rhuPA$_{17-34}$-KPI on the growth of ovarian cancer cells. (B) Dose-effect curve of rhuPA$_{17-34}$-KPI on ovarian cancer SKOV3 cells. The cells were treated with H-DMEM (as control), 0.075, 0.15, 0.3, 0.6, 1.2 and 2.4 mg/ml rhuPA$_{17-34}$-KPI, respectively. KPI, kunitz-type protease inhibitor.
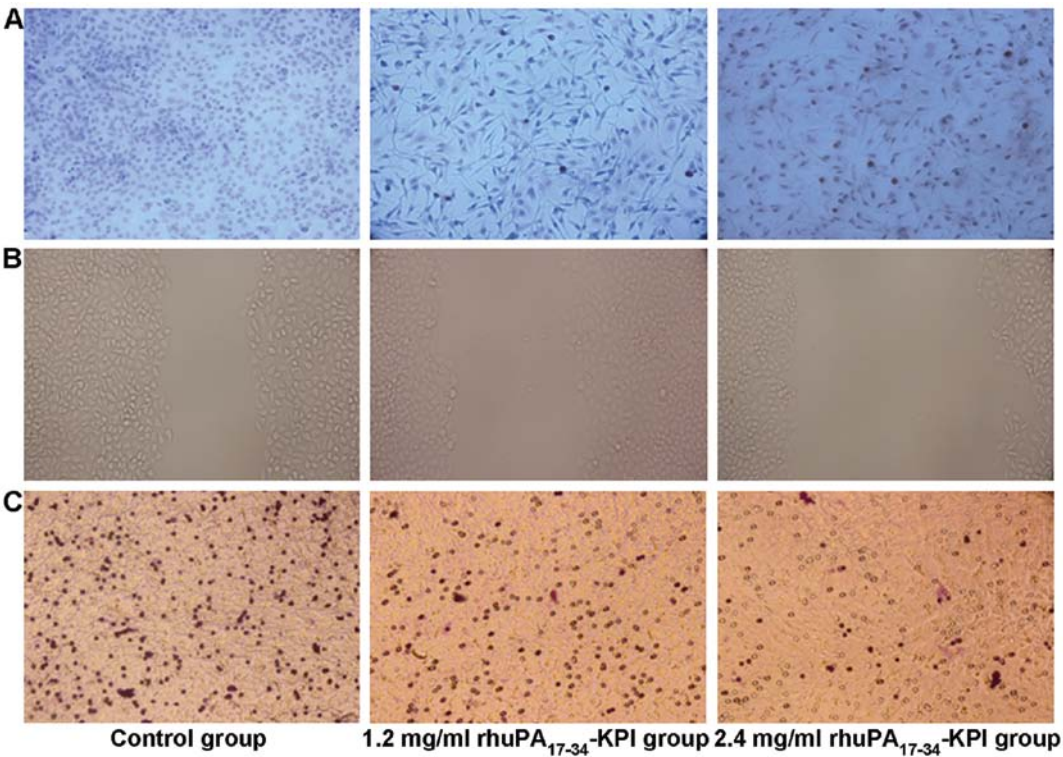

Figure 6. Anticancer effects of rhuPA$_{17-34}$-KPI on ovarian cancer cells. (A) Morphology of TUNEL assay for apoptosis. (B) Morphology of wound-healing assay. (C) Morphology of Matrigel invasion assay. TUNEL, wound-healing and Matrigel invasion assays were detected after SKOV3 cells were treated with different concentration of rhuPA$_{17-34}$-KPI for 24 h. SKOVS cells were treated with H-DMEM (as control), 1.2 and 2.4 mg/ml rhuPA$_{17-34}$-KPI, respectively. KPI, kunitz-type protease inhibitor.

membrane shrinking and cytoplasm spilling were evident. The CCK-8 assay was used to detect the quantity of cells. As a result, rhuPA$_{17-34}$-KPI treatment resulted in a dose-dependent inhibition of growth on SKOV3 cells at 48 h. The inhibition rate was ~80% at the dose of 2.4 mg/ml (Fig. 5).

The TUNEL assay demonstrated that ovarian cancer cells underwent apoptosis following treatment with rhuPA$_{17-34}$-KPI. Data from all the designated fields were pooled to obtain the apoptosis index, which is the percentage of TUNEL-positive cells in total cells manually counted in six random fields. The apoptotic indices of rhuPA$_{17-34}$-KPI treatment groups were markedly increased compared with the normal control group. As a result, rhuPA$_{17-34}$-KPI treatment caused a dose-dependent increase apoptosis in SKOV3 cells within 24 h (Fig. 6A).

Inhibition of invasion and metastatic effects of rhuPA$_{17-34}$-KPI. An *in vitro* wound-healing assay was performed to examine whether rhuPA$_{17-34}$-KPI inhibited the migration of ovarian cancer cells. As shown in Fig. 6B, the cells in the control group that were not treated with rhuPA$_{17-34}$-KPI had migrated into and largely covered the original wound area at 24 h after the scratch. However, the cells treated with rhuPA$_{17-34}$-KPI at different doses failed to migrate substantially into the wound area.

The effects of rhuPA$_{17-34}$-KPI on the invasion of ovarian cancer cells were evaluated using the Matrigel model. RhuPA$_{17-34}$-KPI reduced the number of invaded cells in a dose-dependent manner (Fig. 6C). These findings indicated that rhuPA$_{17-34}$-KPI significantly inhibited the invasion and metastasis of SKOV3 cells.

Discussion

Effective anticancer drugs are developed by clinical doctors and investigators with great effort. Surgery, chemotherapy and radiotherapy are conventional treatments used in the clinic. However, for patients considered unsuitable for surgical treatment and who do not tolerate the side effects of chemotherapy and radiotherapy, new therapeutic drugs and measures are of utmost importance. With the rapid development of molecular biology, immunology and genetic engineering technology in recent years, the focus has been on new types of drugs that have potential anticancer effects. The sequence of 17-34 amino acids of uPA is the key to uPA/uPAR binding, which makes the enzymatic activity of uPA concentrated on the cell surface (9,10). Therefore, in the present study, by considering uPA 17-34 amino acids specifically binding to uPAR that is overexpressed in human ovarian cancer tissues, we determined whether the fusion protein was able to bind to target cells accurately.

Previous findings have shown that the role of the proteolytic enzyme is indispensable in the invasion and metastasis of tumor cells, and the concentrations of many serine proteases are positively correlated with tumor invasion. KPI, which was selected to utilize anticancer effects, is a type of stable serine protease inhibitor. It inhibits the activity of serine protease and its decomposition on proteins. Therefore, it is possible to block the invasive and metastatic processes of tumor cells and recover the cell barrier (19).

The present study aimed to construct the fusion protein that can be applied to effective targeted treatment for ovarian cancer. In the present study, uPA$_{17-34}$-KPI-pPICZαC eukaryotic expression vector was constructed successfully. After being transformed into *P. pastoris* and induced by methanol, rhuPA$_{17-34}$-KPI was detected using SDS-PAGE and western blot analyses. We established a stable and effective rhuPA17-KPI expression system in *P. pastoris*. The α-factor signal peptide in the yeast expression vector was effective in guiding the secretion of rhuPA$_{17-34}$-KPI into the culture broth, which facilitates the production of large-scale fermentation for direct industrial application without complicated purification procedures (20).

Trypsin inhibition analysis showed that rhuPA$_{17-34}$-KPI presented trypsin inhibition activity similar to the serine protease inhibitor. Moreover, rhuPA$_{17-34}$-KPI inhibited the proliferation of SKOV3 cells and induced apoptosis of ovarian cancer cells. *In vitro* wound healing assay and Matrigel model analysis revealed the inhibitory effects of rhuPA$_{17-34}$-KPI on the invasion and metastasis of ovarian cancer cells. Thus, the present study suggests an early-stage experimental basis for the further study of the treatment of ovarian cancer using rhuPA$_{17-34}$-KPI.

Acknowledgements

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