Expression of urokinase plasminogen activator and plasminogen activator inhibitor type-1 in ovarian cancer and its clinical significance

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Abstract. The urokinase plasminogen activator system, which consists of urokinase plasminogen activator (uPA), plasminogen activator inhibitor type-1 (PAI-1) and urokinase plasminogen activator receptor (uPAR), plays an important role in tumor invasion and metastasis, and it may be a potential diagnostic biomarker and therapeutic target in cancer. It has been found that the expression of uPA and PAI-1 in ovarian cancer is related to clinical pathologies, while their effects on the biological behavior of tumor cells and their clinical significance are still unknown. In this study, 100 tissue samples (60 samples from malignant tumors, 20 from benign tumors and 20 from controls) and 147 blood samples (49 samples each from patients with malignant tumors, benign tumors and control group, respectively) were analyzed. The positive expression levels of uPA and PAI-1 in the malignant tumor samples and their serum concentrations in the malignant group were all significantly higher than these levels in the benign tumors and controls. In addition, the levels in patients with poorly differentiated and stage III-IV cancers, cancers with metastases as well as residual tumors >2 cm after surgery, were all obviously increased, consistent with their concentrations in serum. The Cox model analysis showed that expression of uPA at the transcription level had significant associations with prognosis. In addition, uPA greatly enhanced the abilities of cell invasion, migration and adhesion through its overexpression in SKOV3 cells. Collectively, our results showed that uPA and PAI-1 play important roles in ovarian cancer development; therefore, their expression in tissues and their concentrations in serum would greatly assist the diagnosis and prediction of the prognosis in ovarian cancer.

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Introduction

Ovarian cancer is one of the three most common female reproductive tract cancers and it is the most lethal gynecological cancer (1,2). Due to few early symptoms, most patients are diagnosed with an advanced stage, accompanied by metastasis. It has been proven that the urokinase plasminogen activator system, consisting of urokinase plasminogen activator (uPA), urokinase plasminogen activator receptor (uPAR) and plasminogen activator inhibitor type-1 (PAI-1), plays an important role in tumor invasion and metastasis, and may be a potential diagnostic biomarker and therapeutic target. PA is one of the most important proteolytic enzymes in extracellular matrix dissolutions, and participates in many pathophysiological behaviors, such as cell metastasis, tissue repair and vasculature, in particular, tumor invasion and migration (3,4). PAI-1, a serine protease inhibitor and a member of the serpin family, is an important regulator of fibrinolysis, and plays a role in signal transduction, cell invasion and metastasis (5,6). Previous studies have shown that the expression of uPA and PAI-1 in ovarian, breast and colorectal cancer was closely related to clinical pathologies (7-9).

It has been demonstrated that the expression of uPA and PAI-1 is normally associated with advanced stage and distant metastasis in ovarian cancer, while their influence on the biological behavior of tumor cells and their clinical importance are still unknown. In this study, the expression of uPA and PAI-1 in tumors and their concentrations in peripheral blood were measured to analyze their relationship with clinical pathologies in ovarian cancer. In addition, the changes in the biological behavior of tumor cells caused by the overexpression of uPA in ovarian epithelial carcinoma cells was investigated. This study aimed to illustrate the clinical importance of uPA and PAI-1 in ovarian cancer.

Materials and methods

Samples. All ovarian tissue samples were collected from patients after surgery at the Department of Gynecologic Oncology, Affiliated Tumor Hospital of Guangxi Medical University and were evaluated by pathologists. Of the 60 cases presenting with malignant ovarian tumors, 38 were serous

cystadenocarcinomas, 12 were mucinous cystadenocarcinomas and 10 were poorly differentiated adenocarcinomas according to criteria of the World Health Organization (WHO, 1973); and 30 cases were stages I-II and 50 cases were stages III-IV according to FIGO standard (2004). The patients with ovarian cancer were aged 30 to 66 years (average age, 48.3), and received cisplatin-based chemotherapy after cytoreductive surgery. They were followed up for 2.4 to 62.16 months (mean, 41.10). There were 20 cases of benign ovarian tumors, including 17 serous cystadenomas, 3 mucinous cystadenomas, and these patients were aged 13 to 60 years (average age, 39.5). Twenty normal ovarian tissues were excised from patients undergoing myomectomy or total hysterectomy, following receipt of informed consent and were confirmed to be normal by a pathologist. These patients were aged 48-67 years (average age, 48.7). The ovarian sample for cDNA cloning of uPA was a mucinous adenocarcinoma diagnosed by pathologists. The study was endorsed by the Ethics Committee of the Guangxi Medical University. All patients received an explanation concerning the aims of the study and provided signed informed consent. All of the samples were collected from primary lesions during surgery and stored in a liquid nitrogen tank. The stored samples were then ready for mRNA isolation and histopathological examination.

Blood samples were collected from either patients who were diagnosed with malignant/benign ovarian tumors or healthy females undergoing routine physical examinations at the Department of Gynecologic Oncology, Affiliated Tumor Hospital of Guangxi Medical University. The malignant group consisted of 49 cases with ovarian cancer, including 27 cases of serous cystadenocarcinoma, 16 of mucinous cystadenocarcinoma and 6 of undifferentiated carcinoma. Among these, 15 cases were stage I-II and 34 cases were stage III-IV according to FIGO standard (2004). The age of the patients ranged from 16 to 67 years (average age, 44.6), and all of the patients were followed up for 2.4 to 62.16 months (mean, 41.10). The benign group consisted of 49 cases, including 30 serous cystadenomas, 19 mucinous cystadenomas. These patients ranged in age from 14 to 64 years (average age, 35.6). The control group consisted of 49 healthy females aged from 25 to 53 years (average age, 43.4). All blood samples were taken prior to any treatments, and then 2 ml of blood was centrifuged at 3000 rpm for 5 min, and the supernatant was maintained at -80˚C.

Streptavidin-biotin complex assay. Protein expression of uPA and PAI-1 was measured by a streptavidin-biotin complex (SABC) assay. The monoclonal antibodies of the two proteins were purchased from Wuhan Boster Bio-Engineering, Inc. (Wuhan, China), and the SABC kit was purchased from Maxim Biotech, Inc. (Fuzhou, China). For each assay, the positive image supplied by the company and the result without primary antibody incubation were used as positive and negative control, respectively. The images were reviewed in a blinded manner by two experienced pathologists. The determination of staining intensity was as following: the cytoplasm of ovarian cancer cells exhibiting brown granular staining (Fig. 1C and D) was considered positive staining and samples showing the absence of staining (Fig. 1A and B) were considered negative. The intensity of protein expression was identically related to the rate of positive cells. The cells with cytoplasmic or membranous staining showing dark brown granules were determined to exhibit strong positivity (score 3). Cells staining light brown indicated weakly positive (score 1) staining, and cells with no brown granules were scored 0. Intensity between strong positivity and weak positivity was considered as medium positive (score 2) intensity. The positive staining of cells was determined by the number of positive cells vs. the number of total cells at high magnification. A percentage of <5% cells was scored as 0, 6-25%, 1; 26-50%, 2; 51-5%, 3 and >75%, 4. The product of the staining intensity and the positive rate of cells in each field was determined to be the immunity score, and average score of 5 visions in each section was the final immunity score. A final immunity score ranging from 0-2 was determined as negative and a score ≥2 was determined as positive.

RT-PCR. The expression of uPA and PAI-1 at the transcription level was measured by RT-PCR. Total RNA was isolated using TRIzol reagents (Gibco, USA) and first strand cDNA was synthesized using the M-MuLV system from 2 μg RNA. Primers were designed according to the nucleotide sequence that had been deposited in the GenBank database. RT-PCR gene-specific primers were the following: for uPA forward primer, 5'-agaattcaccaccatcgaga-3' and reverse primer, 5'-atc agcttcacaacagtcat-3'; for PAI-1 forward primer, 5'-gctcagaag caaccgggtg-3' and reverse primer, 5'-gcaaagatggcagcctgcc-3'. β-actin was used as the control and the forward primer was 5'-ctccatcctggcctcgctgt-3' and the reverse primer, 5'-gctgtc accttcaccgttcc-3'. Polymerase chain reaction amplification was performed using the following protocol: initial denaturation at 94˚C for 5 min, followed by a variable number of 35 cycles: 94˚C for 30 sec, specific annealing temperature for 30 sec, elongation at 72˚C for 45 sec; then a final elongation at 72˚C for 5 min. PCR products were visualized on 2% agarose gels containing ethidium bromide and photographed using an imaging system. PCR products of uPA and PAI-1 were purified and sequenced.

Determination of the concentration of uPA and PAI-1 in serum. The concentration of uPA and PAI-1 in serum was determined by ELISA. The ELISA kit for uPA was purchased from AssayPro, Inc. (St. Charles, MO, USA), and the kit for PAI-1 was purchased from Sunbio, Inc. (Shanghai, China). Standard curves were established using CurveExpert 1.3 software through absorbance value at OD_{450} vs. the logarithm of the concentration of standard uPA and PAI-1. The equation of standard curves for uPA and PAI-1 were $y = -0.043 + 0.587x - 0.045x^2$ and $y = 0.312 + 0.037x - 0.0001x^2$, respectively.

Transfection and selection of stable cell lines. cDNA of uPA cloned from tissues of epithelial ovarian cancer by PCR was subcloned into *Bgl*II sites of pGEM-T Easy Vector (Promega) for sequencing. Then the *Xho*I and *Hin*dIII fragments were inserted into *Xho*I and *Hin*dIII-digested pcDNA3.1/myc-his(-) B vector to generating uPA recombinant expression system and the insert was confirmed by sequencing. The pcDNA3.1/myc-his(-) B-uPA (+) was transfected into SKOV3 cells using Lipofectamine 2000 (Invitrogen, USA). Individual clones were screened for G418-based induction of recombinant

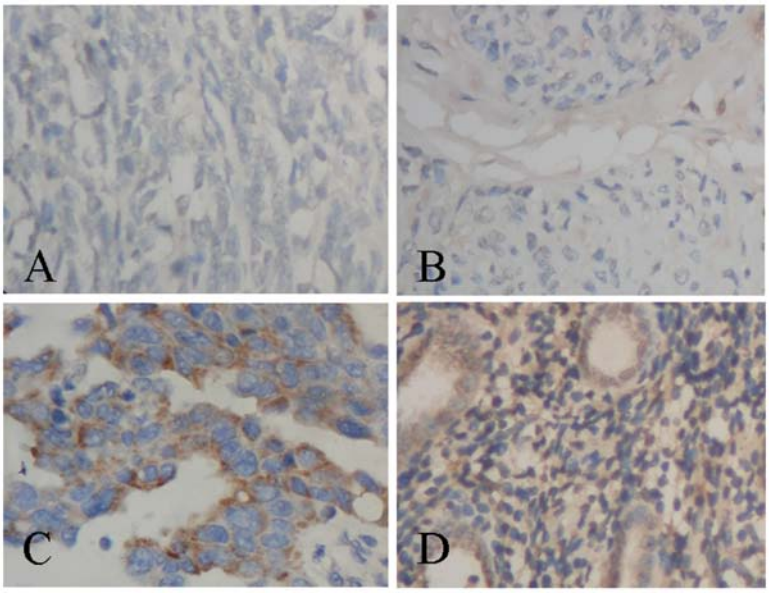

Figure 1. Immunohistochemical staining images of uPA and PAI-1 expression in different ovarian tissues (x40). (A) Expression of uPA (-) in benign ovarian tumors. (B) Expression of uPA (-) in normal ovarian tissues. (C) Expression of uPA in malignant ovarian tumors (+). (D) Expression of PAI-1 in malignant ovarian tumors (+).

Table I. The expression of uPA and PAI-1 at both the protein and mRNA levels in ovarian tissues.

Ovarian tissue samples	No.	Positive expression rates of uPA, n (%)		Positive expression rates of PAI-1, n (%)	
		Protein	mRNA	Protein	mRNA
Malignant ovarian tumor tissue	60	41 (68.3)	48 (80.0)	45 (75.0)	46 (76.7)
Benign ovarian tumor tissue	20	4 (20.0)	7 (35.0)	6 (30.0)	3 (15.0)
Normal ovarian tissue	20	3 (15.0)	5 (25.0)	5 (25.0)	3 (15.0)

uPA expression by western blot analysis using anti-uPA antibody (Pierce, USA).

Methods to determine the cell biological behavior. Cell growth inhibition was determined by MTT assay, with 3 replications. The cell proliferation rate was determined using the colony forming assay as reported (5). Cell cycle distribution was evaluated by flow cytometry, and the proportion of cells in the G1, G2 and S phases of the cell cycle was analyzed using Multicycle software. Cell invasion *in vitro* was measured by Matrigel invasion assay, and the kit was purchased from the Biological Centre of Peking University (Beijing China). Cell migration *in vitro* was measured by Transwell migration assay, and the kit was purchased from Corning Costar (Cambridge, MA, USA). Cell adhesion *in vitro* was measured by an adhesion assay, and the kit was purchased from the Biological Centre of Peking University. All steps were carried out according to the manufacturer's instructions.

Data analysis. The data were analyzed by SPSS 13.0 software. The result of ELISA was presented as means ± SD. The measurement data were analyzed using one-way ANOVA, complemented with the Kruskal-Wallis tests. The statistical data were analyzed with the χ^2 tests; comparison within group was analyzed using the t-test. $P<0.05$ was considered to indicate a statistical significant difference. Prognosis was analyzed using the Cox model.

Results

Expression of uPA and PAI-1 and their relationship with clinicopathological factors and prognosis in ovarian cancers. As shown in Table I, the positive expression rates of uPA and PAI-1 at both the protein and mRNA levels in malignant ovarian tumors were obviously higher than levels in benign tumors and normal tissues, and showed a statistically significant difference (P=0.00 and 0.00, respectively), while the rates in benign tumors and that in normal tissues showed no statistically significant difference (P>0.05).

The expression rates of uPA and PAI-1 and their relationship with clinicopathological factors were evaluated. As shown in Table II, the positive expression rates of uPA and PAI-1 at both the protein and mRNA levels were increased in poorly differentiated tumors in comparison with rates in high or medium differentiated tumors (P=0.00, 0.03 and P=0.00, 0.01, respectively), while the rates within pathological types showed

Table II. Relationship between expression of uPA and PAI-1 and clinicopathological factors in malignant ovarian cancers.

		uPA (+), n (%)		PAI-1 (+), n (%)	
Clinicopathological factors	No.	Protein	mRNA	Protein	mRNA
Pathological type					
Serous adenocarcinoma	38	26 (68.4)	31 (81.2)	29 (76.3)	28 (73.7)
Mucinous adenocarcinoma	12	8 (66.7)	8 (66.7)	9 (75.0)	9 (75.0)
Poorly differentiated adenocarcinoma	10	7 (70.0)	9 (90.0)	7 (70.0)	7 (70.0)
Histological grade					
High, medium differentiated	22	9 (40.9)	13 (59.1)	13 (59.1)	13 (59.1)
Poorly differentiated	38	32 (84.2)	35 (92.1)	32 (84.2)	33 (86.8)
Clinical stage					
I-II	25	10 (40.0)	12 (48.0)	13 (52.0)	15 (60.0)
III-IV	35	31 (88.6)	33 (85.7)	32 (91.4)	31 (88.6)
Peritoneal fluid (U/ml)					
<500	36	25 (69.4)	28 (77.7)	26 (72.2)	28 (77.7)
≥500	24	16 (66.7)	20 (83.3)	19 (79.2)	18 (75.0)
Cancer metastasis					
Lymph node	35	31 (88.6)	33 (85.7)	32 (91.4)	31 (88.6)
No lymph node	25	10 (40.0)	12 (48.0)	13 (52.0)	15 (60.0)
Distant[a]	11	10 (90.9)	11(100.0)	10 (90.9)	10 (90.9)
No distant[a]	49	31 (65.9)	37 (75.5)	35 (71.4)	36 (73.4)
Size of residual tumors (diameter/cm)					
No residual tumor	28	14 (50.0)	17 (60.7)	14 (50.0)	18 (64.3)
Residual tumors ≤2 cm	16	12 (75.0)	15 (93.7)	13 (81.2)	15 (93.7)
Residual tumors >2 cm	16	15 (93.7)	16(100.0)	15 (93.7)	16 (100.0)

[a]Metastasis to lung, brain, bone and liver.

Table III. Analysis of the prognostic factors by the Cox proportional risk model.

							95% CI	
Clinicopathological factors	B	ES	Wald	df	Sig	Exp (B)	Lower	Upper
uPA	-2.203	0.679	10.517	1	0.001	0.11	0.029	0.418
PAI-1	-0.133	1.236	0.012	1	0.915	0.876	0.078	9.871
Clinical stage	0.341	0.623	0.299	1	0.584	1.406	0.414	4.773
Pathological type	-15.397	294.646	0.003	1	0.958	0.000	0.000	1.306
Pathological differentiation	-0.836	0.417	4.013	1	0.045	0.434	0.191	0.982
Lymph node metastasis	0.448	0.705	0.404	1	0.525	1.565	0.393	6.227
Age	0.007	0.03	0.061	1	0.804	1.007	0.95	1.068
Residual tumor after surgery	0.937	0.556	2.844	1	0.092	2.553	0.859	7.589
Distant metastasis	2.877	0.966	8.876	1	0.003	17.753	2.676	117.793

no significant difference (P=0.97, 0.76 and P=0.38, 0.96, respectively). The rates in stage III-IV cancers were apparently higher than that in stage I-II cancers (P=0.00, 0.00 and P=0.00, 0.01, respectively), and the positive expression rates in cancers with metastasis or distant metastasis were also higher than the rates in patients with no metastasis (P=0.00, 0.00 and P=0.00, 0.01, respectively). In addition, compared with the positive expression rates in tissues excised from patients with residual tumors (>2 cm) after surgery, the positive expression rates of uPA and PAI-1 were notably decreased in tissues excised from patients without residual tumors after surgery (P=0.00, 0.00 and P=0.03, 0.00, respectively).

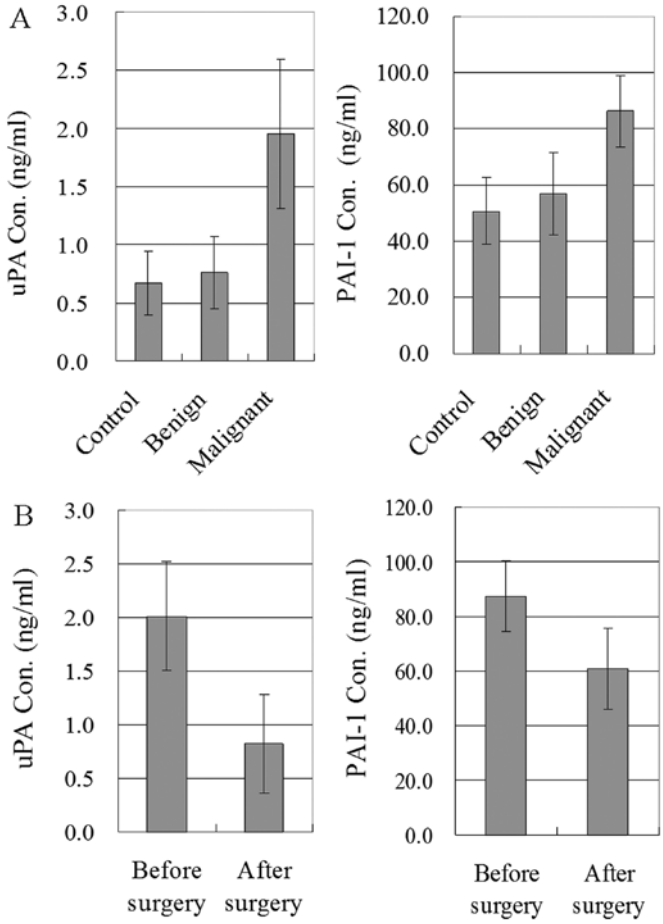

Figure 2. Concentration of uPA and PAI-1 in serum. (A) The concentrations of uPA and PAI-1 in peripheral blood of normal individuals (control) and patients with ovarian tumors. (B) The concentrations of uPA and PAI-1 in the serum of 22 ovarian cancer patients before and after surgery.

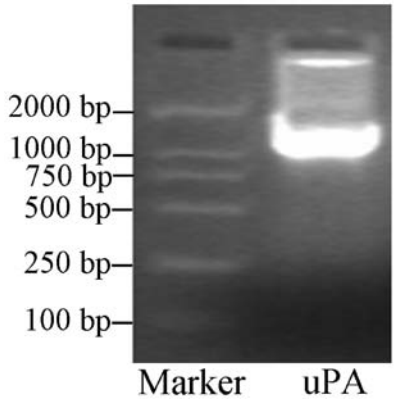

Figure 3. cDNA cloning of uPA from the tissue of epithelial ovarian cancer by PCR. The products were visualized on 1.6% agarose gels.

It was also revealed that the positive expression rate of uPA at the transcription level was an independent factor for unfavorable prognosis. As shown in Table III, according to the COX multivariate analysis (χ^2=31.093, P=0.001), among the factors of age, pathological type, pathological differentiation, stage, lymph node metastasis, distant metastasis, residual size after surgery, expression of uPA and PAI-1, we found that pathological differentiation, distant metastasis and the expression of uPA at the mRNA level were significantly associated with prognosis.

Concentrations of uPA and PAI-1 in the serum of patients prior to surgery and their significance in ovarian cancers. The concentrations of uPA and PAI-1 in the peripheral blood of patients with malignant ovarian tumors were markedly higher than the levels in patients with benign ovarian tumors and that of normal controls (P<0.001) (Fig. 2A). The levels of uPA and PAI-1 in the serum of 22 patients with epithelial ovarian cancer following surgery were apparently decreased compared to that prior to surgery (P<0.0001) (Fig. 2B). These results suggest that the concentrations of uPA and PAI-1 in the peripheral blood of patients may be a biomarker for diagnosing and predicting prognosis in ovarian cancer.

As expected, we found that for ovarian cancer patients before surgery, the concentrations of uPA and PAI-1 in the blood of patients with poorly differentiated carcinomas (2.1780±0.1302 and 91.84±2.652 ng/ml, respectively) were notably higher than the concentrations in patients with high and/or medium differentiated carcinomas (1.6280±0.1351 and 78.98±3.111 ng/ml, respectively) (P=0.0180 and 0.0085), although there was no difference between the pathological types (P=0.2683 and 0.1182). We also found that the levels of uPA and PAI-1 in the serum of patients with stage III-IV cancers (2.0990±0.1102 and 88.52±2.453 ng/ml, respectively)

Table IV. Determination of tumor malignancy according to the levels of uPA and PAI-1 in serum of patients prior to surgery.

Factors	Pathological stage	Sensitivity (%)	Specificity (%)	Accuracy (%)	Positive predictive value (%)	Negative predictive value (%)	Positive likelihood ratio	Negative likelihood ratio
uPA	I-II	73.0 (11/15)	89.8 (44/49)	77.8 (88/113)	55.0 (11/22)	95.6 (87/91)	7.16	0.3
	III-IV	93.3 (28/30)	89.8 (44/49)	89.8 (115/128)	71.8 (28/39)	97.7 (87/89)	9.15	0.07
	Total	86.7 (39/45)	89.8 (44/49)	88.1 (126/143)	88.1 (39/50)	93.5 (87/93)	8.50	0.15
PAI-1	I-II	73.0 (11/15)	93.9 (46/49)	77.8 (88/113)	61.1 (11/18)	95.8 (91/95)	11.97	0.28
	III-IV	76.7 (23/30)	93.9 (46/49)	89.1 (114/128)	76.7 (23/30)	92.9 (91/98)	12.60	0.25
	Total	75.6 (34/45)	93.9 (46/49)	87.4 (125/143)	84.7 (39/46)	89.2 (91/102)	12.30	0.26

were increased compared to levels in patients with stages I-II (1.6170±0.1344, 80.31±2.588 ng/ml and P=0.0117, 0.0045, respectively); and the levels in patients with lymphatic metastases (2.2250±0.1588 and 93.87±3.580 ngml, respectively) were higher than those in patients with no lymphatic metastases (1.7790±0.1057 and 82.13±1.983 ng/ml, respectively) (P=0.0236 and 0.0035). The levels in patients with distant metastases (including metastases to liver or lung or spleen or bones) (2.1070±0.1483 and 90.03±3.929 ng/ml, respectively) were much higher than levels in patients with no metastases (1.7320±0.1033 and 33.44±1.974) (P=0.0338 and 0.01013). However, no relationship was noted between peritoneal fluid amounts (>500 ml vs. ≤500 ml) and the serum concentrations of uPA and PAI-1 (2.1640±0.1877 vs. 1.8790±0.1036, 89.90±3.531 vs. 84.99±2.064) (P=0.1824 and 0.2423).

The levels of uPA and PAI-1 in the serum of ovarian cancer patients before surgery may be a useful marker to identify whether the tumor is benign or malignant. As shown in Table IV, we found that values of uPA and PAI-1 at 1.2161 ng/ml (mean ± 2SD) and 74.76 ng/ml (mean ± 2SD) was the cutoff point; patients with serum levels of uPA and PAI-1 higher than these values were diagnosed with malignant ovarian tumors.

Influence on the biological behavior of tumor cells mediated by the expression of uPA. The construct of pcDNA3.1/myc-his(-) B-uPA(+) for recombinant expression of uPA was transfected into ovarian cancer SKOV3 cells to study its influence on the biological behavior of tumor cells caused by uPA. cDNA of uPA was cloned from the tissue of epithelial ovarian cancer by PCR, and the length was ~1300 bp in accordance with a standard DNA marker (Fig. 3), consistent with the theoretical length of uPA. The cDNA was subcloned into the pGEM-T Easy Vector and sequenced to have 100% identity to the sequence of uPA which was previously deposited in GeneBank. Then the *Xho*I and *Hin*dIII fragments were inserted into *Xho*I and *Hin*dIII-digested pcDNA3.1/myc-his(-) B vector to generate uPA recombinant expression system, and the insert was confirmed again by sequencing. Finally, the pcDNA3.1/myc-his(-) B-uPA(+) construct primer DNA was transfected into SKOV3 ovarian cancer cells. As shown in Fig. 4, compared with pcDNA3.1/myc-his(-) B-uPA(-)/SKOV3 [uPA(-)/SKOV3] cells and normal SKOV3 cells, uPA was only expressed in pcDNA3.1/myc-his(-) B-uPA(+)/SKOV3 [uPA(+)/SKOV3] cells.

The influence on the biological behavior of SKOV3 cells caused by the increased expression of uPA was as follows: i) The expression of uPA in SKOV3 cells had less contribution to cell growth (Fig. 5). Compared to normal SKOV3 cells and uPA(-)/SKOV3 cells, the uPA(+)/SKOV3 cells proliferated more rapidly, but with no statistical significant difference between them (P=0.402 and 0.463, respectively). ii) Based on the results of soft agar colony formation, we found that more colonies were formed by uPA(+)/SKOV3 cells compared to those formed by normal SKOV3 and uPA(-)/SKOV3 cells (Table V), and the difference between them was significant (P<0.05). iii) As shown in Fig. 6 and Table VI, the percentage of cells in the S phase of the cell cycle in uPA(+)/SKOV3 cells was apparently more than that in the uPA(-)/SKOV3 and normal SKOV3 cells (P<0.05); meanwhile, the percentage of cells in the G0-G1 phases in uPA(+)/SKOV3 was decreased compared to that in uPA(-)/SKOV3 and normal SKOV3 cells, but the difference was not significant (P>0.05). Furthermore, analysis of the S-phase fraction (SPF) and proliferation index (PI) revealed that the SPF and PI of uPA(+)/SKOV3 cells were both higher than these values in uPA(-)/SKOV3 and normal cells (P<0.05) (Table VI). iv) As shown in Table VII, the invasive, metastatic and adhesive abilities of the uPA(+)/SKOV3 cells were all notably higher than those of uPA(-)/SKOV3 and

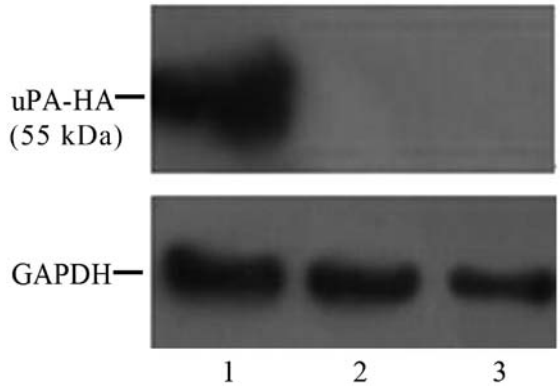

Figure 4. Detection of protein expression of uPA in SKOV3 cells by western blotting. Lane 1, protein extracted from uPA(+)/SKOV3 cells; lane 2, protein extracted from uPA(-)/SKOV3 cells; lane 3, protein extracted from normal SKOV3 cells. Primary antibody, anti-uPA antibody (Pierce); dilution, 1:3,000.

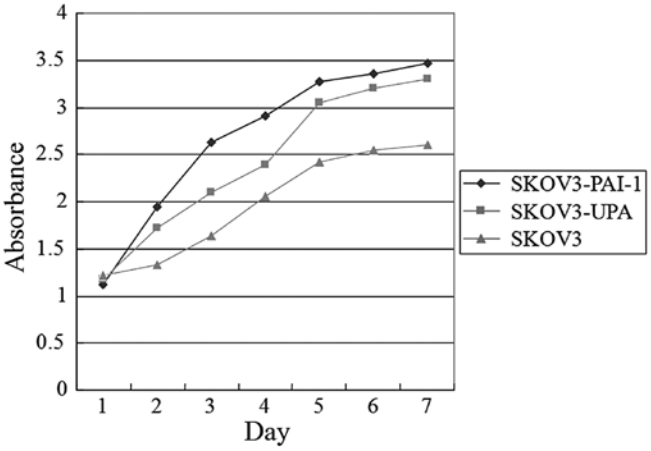

Figure 5. The cell growth curve of SKOV3 cells before and after transfection with uPA and PAI-1.

Table V. Influence on cell colony formation mediated by the expression of uPA.

	Soft agar colony formation (no.)		
Replication	uPA(+)/SKOV3	SKOV3	uPA(-)/SKOV3
1	12	9	8
2	17	7	6
3	20	6	9
Mean ± SD	16.3±4.04	7.3±1.5	7.7±1.5

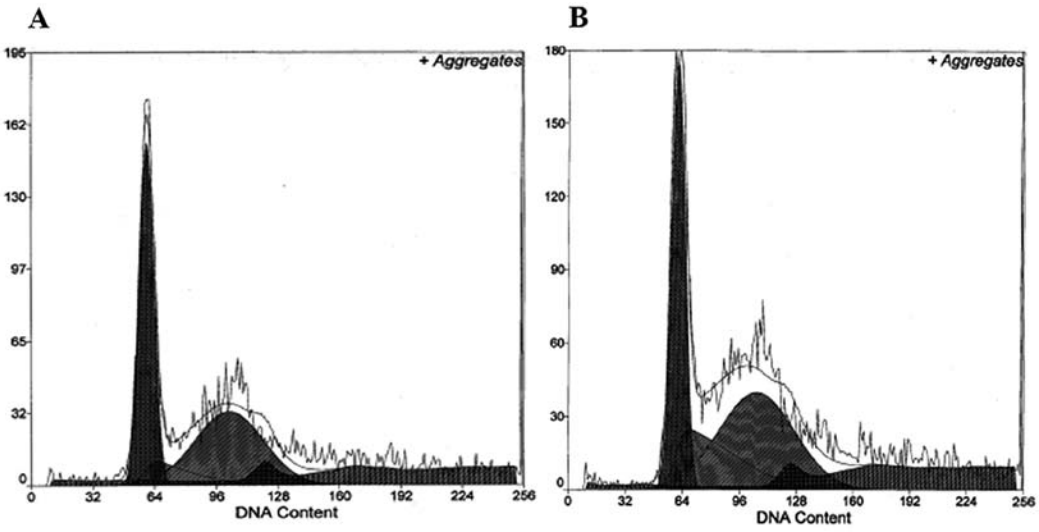

Figure 6. Detection of the cell cycle phase of uPA(-)/SKOV3 cells (A) and uPA(+)/SKOV3 cells (B) by flow cytometry.

Table VI. Analysis of the percentage of SKOV3 cells in the S, G0-G1, G2-M phases of the cell cycle and their S-phase fraction (SPF) and proliferation index (PI).

Cells	S%	G0-G1 (%)	G2-M (%)	PI	SPF
uPA(+)/SKOV3 cells	20.4 (P=0.019)[a]	35.2 (P=0.179)[a]	44.3 (P=0.454)[a]	64.7±2.46 (P=0.041)[a]	20.4±1.35 (P=0.001)[a]
uPA(-)/SKOV3 cells	12.67	40.7	47.60	53.9±2.30	11.67±0.97
Normal SKOV3 cells	11.43	43.0	45.57	56.8±3.87	10.43±1.10

[a]Compared with normal SKOV3 cells.

Table VII. The influence on ability of cell invasion, metastasis and adhesion mediated by the expression of uPA.

	Absorbance (mean ± SD)		
Cells	Invasive ability	Metastatic ability	Adhesive ability
uPA(+)/SKOV3 cells	2.29±0.041	2.140±0.078	0.109±0.022
uPA(-)/SKOV3 cells	1.77±0.071	0.774±0.056	0.029±0.009
Normal SKOV3 cells	1.84±0.088	0.853±0.077	0.031±0.006

normal SKOV3 cells, and the differences were statistically significant (P=0.0002, 0.0001 and 0.0049, respectively).

Discussion

The process of metastasis in malignancies involves numerous steps. It is involved in the dissolution of the extracellular matrix and basement membrane components mediated by numerous proteolytic enzymes. It has been proven that the uPA system plays an important role in many processes of pathophysiology, such as cell differentiation, migration, tissue reconstruction and matrix dissolution. Activated specifically by uPA, the plasminogen can convert to active plasmin which finally results in the dissolution of the extracellular matrix and the components of the basal membrane (fabrin, fibronectin, proteoglycans and laminin). Thus, overexpression of uPA is considered related to tumor invasion and metastasis (10). In this study, we found that the positive expression rates of uPA and PAI-1 in ovarian cancers and poorly differentiated carcinomas were notably higher than the rates in benign ovarian tumors and normal ovarian tissues, with no difference between the latter two. Meanwhile, the concentrations of uPA and PAI-1 in the peripheral blood of patients with malignant ovarian tumors were also obviously more than these in patients with benign tumors or healthy females. In addition, the levels of uPA and PAI-1 in peripheral blood of patients after surgery were decreased markedly compared to levels before surgery. These results were consistent with previous studies (11-13),

indicating that the overexpression of uPA and PAI-1 is closely related to aggressive biological behaviors and may be key parameter for identifying tumor type (malignant or benign). The consistency of uPA and PAI-1 expression in tissues and their levels in peripheral blood can be explained as follows. Firstly, uPA and PAI-1 possibly regulate each other and may co-act in the generation and development of ovarian cancers since the expression of the two proteins was correspondingly upregulated in malignant ovarian tumors. It has been previously confirmed that uPA and PAI-1 can regulate each other; the binding of inactive urokinase with uPAR located on the surface of tumor cells leads to a conversion from uPAR to active uPA which results in a conversion from plasminogen to plasmin. Then plasmin gives rise to the dissolution of the extracellular matrix, and finally facilitates cell metastasis (14). Secondly, as an inhibitor of uPA produced by ovarian cancer cells, PAI-1 can bind to uPA/uPAR specifically to form a complex. By internalization and degradation, the complex results in a heterogeneous distribution of proteolytic activities on the surface of cells, and this eventually promotes cell proliferation, invasion and metastases. After this process is completed, uPAR recycles back to the cell surface, forming a complex (15). Regardless of the mechanism, the consistency of uPA and PAI-1 expression in tissues and their amounts in blood confirmed their importance in ovarian cancers. In addition, we also found that the concentrations of uPA and PAI-1 in serum were an accurate diagnostic marker for malignant ovarian tumors, and their total specificity and sensitivity were 89.8 and 86.7% and 93.9 and 75.6%, respectively, even up to 73.0 and 89.8% and 73.0 and 93.9% in stage I-II patients.

We also discovered that for uPA and PAI-1, their positive expression rates in tissues and their concentrations in serum of stage III-IV patients with ovarian cancer and patients having residual tumors >2 cm after surgery were all obviously higher than these values in stage I-II patients and patients with residual tumors ≤2 cm or without residuals. This result was basically consistent with previous studies (11,12). The positive expression rates of uPA and PAI-1 at the mRNA level in tissues and their concentrations in the serum of patients with distant metastases were apparently higher than those in patients with no metastases, suggesting that uPA and PAI-1 are related to cell invasion and metastasis. This finding agrees with the results reported by Schmalfeld *et al* (16). Upon the upregulation of expression in tissues accompanied by the activation of activities, uPA and PAI-1 also facilitate tumor cell implantation and tumor matrix restructuring (7). Thus, on the basis of previous studies and our new findings, we conclude that uPA and PAI-1 are diagnostic factors for tumor invasion and metastases.

The relationship between prognosis and the expression of uPA and PAI-1 in ovarian cancers is controversial. In this study, the COX proportional hazards model revealed that the positive expression rate of uPA at the mRNA level is an independent factor for prognosis. The survival rate of ovarian cancer patients with high expression of uPA at the mRNA level was notably decreased, which was confirmed by previous research (17,18).

The constructed recombinant expression vector of uPA was transfected into SKOV3 cells to investigate the influence on the biological behaviors such as cell invasion and metastasis of ovarian cancer cells mediated by the expression of uPA. Firstly, the expression of uPA in SKOV3 cells markedly enhanced colony formation. Secondly, the SPF and PI of the cell cycle in uPA(+)/SKOV3 cells were evidently higher than these values in control cells, suggesting that the ability of cell proliferation and the degrees of malignancy were both elevated as mediated by the expression of uPA. This result was consistent with a previous study (9) that reported that the metastatic ability of malignant melanoma cells was enhanced markedly after cells were transfected by pro-uPA recombinant expression vector.

In this study, the adhesive ability to a culture plate of uPA(+)/SKOV3 cells was significantly increased compared with that of control cells, indicating that uPA may play a role in cell adhesion. In addition, the expression of uPA elevated the ability of cell invasion and migration in SKOV3 cells, consistent with a previous study (19) demonstrating that the expression of uPA in PC3 kidney cancer cells of mice enhances cell migration, indicating that uPA is closely related to cell invasion in ovarian cancer cells. The mechanism involved in uPA-mediated cell invasion can be explained (20). Cell migration is defined as locomotion of a cell over an ECM substratum, and involves extension and binding of integrins and other adhesion receptors to their ECM ligands at the leading cell edge and the dissociation of integrin/ligand complexes at the trailing edge (21). The complex of uPA, uPAR and vitronectin can regulate the interaction between uPAR and integrin, promote cell adhesion at the leading edge, and finally lead to cell migration. Meanwhile, catalyzed by uPA, plasminogen is converted to plasmin, resulting in the dissociation of ECM protein/ligand complexes, leading to cell shrinkage at the trailing edge (22). Additionally, other important enzymes are also needed to assist tumor cells to penetrate the ECM during cell migration and invasion, and several are regulated by uPA. Among others, matrix metalloproteinases (MMPs) are activated by plasmin which is regulated by the binding of uPA with uPAR (13,14). In addition, uPAR condenses uPA on cell surface via binding to the single-strand uPA (Scu2PA) with high affinity, which eventually increases the chance of conversion from plasminogen to plasin (22-24). In turn, plasin activates the production of single-strand uPA, and these processes form a continuous cycle.

In conclusion, tumor invasion and metastasis are complicated biological processes regulated by many different factors such as proteolytic enzymes, ability of cell adhesion, the microenvironment, the overexpression of oncogenes and the silencing of tumor-suppressor genes. Among all these factors, it is no doubt that the uPA system is one of the important mechanism involved in tumor invasion and metastasis, and further research on uPA needs to be carried out to clarify its importance in ovarian cancers.

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