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Expression of urokinase plasminogen activator and its receptor in advanced epithelial ovarian cancer patients

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ABSTRACT

Background. The urokinase plasminogen activator (uPA) system has been implicated in progression and poor prognosis in epithelial ovarian cancer (EOC) patients. The present study investigated the distribution of uPA and its receptor (uPAR) in EOC cell lines, primary and metastatic tumors, and the relationship between uPA/uPAR and matrix metalloproteinase (MMP) expression using immunohistochemistry. We also studied the association between uPA/uPAR expression and clinical and pathological parameters including disease progression free survival (PFS).

Methods. The expression of uPA/uPAR was examined on paraffin-embedded tissue sections from primary EOC ($n=100$), and matched metastatic lesions ($n=30$) of untreated patients, normal ovarian tissues ($n=20$) as well as 8 primary and metastatic EOC cell lines by immunohistochemistry. Co-immunolabeling of uPA and MMP-1, -2, -9 or MT1-MMP was examined using confocal microscopy.

Results. The expression of uPA/uPAR was found in most primary (92% and 88% positive, respectively), metastatic ovarian tumors (93% and 90% positive, respectively), and all of examined EOC cell lines. The majority of specimens showed moderate to strong immunostaining of tumor and stromal cells; for primary specimens, this was significantly associated with tumor stage, grade and time to relapse ($P<0.01$). Overexpression of uPA/uPAR was found to be associated with an unfavorable prognosis with significantly reduced median disease PFS of 16 vs. 33 months for uPA ($P<0.001$), and 15 vs. 28 months for uPAR ($P<0.001$). Co-localization of uPA with MMP-1, -2, -9 or MT1-MMP was also seen in primary tumors and metastatic lesions.

Conclusions. The expression of uPA/uPAR was associated with EOC progression. uPA/uPAR are useful markers for EOC prognosis and could be promising therapeutic targets for treating incurable, recurrent EOC.

Introduction

Ovarian cancer is the most common and fatal gynecological malignancy in industrialized countries, and the second most common cause of death of the gynecological malignancies in China [1]. Although current approaches including surgery and combination chemotherapy, and hormonal therapy yield responses in 60–80% of patients with advanced disease, the majority of ovarian cancer patients eventually relapse and become refractory to additional treatment [2]. Currently, there is no satisfactory adjuvant treatment following surgery and chemotherapy.

Epithelial ovarian cancers (EOC) account for 90% of all ovarian cancers. EOC can spread directly to adjacent organs, and 'seeding' of the peritoneal cavity is frequently associated with ascite formation, the most common feature of ovarian carcinoma, particularly serous carcinoma [3]. Intra-abdominal dissemination is the primary cause of death for patients with EOC, although the exact mechanisms involved in EOC progression remain unclear.

Several proteolytic enzyme systems are reported to be involved in degradation of the extracellular matrix (ECM) and basement membranes, including the urokinase plasminogen activator (uPA) system [4]. uPA is strongly implicated as a promoter of tumor progression in various human malignancies, and is synthesized and

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secreted as a pro-enzyme. uPA is activated by binding with high affinity specific membrane-bound or soluble cell surface uPA receptors (uPAR), which efficiently convert plasminogen, into plasmin, that directly or indirectly cleaves ECM components [5]. Plasmin can also activate latent elastase and matrix metalloproteinases (MMPs), which can also degrade ECM components [6]. uPAR not only localizes uPA activity, but also mediates signaling events essential for tumor cell differentiation and migration within the tumor microenvironment. Overwhelming evidence demonstrates that the cell surface-associated uPA/uPAR complex is causatively involved in tumor invasion and metastasis of many cancers, either via direct or indirect interactions with integrins, endocytosis receptors and growth factors [7].

Elevated serum levels of uPA have been found in patients with EOC compared to controls [8]. Significant elevation of uPA protein levels in primary ovarian cancer tissue associated with poor prognosis and disease progression has been reported [9–11], and increased expression of uPA/uPAR mRNA has also been associated with dedifferentiation of serous EOC from cystic to solid tumors [12]. The clinical impact of uPAR expression in ovarian cancer remains inconclusive. High levels of tissue uPAR protein have been associated with a good prognosis [13], although uPAR or soluble uPAR (suPAR) has been associated with advanced stages of ovarian cancer and poor prognosis [14]. The distribution of uPA/uPAR and the relationship between uPA/uPAR and MMP expression in EOC has not been fully investigated.

We examined primary and metastatic EOC cell lines and specimens from patients with primary untreated EOC and matched metastatic lesions for expression of uPA and uPAR. The expression of uPA and several MMPs was also investigated. High levels of uPA/uPAR were observed in all EOC cell lines, and in advanced stage EOC as compared to normal ovary. uPA/uPAR overexpression was significantly correlated with clinicopathological parameters including time to relapse, tumor grade, and clinical stage; and with reduced progression free survival (PFS). uPA was also found to be co-localized with MMP-1, -2, -9 and MT1-MMP, consistent with the involvement of uPA/uPAR in EOC progression and dissemination.

Materials and methods

Antibodies

The following antibodies were used: mouse anti-human uPA IgG_1 (#394), which recognizes all known forms of human uPA including receptor bound uPA, and mouse anti-human uPAR IgG_{2a} (#3936), which binds to membrane-associated uPAR (American Diagnostica, Greenwich, CT, USA); biotinylated rabbit anti-mouse Ig, streptavidin-horseradish peroxidase (HRP), mouse anti-human IgG_1 and mouse anti-human IgG_{2a} isotype control monoclonal antibodies (MAbs) (Dako, Glostrup, Denmark); mouse anti-MMP-2 MAb, and rabbit anti-human MMP-1, MMP-2, MMP-9 and MT1-MMP (LabVision, NeoMarkers, San Francisco, CA, USA); AlexaFluor-488 goat anti-mouse IgG and AlexaFluor-594 goat anti-rabbit IgG (Molecular Probes Eugene, Oregon, USA).

Table 2
Characteristics of primary EOC patients.

	EOC	Controls
Number of patients	100	20
Mean age ± SD (years)	54 ± 17	51 ± 14
Age range (years)	42–73	40–70
Tumor grade		
Grade 1	10 (10%)	
Grade 2	25 (25%)	
Grade 3	65 (65%)	
Clinical stage (FIGO[a])		
II	13 (13%)	
III	82 (82%)	
IV	5 (5%)	
Histology		
Serous	60 (60%)	
Mucinous	20 (20%)	
Undifferentiated	9 (9%)	
Endometrioid	6 (6%)	
Clear cell	5 (5%)	
Residual tumor after first surgery		
No	58 (58%)	
Yes	42 (42%)	
Relapse		
No	18 (18%)	
Yes	82 (82%)	
Ascites		
No	2 (2%)	
Yes	98 (98%)	

[a] Federation of Gynecology and Obstetrics (FIGO) [15].

Cell lines and cell culture

Primary (OVCAR-3, A2780, IGROV1, CAOV-3, TOV21G and TOV112D) and metastatic (SKOV-3, OV-90) EOC cell lines (American Type Culture Collection (ATCC, Rockville, MD, USA) representative of different histological types were studied (Table 1). All tissue culture reagents were supplied by Invitrogen Australia Pty Ltd., (Melbourne, VIC, Australia), unless otherwise stated. SKOV-3, OVCAR-3, A2780 and IGROV1 cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) heated-inactivated fetal bovine serum (FBS), 50 U/mL penicillin and 50 U/mL streptomycin. OV-90 cells were maintained in a 1:1 mixture of MCDB 105 medium (Sigma, USA) and 199 medium (Sigma, USA), supplemented with 15% FBS, 50 U/mL penicillin and 50 U/mL streptomycin. CAOV-3 cells were maintained in DMEM with 10% FBS. TOV21G and TOV112D cells were maintained in DMEM with 15% FBS. All cell lines were maintained in a humidified incubator at 37 °C and 5% CO_2.

Confocal microscopy for uPA/uPAR in EOC cell lines

OVCAR-3, A2780, IGROV1, CAOV-3, TOV21G, TOV112D, SKOV-3 and OV-90 cells were grown on glass coverslips (10^5 cells) for 24 h. After washing with Tris buffered saline (TBS) (pH 7.5), cells were fixed on coverslips in ice-cold methanol for 10 min at room temperature (RT). After rinsing in TBS, coverslips were incubated for 1 h at RT in anti-uPA (10 μg/mL) or anti-uPAR (10 μg/mL) MAbs, rinsed again in TBS, then

Table 1
Characteristics of EOC cell lines and uPA/uPAR immunofluroescence.

Cell line	Site of origin	Immunofluorescence[a]	
		uPA	uPAR
OVCAR-3	Adenocarcinoma of ovary	3	3
A2780	Adenocarcinoma of ovary	3	3
IGROV1	Adenocarcinoma of ovary	2	3
CAOV-3	Adenocarcinoma of ovary	2	1
TOV21G	Clear cell	2	3
TOV112D	Endometrioid	2	3
SKOV-3	Ascites, papillary serous adenocarcinoma of ovary	3	2
OV-90	Ascites, ovarian adenocarcinoma	2	2

[a] Immunofluorescence staining scores: 0 = negative; 1 = weak; 2 = moderate; 3 = strong.

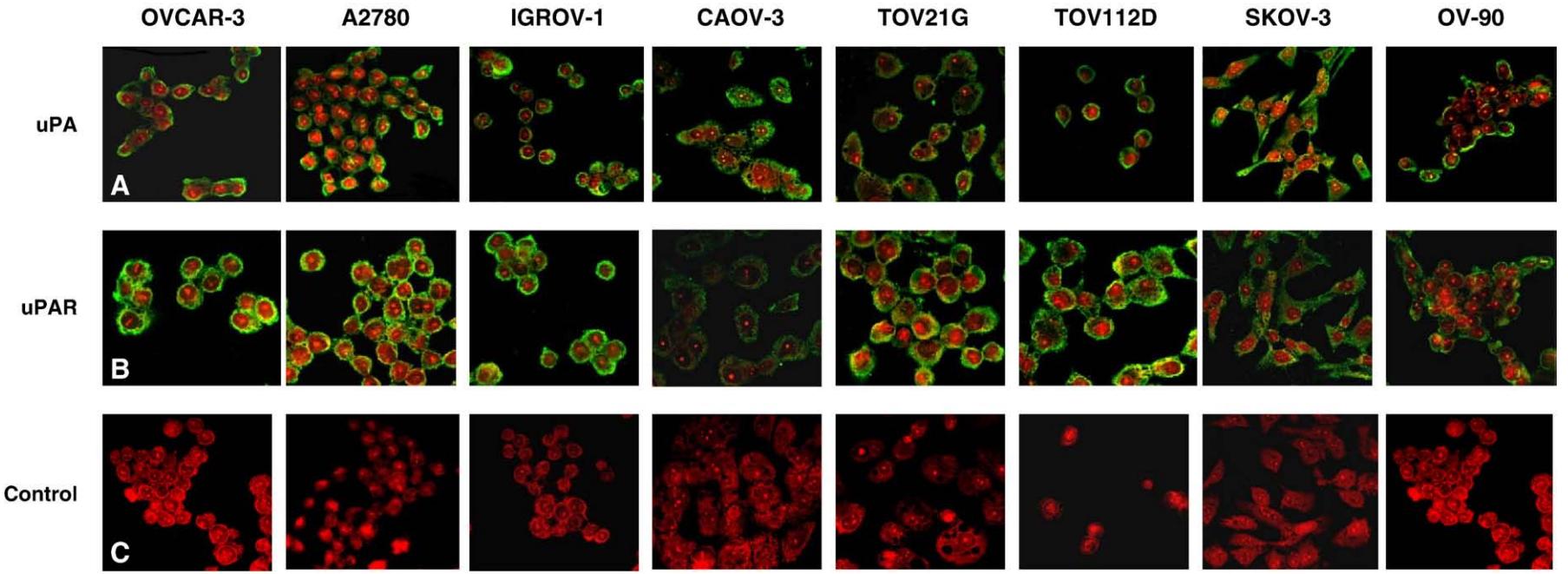

Fig. 1. Representative images showing uPA and uPAR immunofluorescence (green) in EOC cell lines. Variable levels of uPA (A) and uPAR (B) immunostaining are seen in primary and metastatic EOC cell lines. No immunostaining is seen for isotype IgG1 and IgG 2a controls for all cell lines (C). Nuclei are visualized using propidium iodide (red). Magnification: A, B, C ×400.

incubated for 1 h at RT in Alexa goat anti-mouse 488, with a further rinse in TBS. Cell nuclei were visualized with propidium iodide (PI) (1:2000 dilution) for 1 min, before coverslips were mounted on glass slides using glycerol (Sigma-Aldrich Pty Ltd, Castle Hills, NSW, Australia), and examined using a confocal microscope (FV 300/FV500 Olympus, Japan).

Patients and clinical data

100 primary EOC and 30 corresponding intraperitoneal metastatic lesions were obtained from the surgical pathology files of the Department of Pathology, Henan Tumor Hospital, China. All patients underwent primary surgery at the Department of Gynecological Oncology between 2001 and 2006. None of the patients had received chemotherapy before surgery. Clinical data were obtained by retrospective review of the medical records. The study was approved by the Institutional Review Board, Henan Tumor Hospital. Tumors were staged according to the criteria of the International Federation of Gynecology and Obstetrics (FIGO) [15]. Details of the patients' characteristics are summarized in Table 2. There were 60 serous carcinomas, 20 mucinous carcinomas, 9 undifferentiated carcinomas, 6 endometrioid carcinomas and 5 clear cell carcinomas confirmed by pathology. The mean age was 54 ± 17 years (range, 42–73). Thirteen tumors were stage II, 82 tumors were stage III, and 5 tumors were stage IV; 98% of patients had ascites. Twenty normal ovarian specimens (controls) were obtained from early stage cervical cancer patients with a mean age of 51 ± 14 years (range, 40–70), who underwent surgery during the same period (Table 2). The criteria for tumor relapse were serum levels of CA125 > 35 μg/mL, that continued to increase, with concurrent evidence of tumor recurrence by ultrasound, computed tomography (CT), magnetic resonance imaging (MRI) or positron emission tomography (PET).

Tumor tissue collection

Surgical specimens (EOC tissues and normal ovarian tissues) were fixed in 10% neutral buffered formalin, routinely processed, whole-mount-embedded in paraffin, and 4 μm sections were collected. Hematoxylin and eosin (H & E)-stained sections were examined and tumor foci identified, circled in ink, and graded. All tissue specimens from primary tumors and metastatic lesions were verified by histology to confirm the diagnosis, histological type, and tumor grade (Dr. QingKai Yu, Pathologist, Director of Department of Pathology, Henan Tumor Hospital, China).

Immunohistochemistry

uPA and uPAR were detected in EOC sections and normal ovarian tissues using published methods [16]. Briefly, samples were deparaffinized in xylenes and graded alcohols and rehydrated in TBS. Following antigen retrieval in citrate buffer (0.01 M, pH 6.0) for 15 min at 120 °C, sections were rinsed in TBS, treated with 3% hydrogen peroxide in TBS, rinsed and incubated with 10% normal rabbit serum for 10 min. Sections were incubated in mouse MAbs #394 (uPA) (20 μg/mL) or #3936 (uPAR) (20 μg/mL) overnight at 4 °C, rinsed with TBS, then incubated in rabbit anti-mouse biotinylated IgG antibody (1:200 dilution) for 45 min at RT. After incubating in streptavidin-HRP (1:300 dilution) for 30 min at RT, sections were developed with 3,3′ diaminobenzidine (DAB) substrate and counter-stained with hematoxylin. Negative controls were treated identically but incubated in isotype control MAbs (mouse anti-human IgG_1 or IgG_{2a}) or the primary antibody was omitted. The PC-3 prostate cancer cell line (uPA and uPAR positive) was used as a positive control for sections or cells.

For co-immunolabeling with uPA and MMP-1, -2, -9 or MT1-MMP antibodies, sections were deparaffinized, rehydrated and following antigen retrieval as above, rinsed in TBS, and blocked in 10% normal goat serum (NGS) in TBS for 30 min. Sections were incubated overnight at 4 °C in primary mouse anti-uPA MAb (20 μg/mL), and rabbit anti-MMP-1, -2, -9 or MT1-MMP MAbs (4 μg/mL), respectively. After washing with TBS, sections were incubated in goat anti-mouse Alexa 488 (for uPA) and goat anti-rabbit Alexa 594 (for MMPs) for 1 h at RT, and rinsed in TBS. Negative controls were treated identically, using non-specific immunoglobulins (IgG_1 or rabbit IgG). Sections were examined using confocal microscopy (FV 300/FV500 Olympus, Japan). Multitracking and sequential image capture was used to correct signal emission crosstalk between neighboring channels, and the images combined.

Assessment of immunostaining

Immunostaining was assessed and scored using light microscopy and previously reported criteria [17], where: 0 = negative, or <25% tumor stained; 1+ = weak, 25–50% tumor stained; 2+ = moderate, 50–70% tumor stained and 3+ = strong, >75% tumor stained. Immunostaining was independently assessed by two experienced observers (LW and YL), and specimens were scored blind, and averaged. If results were discordant, differences were resolved by joint review and consultation with other experienced observers. For statistical analysis, EOC cases were divided into 2 groups: a low-expression group (LEG; 0 and 1+ immunostaining), and a high-expression (overexpression) group (HEG; ≥2+ immunostaining).

Statistical analysis

Associations between uPA and uPAR immunostaining and the clinicopathological data were tested using a Chi-squared test. Comparison

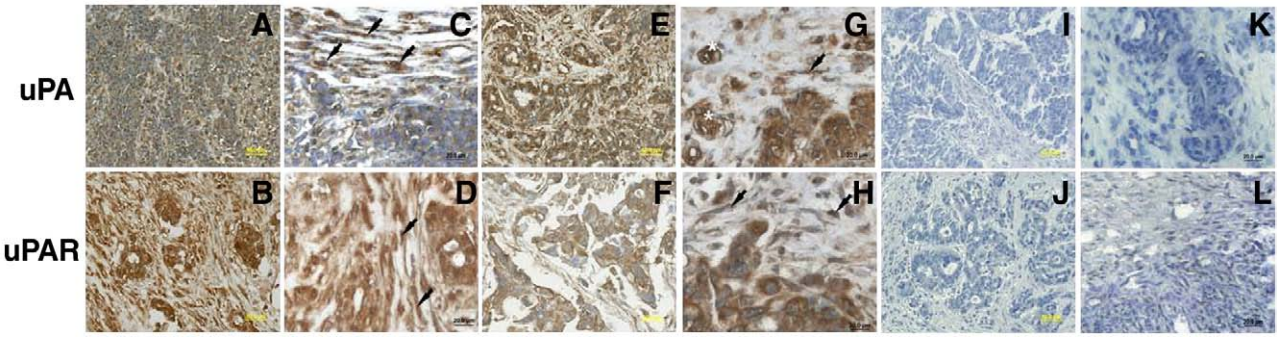

Fig. 2. Immunoperoxidase staining for uPA and uPAR in primary and metastatic EOCs. Representative images taken from different patient specimens showing. uPA in primary tumors (A, C) and metastatic lesions (E, G); and uPAR in primary tumors (B, D), and metastatic lesions (F, H). Brown staining indicates immunoreactive cells. Both uPA and uPAR are seen in stromal cells in primary tumors and metastatic lesions (small arrows). Small clusters of uPA immunoreactive tumor cells are also seen in the stroma (G, *). No immunoreactivity is seen in negative controls from primary tumors (I) and metastatic lesions (J). Normal ovarian tissue displayed no uPA (K) or uPAR (L) immunostaining. Magnification: A–C, E, I, J, L ×200 and D, F–H, K ×400.

Table 3
uPA and uPAR immunoreactivity in primary EOC and metastatic lesions.

Antigen	uPA (#394 MAb)						uPAR (#3936 MAb)					
Specimens	Immunostaining[a]				% Pos[b]	% HEG[c]	Immunostaining[a]				% Pos[b]	HEG[c]
Score	0	1+	2+	3+			0	1+	2+	3+		
PT ($n=100$)	8	6	46	40	92	**86**	12	15	40	33	88	**73**
MT ($n=30$)	2	4	10	14	93	**80**	3	4	12	11	90	**77**

Bold data indicates no significance found between PT and MT for uPA/uPAR expression ($P>0.05$).
[a] Immunostaining staining score: 0 = negative; 1+ = weak; 2+ = moderate; 3+ = strong.
[b] % immunopositive tumors (score 1+ to 3+) in each subgroup.
[c] % of tumors ≥ score 2+ immunostaining in each subgroup (high expression group: HEG); PT: primary tumors; MT: metastatic tumors.

of immunostaining for uPA and uPAR in EOC tissues and normal ovarian tissues or between primary EOC and metastatic lesions was performed using a Chi-squared test, where $P<0.05$ (2-tail) was considered significant. The distribution of disease PFS was calculated using the Kaplan–Meier method and log-rank test. The significance level was $P<0.05$. All statistical analyses were performed using GraphPad Prism 4.00 (GraphPad, San Diego CA) and Stata/SE 10.0 (StataCorp LP, TX, USA).

Results

uPA and uPAR expression in primary and metastatic EOC cell lines

All EOC cell lines (OVCAR-3, A2780, IGROV1, CAOV-3, TOV21G, TOV112D, SKOV-3, OV-90) were uPA (Fig. 1A) and uPAR (Fig. 1B) imunoreactive. No immunolabeling was detected with isotype MAbs (Fig. 1C) or when primary MAbs were omitted (not shown). The immunostaining results are summarized in Table 1.

Expression of uPA/uPAR in primary EOC and metastatic lesions

In primary EOC, 92% and 88% displayed uPA and uPAR immunoreactivity respectively (1+ to 3+). In the matched metastatic lesions, 93% (2/30) and 90% (27/30) were uPA and uPAR immunoreactive (1+ to 3+), respectively. For uPA-positive EOC sections, weak staining (1+) was found in 7% (6/92) of primary tumors and 14% (4/28) metastatic lesions; moderate staining (2+) in 50% (46/92) of primary tumors (Figs. 2A and C) and 36% (10/28) metastatic lesions; and strong staining (3+) in 43% (40/92) of primary tumors and 50% (14/28) of metastatic lesions (Figs. 2E and G). No immunostaining was observed for isotype controls or when the primary antibody was omitted (Fig. 2I), and no uPA staining was found in normal ovarian tissues (Fig. 2K).

In uPAR-positive EOC sections, weak staining (1+) was found in 15% (15/88) of primary tumors and 17% (4/27) of metastatic lesions; moderate staining (2+) in 44% (40/88) of primary tumors and 46% (12/27) of metastatic lesions (Fig. 2F); and strong staining (3+) in 41% (33/88) of primary tumors (Figs. 2B and D) and 37% (11/27) of metastatic lesions (Fig. 2H). No immunostaining was observed for isotype controls or when the primary antibody was omitted (Fig. 2J), and no uPAR staining was seen in normal ovarian tissues (Fig. 2L).

Immunostaining for uPA and uPAR in primary and metastatic lesions is summarized in Table 3. The concordance rate between uPA and uPAR expression was 87% and 90% in primary EOC and metastatic lesions, respectively. In primary tumors and matched metastatic lesions from the same patients, moderate to strong uPA/uPAR immunostaining was found.

Expression of uPA and uPAR was generally uniform in most tumors, and mainly cell membrane-associated, although distinct positive cytoplasmic staining was also observed in some specimens. In high-grade EOC (≥tumor grade 2), areas of tumor stroma showed strong immunoreactivity for uPA and uPAR in most primary tumors (Figs. 2C and D) and metastatic lesions (Figs. 2G and H). However, stromal staining for uPA and uPAR was generally not observed in low grade EOC (tumor grade 1).

Correlation between clinical parameters and uPA or uPAR expression

Relapse with metastases was seen in 82% of EOC patients. Residual tumors were found in 42% of patients after the first surgery and 83% of

Table 4
Clinicopathological characteristics associated with uPA and uPAR expression in primary EOCs.

Variable	No. uPA/uPAR/Total No (%)					
	uPA (92%)			uPAR (88%)		
	LEG[a]	HEG[b]	*P*-value*	LEG	HEG	*P*-value*
Tumor grade						
Low (1)	4/10 (40%)	2/10 (20%)	<**0.0001**	9/10 (90%)	3/10 (30%)	<**0.0001**
High (2–3)	2/90 (2%)	84/90 (93%)		4/90 (4%)	70/90 (78%)	
FIGO stage						
Low (I–II)	5/13 (39%)	6/13 (46%)	<**0.0001**	8/13 (62%)	3/13 (23%)	<**0.0001**
High (III–IV)	1/87 (1%)	80/87 (91%)		5/87 (6%)	70/87 (81%)	
Histological type						
Serous	3/60 (5%)	50/60 (83%)	0.697	7/60 (12%)	41/60 (68%)	0.999
Non-serous	3/40 (8%)	36/40 (90%)		6/40 (15%)	32/40 (80%)	
Residual tumor						
No	5/42 (12%)	30/42 (71%)	0.289	8/42 (19%)	31/42 (74%)	0.805
Yes	1/58 (2%)	51/58 (88%)		5/58 (7%)	42/58 (72%)	
Relapse						
No	4/18 (22%)	11/18 (61%)	<**0.0006**	9/18 (50%)	8/18 (44%)	<**0.0001**
Yes	2/82 (2%)	75/82 (92%)		5/82 (6%)	65/82 (79%)	
Ascites						
<500 mL	5/15 (33%)	9/15 (60%)	<**0.0001**	8/15 (53%)	7/15 (47%)	<**0.0001**
≥500 mL	1/83 (1%)	77/83 (93%)		7/8 (38%)	66/83 (80%)	

[a] LEG, low expression group.
[b] HEG, high expression group.
* Chi-squared test, $P<0.05$ significant.

patients had >500 mL ascites. Table 4 summarizes the correlations between uPA and uPAR expression and tumor grade, clinical stage, histological type, residual tumor following surgery, relapse and ascites for patients with primary tumors. Overexpression (HEG) of uPA and uPAR was correlated with relapse ($P<0.01$) and increased with progression of EOC (tumor grade, $P<0.01$; clinical stage, $P<0.01$; ascites, $P<0.01$). No correlation was found between uPA and uPAR overexpression and histological type or residual tumor following surgery.

A comparison of disease PFS was made between patients with high levels of uPA/uPAR (HEG) and low levels of uPA/uPAR (LEG) (Fig. 3). The patients with high level uPA expression achieved 16 months median survival (95% CI 15–17), whereas those with low level uPA expression achieved a median survival of 33 months (95% CI 26–36, $P<0.001$, Fig. 3A). The patients with high level uPAR expression achieved 15 months median survival (95% CI 14–16), whereas those with low level uPAR expression achieved a median survival of 28 months (95% CI 27–33, $P<0.001$, Fig. 3B).

Co-immunolabeling of primary tumors and metastatic lesions with uPA and MMP-1, -2, -9, or MT1-MMP

The immunostaining pattern and distribution of uPA and uPAR were very similar (Fig. 2), and we only further investigated uPA/MMP co-immunolabeling (Fig. 4). uPA and MMP co-immunolabeling were seen in primary and metastatic specimens (Figs. 4A–D), but not in normal ovarian tissues (data not shown) and IgG controls (Fig. 4E). uPA immunofluorescence and immunoperoxidase displayed a similar distribution in EOC tissues (Fig. 2 cf. Fig. 4), generally localized to epithelial cell membranes. More obvious uPA immunolabeling of epithelial membranes and stromal cells was observed in EOC specimens (Figs. 4A–D). Distinct heterogeneous cytoplasmic MMP-1, -2 and -9 immunostaining were seen in tumor cells and adjacent stromal cells in EOC (Figs. 4A–C). MT1-MMP immunolabeling was seen consistently on epithelial cell membranes (Fig. 4D), often co-localized with uPA (Fig. 4D).

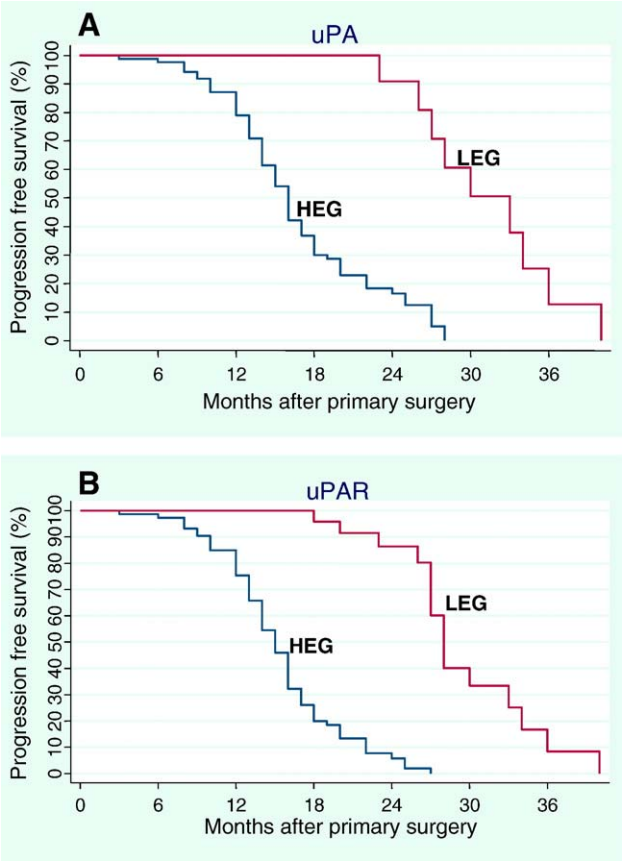

Fig. 3. Kaplan–Meier progression free survival curves for uPA (A) and uPAR (B) expression in patients with advanced stage EOC. These plots illustrate the estimated progression free survival for patients with high level expression (HEG) compared with low levels expression (LEG). The significance of the log-rank test to evaluate the equality of progression free survival distribution was $P<0.001$ for uPA and uPAR, respectively.

Discussion

Previous reports observed that uPA and uPAR expression is related to EOC progression and prognosis; however, their expression and distribution in primary EOC and in the metastatic microenvironment, including their relationship to MMPs, has not been fully investigated. In the present study, we demonstrate high levels of uPA and uPAR expression in 8 EOC cell lines and in advanced EOC specimens, but not in normal ovarian tissues. The majority of primary EOC and matched metastatic lesions from the same patients expressed both uPA and uPAR (Table 3).

Overexpression of uPA and uPAR has been shown to be important in many cancers including breast, colorectal, bladder, pancreatic, gastric and ovarian carcinomas. The prognostic value of components of the uPA system has been extensively studied, particularly in breast and colorectal cancer, where high levels of uPA and uPAR have been associated with poor prognosis and shorter survival [18,19]. Tumor content of uPA and uPAR has also been reported to be increased with loss of histological differentiation, and with advanced FIGO stage in ovarian cancer [20]. Our study confirms this, with high levels of uPA and uPAR expression found to correlate with increasing tumor grade, clinical stage, disease relapse and presence of ascites. However, we found no difference in histological type and residual tumor following surgery between groups, the latter most likely due to the relatively short follow-up period in this patient cohort. Our results indicate that the overexpression of uPA/uPAR is associated with EOC progression and metastasis. The previous studies evaluated uPA/uPAR using either enzyme-linked immunosorbent assay or in situ hybridization. To our knowledge, this is the first report that describes the expression of uPA/uPAR in clinical EOC using immunohistochemistry, and investigates the association between uPA/uPAR expression and prognosis parameters. These results suggest that uPA/uPAR may be useful markers of primary EOC prognosis after surgery, or in evaluating the patient's outcome after chemotherapy.

Interestingly, uPA and uPAR were expressed not only in EOC cells, but in the surrounding stroma of most primary tumors and metastatic lesions. Similar observations have been made in other cancers, including lung, breast, colon and prostate, where the stromal cells predominantly express uPA and uPAR [17,21,22]. We also found similar staining patterns for uPA and uPAR expression in prostate cancer, using the same antibodies (MAbs #394 and #3936) [17]. Increased expression of uPA and uPAR mRNA mainly within the stroma of poorly differentiated tumors and metastases in EOC has also been reported, with uPAR mRNA expressed by tumor cells located along the stromal/epithelial border [12]. Increased stromal release of uPA may result in increased binding to uPAR in tumor cells and endothelial cells. Ligand binding to uPAR also increases its affinity to other cell surface proteins, e.g. vitronectin and integrin receptors, and initiates assembly of these proteins together with caveolin and src kinases at focal adhesion sites [23,24]. Focal adhesion sites mediate connections between the cytoskeleton and ECM during cell migration. Both migration and pericellular proteolysis are initiated by uPA/uPAR binding, as expressed by invasive cell phenotypes such as tumor cells and endothelial cells during new vessel growth [25].

Growth and invasion of EOC cells also involves proteolytic cleavage of ECM by proteinases including MMPs, which are involved in tumor growth, invasion, angiogenesis and metastasis [26]. uPA converts

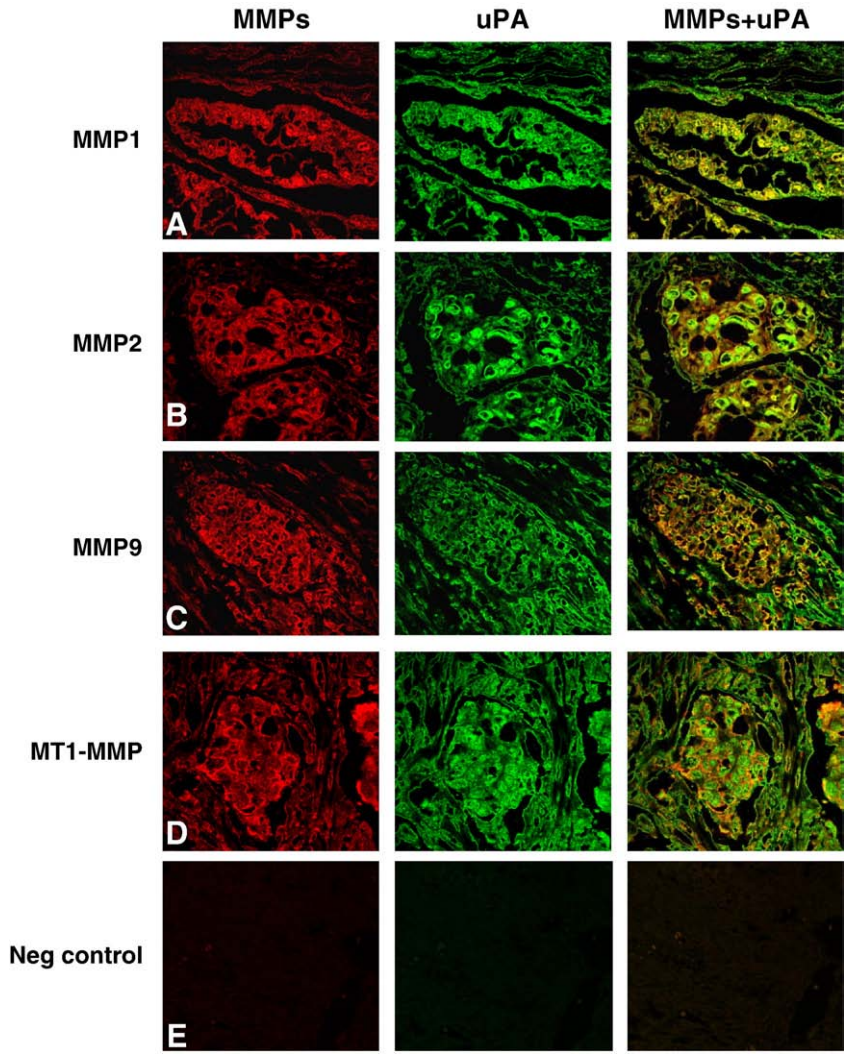

Fig. 4. Co-immunolabeling of uPA and MMPs in EOC. Representative confocal images of uPA (green; Alexa-488) and MMPs (red; Alexa-594) expression in EOC primary tumors and matched metastatic lesions are shown. Merged images, and red, and green channels are shown separately. (A) co-localization of uPA with MMP-1; (B) co-localization of uPA with MMP2; (C) co-localization of uPA with MMP-9; (D) co-localization of uPA with MT1-MMP; (E) IgG negative control. uPA immunolabeling is homogeneous, and generally seen on epithelial and stromal cells. Magnification: A–E ×400.

plasminogen to plasmin, which then activates MMPs [27]. Interactions between cancer cells and stromal fibroblasts are required for activation of the uPAR-uPA-MMP-2 cascade in pancreatic cancer metastasis [28]. Furthermore, the ascite-derived membrane vesicles from ovarian cancer, which include activated MMP-2, -9 and uPA, can stimulate proteinase activation, facilitating tumor cell invasion and metastasis [29]. Increased MMP-2, -9 and uPA have been reported to be associated with progression from benign to advanced EOC [30], although, the relationship between uPA and MMPs in EOC tissues has not been fully explored. We firstly observed evidence of co-immunolocalization of uPA with MMP-1, -2, -9 or MT1-MMP in advanced stage primary and metastatic EOC, but not in normal ovarian tissue. MMPs secreted by stromal cells can be recruited to the cancer-cell membrane to stimulate cancer cells [31], suggesting that interactions of uPA/uPAR and MMPs may promote EOC growth and invasion. Consistent with these observations, MMP-2, -9 and MT1-MMP expression has been found in both epithelial and stromal cells, with high expression of stromal MMPs being correlated to aggressive clinical features in EOC [32]. MMP-2 overexpression by cancer cells in implants, but not in the primary ovarian tumor, was also demonstrated to be an independent prognostic factor in FIGO stage III serous ovarian carcinoma [33]. Increased expression of MMP-2 and -9 has also been reported to be related to earlier stage ovarian tumorigenesis [34, 35]. Although MMPs appear to be involved in EOC progression and metastasis, clinical trials using synthetic MMP inhibitors are generally disappointing for advanced cancers, most likely reflecting earlier involvement of MMPs, before advanced disease is well established [36–38]. The concordance between uPA/uPAR expression in primary and metastatic specimens suggests that targeting uPA/uPAR pathways (upstream of MMPs) may be more effective in controlling both advanced and metastatic EOC.

Several approaches that interfere with uPA/uPAR activity, ranging from neutralizing antibodies, soluble receptors, catalytically inactive uPA fragments, and synthetic peptides/peptidomimetics to antisense approaches, RNAi vector, and DNAzymes have been tested to date [39]. In these systems, the selective targeting of cells expressing uPA/uPAR was not associated with major side effects. We have demonstrated that treatment with ^{213}Bi labeled PAI2 (targeting membrane-bound uPA) inhibits single EOC cells and spheroid growth *in vitro* [40]. Recently, a uPA-derived peptide, A6, which in animal models reduced tumor growth, metastasis, and angiogenesis alone or in combination with other therapies, was evaluated in a phase I clinical trial in patients with gynecological cancers, especially ovarian cancer. This study demonstrated the safety of A6 and some clinical potential [41], and a phase II trial of A6 is now underway in ovarian cancer. A combination therapy targeting uPA/uPAR proteolytic pathways also

may be useful in the control of metastatic EOC disease. In addition, targeting uPA/uPAR may provide additive or synergistic treatment benefits if used in combination with conventional therapeutics (chemotherapy or radiation), particularly in late-stage, metastatic, drug-resistant EOC, for which potent conventional regimens already exist.

Conclusions

In summary, compared with normal ovarian tissues, we observed uPA/uPAR expression in advanced stage EOC, in primary tumors and in matched metastatic lesions. Overexpression of uPA/uPAR in primary EOC correlated significantly with progression parameters including tumor grade, relapse, ascites, and progression free survival and may be used to predict patient prognosis. The co-localization of uPA with MMPs (MMP-1, -2, -9 and MT1-MMP) in tumor and stromal cells highlights the importance of the tumor microenvironment in the pathogenesis of EOC. Our results suggest uPA/uPAR as potential therapeutic targets for treating late-stage recurrent metastatic EOC.

Conflicts of interest statement
None declared.

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