Peptide P7 inhibits the bFGF-stimulated proliferation and invasion of SKOV3 cells

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Abstract. Peptide P7 specifically binds with basic fibroblast growth factor (bFGF) to inhibit the proliferation and invasion of numerous types of cancer cell. However, this effect has remained to be demonstrated in ovarian cancer-derived cell lines. In the present study, the protein P7 was used treat bFGF-stimulated SKOV3 epithelial ovarian cancer cells to explore the therapeutic potential of P7. An MTT and a scratch wound assay were used to respectively evaluate the proliferation and migration of bFGF-stimulated SKOV3 cells treated with P7. Reverse transcription-quantitative polymerase chain reaction analysis was used to detect the gene expression of urokinase-type plasminogen activator (uPA), as well as matrix metallopeptidase (MMP)-2 and -9, which have a role in cell migration/invasion. The morphology and proliferation of SKOV3 cells were not significantly affected by different concentrations of P7. However, P7 had an obvious inhibitory effect on the proliferation and migration of bFGF-stimulated SKOV3 cells. Treatment with P7 significantly lowered the gene expression of uPA, MMP-2 and MMP-9 compared with that in the control group. In conclusion, the present results suggested that P7, which, at least in part, acts through inhibition of bFGF, may have a potential therapeutic application in epithelial ovarian cancer.

Introduction

Ovarian cancer is one of the three most common malignant tumor types of the female reproductive system; it poses a serious threat to women's health and is associated with high mortality (1). The symptoms of the disease are frequently non-specific, which hampers early detection, so that the majority of patients present with advanced-stage disease at the time-point of diagnosis (2). Therefore, the treatment of advanced ovarian cancer is particularly important in the clinic.

Studies have confirmed that tumor growth is highly dependent on blood vessels, and tumor metastasis and prognosis are also closely linked to angiogenesis (3-6). Although anti-angiogenic agents, e.g. bevacizumab, are used for almost all patients with ovarian cancer, the cure rate is not increased (7,8). Furthermore, predictive biomarkers are currently insufficient and are urgently required. Basic fibroblast growth factor (bFGF), belonging to the family of FGFs, is one of the strongest vascular growth factors involved in the processes of proliferation and differentiation of a wide variety of cell types. Previous studies have indicated that the P7 high-affinity bFGF-binding peptide is able to inhibit the proliferation and invasion of various cell types induced by bFGF (9-12). Angiogenesis has a fundamental role in normal ovarian physiology as well as in the pathogenesis of ovarian cancer, promoting tumor growth and progression through ascites formation and metastatic spread (7). However, whether P7 also inhibits bFGF-induced proliferation and angiogenesis of human epithelial ovarian cancer cells has not been previously reported, to the best of our knowledge. In the present study, the effect of P7 on bFGF-induced proliferation and invasion of SKOV3 ovarian cancer cells was assessed *in vitro*, providing an experimental basis for the targeted treatment of epithelial ovarian cancer.

Materials and methods

Materials. The SKOV3 human ovarian cancer cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). P7 peptides (PLLQATLGGGS) with a purity of >98% was synthesized by Beijing SBS Genetech Corp. (Beijing, China) and recombinant human bFGF was obtained from Peprotech Inc. (Rocky Hill, NJ, USA). RPMI-1640 medium and fetal bovine serum (FBS) were from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

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Morphological examination. SKOV3 cells in the exponential growth phase were seeded into 96-well culture plates in 100 μl RPMI-1640 medium containing 0.4% FBS at a density of $1x10^4$ cells/well at 37˚C in a humidified atmosphere containing 5% CO_2. After addition of 100 μl medium containing P7 at different final concentrations (0.25, 1, 4 and 16 μM) and culture for 48 h, the cell morphology was observed with an inverted microscope (Olympus IX70; Olympus, Tokyo, Japan). SKOV3 cells with addition of 100 μl complete medium were used as a control group.

MTT cell proliferation assay. Cell viability was determined using an MTT assay. SKOV3 cells in the exponential growth phase were seeded into 96-well culture plates in 100 μl medium at a density of $1x10^4$ cells/well. The cells were serum-starved for 24 h. In the P7-treated group, 100 μl medium containing different concentrations of P7 was added to each well, followed by incubation for 48 h. In the bFGF-treated group, 100 μl medium containing different concentrations of bFGF (0.1, 1, 10 and 100 ng/ml) was added. Furthermore, in the P7+bFGF group, 100 μl medium containing different concentrations of P7 mixed with 10 ng/ml bFGF (IC_{50}) was added to the wells in quadruplicate. SKOV3 cells with addition of RPMI-1640 medium containing 0.4% FBS were used as the control group, while 100 μl bFGF (10 ng/ml) was added for the positive control group. After 48 h of incubation, the MTT assay was performed by adding 20 μl MTT solution (5 mg/ml in PBS; Beyotime Institute of Biotechnology, Haimen, China) to each well, followed by further incubation for 4 h. Subsequently, 150 μl dimethyl sulfoxide (Beijing Chemical Industry Co. Ltd., Beijing, China) was added. After shaking for 10 min, the optical density (OD) value was measured at a wavelength of 570 nm using a microplate reader (BIO-TEK800; Biotech Instruments, Winooski, VT, USA). Each treatment was performed in triplicate and the results were expressed as a percentage of the control: Cell proliferation rate (%)=$(OD_{bFGF}-OD_{Control})/OD_{Control}$ x100%. The inhibition rate of P7 on bFGF-induced SKOV3 cell proliferation was calculated as follows: Inhibition rate (%)=$[(OD_{bFGF}-OD_{Control})-(OD_{P7}-OD_{Control})]/(OD_{bFGF}-OD_{Control})$ x100%.

Scratch wound cell migration assay. Exponentially growing SKOV3 cells were seeded into 6-well culture plates at a density of $1x10^4$ cells/well, followed by starved incubation for 24 h. In the middle of each well, a 0.5 cm-wide linear scratch was generated with a 20-μl filter tip. The detached cells were rinsed with PBS 3 times so as to prevent their re-attachment in the scratched area. Subsequently, 100 μl DMEM containing 4 μM P7 for the P7 group, 10 ng/ml bFGF for the bFGF group or 50 μl 8 μM P7 + 50 μl 20 ng/ml bFGF for the P7+bFGF group was added to each well separately, while 100 μl DMEM was added to the blank control. Following incubation for 0, 6, 12 and 24 h, an inverted microscope was used to observe wound closure and capture images of the cells. The distance the cells had migrated into the scratched area was measured with ImagePro Express software 6.0 (Media Cybernetics, Rockville, MD, USA). Cell migration was quantified using the following formula: Cell migration (%)=(scratch edge distance at 0 h-migration distance at 6, 12 or 24 h)/scratch edge distance at 0 h x100%.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The mRNA levels of urokinase-type plasminogen activator (uPA), matrix metallopeptidase (MMP)2 and MMP9 were assessed as factors associated with cell invasion. SKOV3 cells were incubated in 100 μl RPMI-1640 medium containing 0.4% FBS with 4 μM P7 for the P7 group or 10 ng/ml bFGF for the bFGF group, or 50 μl 8 μM P7+50 μl 20 ng/ml bFGF for the P7+bFGF group for 24 h. Cells incubated with 100 μl RPMI-1640 medium containing 0.4% FBS were used as the blank control. Total RNA was isolated with a Qiagen RNeasy Micro kit (cat. no. 74004; Qiagen, Hilden, Germany). RT was performed by using a Takara PrimeScript™ RT reagent kit (cat. no. RR037A, Takara Bio Inc., Otsu, Japan). The first-strand complementary DNA synthesis was performed using 500 ng isolated RNA. PCR primers of the genes are displayed in Table I. qPCR was performed using SYBR® Premix Ex Taq™ (cat. no. DRR041A; Takara Bio Inc.) with the following thermocycling conditions: Initial denaturation, 95˚C for 5 min; amplification, 95˚C for 10 sec, 60˚C for 10 sec, 72˚C for 10 sec, gathering fluorescence signal at 72˚C for 40 cycles; dissolve, 95˚C for 5 sec, 65˚C for 1 min, 97˚C termination for 1 cycle; cooled at 40˚C for 30 sec. Each sample was anlysed 3 times and the average value was recorded. The relative expression levels of uPA, MMP2 and MMP9 were normalized to β-actin levels. The $2^{-\Delta\Delta Cq}$ value was used to represent the relative gene expression (13).

Statistical analysis. Each experiment was performed at least three times and values are expressed as the mean ± standard deviation. The statistical data analysis was performed using one-way analysis of variance between groups, and Tukey's multiple-comparisons test was used to compare between pairs of groups by using SPSS 19.0 (IBM Corp., Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

P7 does not affect the morphology of SKOV3 cells. In the presence of P7, the cells grew well and their morphology observed under the inverted microscope was normal compared with that in the control group. P7 had no significant effect on SKOV3 cell morphology within the range of concentrations tested (0.25-16 μM; Fig. 1). These results suggested that, at the concentrations assessed, P7 had no cytotoxic effect on SKOV3 cells.

Effects of P7, bFGF and P7+bFGF on the proliferation of SKOV3 cells. The cell proliferation was expressed as a percentage of the control. Among the P7-treated groups, the inhibition rate of 0.25 μM P7 on SKOV3 cells was 6.47±2.12% and this rate was enhanced with increasing concentrations of P7 (Fig. 2). However, the inhibitory rate between the different groups exhibited no statistically significant difference ($P>0.05$). Overall, the results of the MTT assay indicated that P7 slightly inhibited SKOV3 cell proliferation, but this was not significant (Fig. 2).

Table I. Sequences and amplicon sizes of the oligonucleotide primers used for quantitative polymerase chain reaction.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)
UPA	TGTGAGATCACTCTGGCTTTGGAA	CCTTGGAGGGAACAGACGAG	223
MMP2	AATGCCATCCCCGATAACC	GCTCAGCAGCCTAGCCAGTC	155
MMP9	GGGGGAAGATGCTGCTGTT	GCCGGTCCTGGCAGAAATAG	172
β-actin	CATTGCCGACAGGATGCAG	CTCGTCATACTCCTGCTTGCTG	169

MMP, matrix metallopeptidase; uPA, urokinase-type plasminogen activator.

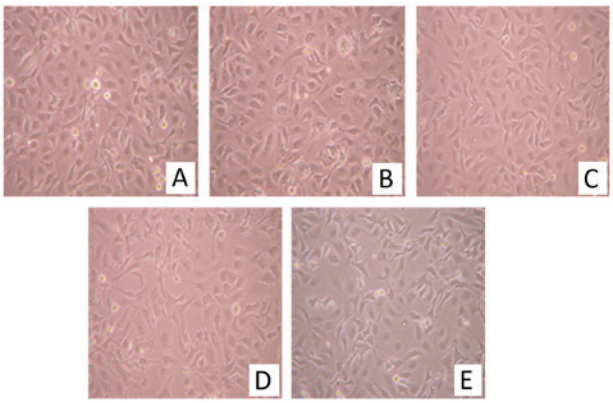

Figure 1. Representative inverted microscopy images indicating that the cell morphology was not significantly affected by P7 at different concentrations (magnification, x400). (A) Control; (B) P7 (0.25 μM); (C) P7 (1 μM); (D) P7 (4 μM); (E) P7 (16 μM).

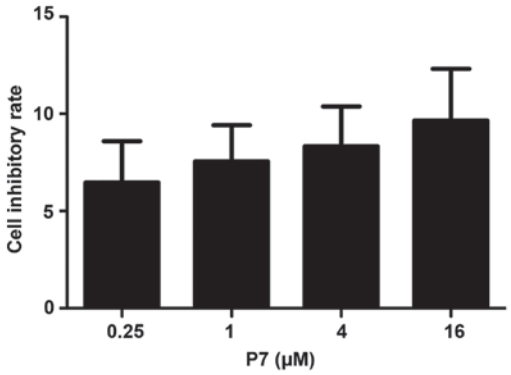

Figure 2. Effect of different concentrations of P7 on cell proliferation. The mean cell inhibitory rate of different concentrations of P7 (0.25, 1, 4 and 16 μM) was 6.47, 7.56, 8.33 and 9.66%, respectively.

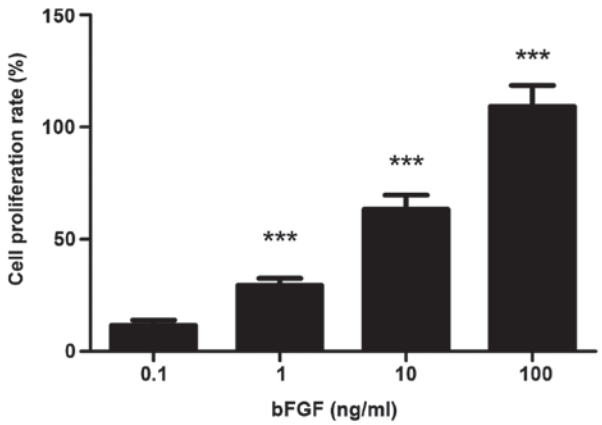

Figure 3. Effect of different concentrations of bFGF on the proliferation of ovarian cancer cells. The mean proliferative rate of SKOV3 cells treated with different concentrations of bFGF (0.1, 1, 10 and 100 ng/ml) was 11.57, 29.40, 63.32 and 109.2%, respectively. ***P<0.001 vs. 0.1 ng/ml. bFGF, basic fibroblast growth factor.

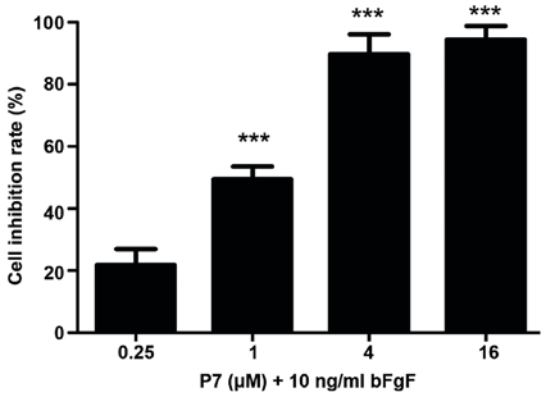

Figure 4. Effect of different concentrations of P7 on bFGF-stimulated cell proliferation. Treatment of 10 ng/ml bFGF-stimulated SKOV3 cells with P7 at concentrations of 0.25, 1, 4 and 16 μM significantly inhibited cell proliferation with mean inhibition rates of 21.83, 49.47, 89.67 and 94.43%, respectively. ***P<0.001 vs. 0.25 μM. bFGF, basic fibroblast growth factor.

In the bFGF-treated groups, the viability of the SKOV3 cells was significantly enhanced in a dose-dependent manner (P<0.001; Fig. 3). When SKOV3 cells were treated with 10 ng/ml bFGF, the proliferation rate reached >60%.

In the P7+bFGF group, P7 significantly reduced the bFGF-stimulated proliferation of SKOV3 cells at concentrations from 0.25 to 4 μM (P<0.001; Fig. 3). In the group treated with 4 μM P7, the mean inhibitory rate of SKOV3 cells was nearly 90% (Fig. 4).

Effects of P7, bFGF and P7+bFGF on the migration of SKOV3 cells. The results of the cell scratch assay indicated that after 24 h, the SKOV3 cells in the bFGF group had almost migrated into the total scratched area, while an obvious gap was still present in the P7+bFGF group (Fig. 5A). Quantitative analysis of cell migration indicated a statistically significant difference between the P7+bFGF group and the bFGF group (P<0.01; Fig. 5B).

Effects of P7, bFGF and P7+bFGF on the expression of uPA, MMP2 and MMP9. RT-qPCR analysis confirmed that

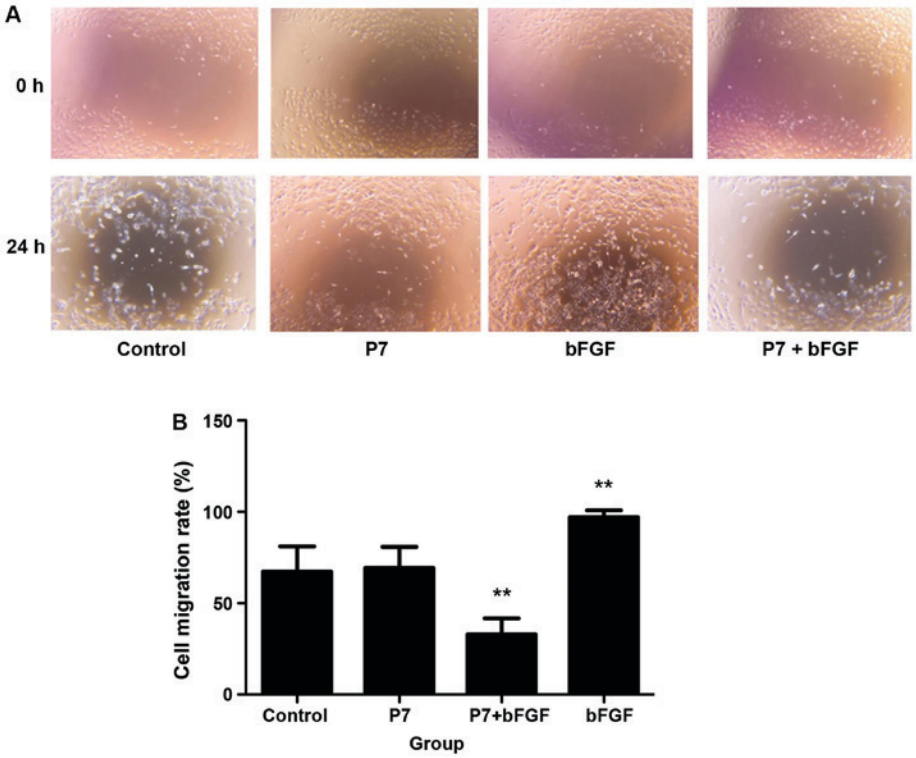

Figure 5. Scratch wound assay. (A) Inverted microscopy images of different groups of SKOV3 cells for comparative analysis of the cell migration (magnification, x100). (B) Quantitative evaluation indicated an obvious difference between the bFGF group, the P7+bFGF group and the control group. However, no significant difference between the P7 group and the control group was identified. **P<0.01 vs. control group. bFGF, basic fibroblast growth factor.

uPA, MMP2 and MMP9 were all expressed in SKOV3 cells. After treatment with P7, the expression of these genes was significantly inhibited. In the bFGF group, the expression of the genes was significantly increased. The expression levels in the P7+bFGF group were low (Fig. 6). In the P7 group, it was demonstrated that P7 had the strongest inhibitory effect on uPA, followed by MMP2, and the weakest inhibitory effect on MMP9. The bFGF group demonstrated that bFGF had the strongest effect to enhance uPA expression, followed by MMP2, and the weakest effect on MMP9 expression.

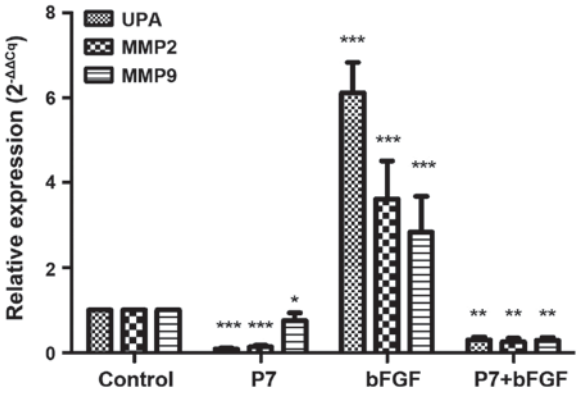

Figure 6. Reverse transcription-quantitative polymerase chain reaction analysis of the expression of uPA, MMP2 and MMP9 in SKOV3 cells treated with P7 and bFGF. The results indicated that the expression rate of the gene was obviously increased by bFGF stimulation and downregulated by P7 and P7+bFGF treatment. *P<0.05 **P<0.01, ***P<0.001 vs. control group. bFGF, basic fibroblast growth factor; MMP, matrix metallopeptidase; uPA, urokinase-type plasminogen activator; Cq, quantification cycle.

Discussion

Epithelial ovarian cancer has been reported to be the leading cause of mortality among gynecologic malignancies worldwide during the last twenty years. Ovarian cancer is characterized by a high degree of malignancy, insidious onset, high invasive capacity and fast growth. Screening based on the detection of cancer antigen (CA)125 and transvaginal sonography did not markedly reduce the mortality rate associated with ovarian cancer (14). Due to the insufficient diagnostic methods for early-onset ovarian cancer, most patients are diagnosed at a late stage. Therefore, chemotherapy is important in the treatment of ovarian cancer, but the adverse effects of drugs and multi-drug resistance affect the therapeutic efficacy of ovarian carcinoma (15,16).

Angiogenesis is required for invasive tumor growth and metastasis. Inhibition of angiogenesis is considered to be a promising approach for antitumor therapy. Bevacizumab is a monoclonal antibody against vascular endothelial growth factor and was approved by the US Food and Drug Administration in February 2004 for the first-line treatment of advanced colorectal cancer; it has been used to successfully treat colorectal cancer and advanced non-small cell lung cancer (7,17). Bevacizumab also has efficacy against recurrent or treatment-resistant ovarian cancer. Combined with paclitaxel, it rapidly reduces serum CA125 levels of patients with advanced chemoresistant ovarian cancer and significantly reduces cancer-associated symptoms (18). bFGF is one of the most potent vascular growth factors, which regulates the expression of various invasion-associated factors, including

uPA and MMPs, to promote the degradation of the extracellular matrix and the destruction of the basal layer to inhibit tumor invasion, metastasis and spread (6). Overexpression of bFGF was reported in the A90 and A121 ovarian cancer cell lines, and cell proliferation was significantly enhanced under exogenous bFGF stimulation (19). The present study also confirmed that bFGF promotes the proliferation of SKOV3 cells, and it significantly increased the amount of viable SKOV3 cells in a dose-dependent manner. This supports the hypothesis that inhibiting tumor cell growth and tumor angiogenesis via a targeting inhibition of bFGF to impair its biological activity may provide a novel approach for the development of drugs (20).

Peptides have become a focus of drug research due to their high activity and specificity, while having relatively few side effects. Phage display technology was used to screen the novel lead peptide P7 from a random peptide library, based on its ability to specifically bind to bFGF (21). The present study indicated that P7 had almost no effect on the morphology, proliferation and migration of SKOV3 cells, but had a significant inhibitory effect on cell proliferation and migration induced by bFGF. It is therefore indicated that P7 has a slight cytotoxic effect on SKOV3 cells, but inhibits SKOV3 cell proliferation and migration by specifically binding to bFGF. This supports the hypothesis that P7 may be utilized as a specific treatment for ovarian cancer.

uPA is a type of serine proteolytic enzyme, and it may activate MMPs and proteolytic enzymes from a variety of precursors in tumor tissue, and degrade extracellular matrix and basement membrane components of the tumor and vessel wall, resulting in an increase of the tumor vessel wall permeability. Fibrinolytic enzymes may stimulate angiogenesis by increasing the activity of vascular endothelial growth factor to promote the growth and spread of tumors (22). Studies have confirmed that the content of uPA is higher in the center of the tumor than that in the normal adjacent tissue, and is correlated with the depth of invasion and metastasis. MMPs are zinc-dependent endopeptidases, and have a high expression and activity in most types of tumor tissue, which promotes cell invasion and migration, and induces angiogenesis via numerous ways, to enhance metastasis of tumor cells. The expression levels of MMP-2 and MMP-9 are associated with the degree of malignancy and angiogenesis of tumors (23). Studies have indicated that MMPs are expressed in ovarian cancer tissues, and their expression level is associated with the clinical stage and prognosis of ovarian cancer (24). The present study indicated that bFGF significantly increases the expression of uPA, MMP-2 and MMP-9 in SKOV3 cells, confirming that bFGF is associated with the invasive ability of ovarian cancer cells. bFGF may promote the degradation of extracellular matrix by upregulating the expression of uPA, MMP-2 and MMP-9, and increase vascular permeability to promote tumor cell proliferation. P7 significantly reduced the expression of these three genes in bFGF-induced SKOV3 cells, which indicates that P7 may specifically inhibit bFGF-induced tumor cell invasion and proliferation by sequestering the increasing expression effect of bFGF on invasion-associated factors. In the present study, it was revealed that P7 had the greatest inhibitory effect on uPA, followed by MMP2 and MMP9. However, the exact mechanisms remain elusive and further studies are required to investigate them.

In conclusion, the present study indicated that P7 inhibits the proliferation and migration of ovarian cancer cells induced by bFGF, and downregulates the expression of invasion-associated factors, including uPA, MMP-2 and MMP-9. It was revealed that treatment with P7 inhibits the proliferation, migration and invasion of bFGF-induced human ovarian cancer SKOV3 cells *in vitro*. Due to its prominent effects, it is recommended that the use of P7 as an anticancer agent for ovarian tumors should be pursued.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WQ initiated and guided the study, and revised the manuscript. QC and ZY performed experiments, and wrote the manuscript. XC and LS contributed to data collection and processing. All authors have read and approved the final version of the manuscript.

Ethical approval and informed consent

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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