Kallikrein 4 Expression Is Up-Regulated in Epithelial Ovarian Carcinoma Cells in Effusions

Ben Davidson, MD, PhD,[1] Zhijun Xi, MD, PhD,[2*] Tove Irene Klokk, MSc,[2] Claes G. Tropé, MD, PhD,[3] Anne Dørum, MD, PhD,[3] Marit Scheistrøen, MD,[3] and Fahri Saatcioglu, PhD[2]*

Key Words: Immunohistochemistry; Immunoblotting; Quantitative RT-PCR; Metastasis; Prognosis

DOI: 10.1309/PTBB5BPCKX8K9V69

Abstract

We immunohistochemically analyzed kallikrein 4 protein (hK4) expression in patients with epithelial ovarian carcinoma (181 malignant effusions and 103 solid carcinoma lesions). Expression of hK4 was also studied in 32 effusions using immunoblotting.

*Carcinoma cells expressed hK4 in 144 (79.6%) of 181 effusions and 85 (82.5%) of 103 solid tumors. Expression was seen in 51% or more of tumor cells in 70 effusions but often was limited to 5% or fewer cells in solid tumors (*P = *.009, primary tumors vs effusions;* P = *.002, metastases vs effusions). Immunoblotting showed hK4 expression in 31 of 32 specimens. Stromal cell hK4 expression, seen in 48 (46.6%) of 103 lesions, was significantly higher in primary tumors than metastases (26/43 vs 22/60,* P = *.019). hK4 expression in tumor cells was significantly lower in International Federation of Gynecology and Obstetrics stage IV than stage III tumors (*P = *.004, all lesions;* P = *.012, primary tumors). hK4 expression in carcinoma cells was associated with longer overall survival (not significant;* P = *.14, peritoneal effusions).*

hK4 is expressed widely in ovarian carcinoma; levels in carcinoma cells are highest in effusions, which might be related to loss of stromal contribution and/or altered microenvironment. hK4 expression in carcinoma cells of effusions or solid tumors does not predict survival.

Ovarian cancer continues to be the leading cause of death from gynecologic cancer for women in industrialized countries.[1] Despite improved chemotherapy regimens, only modest improvement has been achieved in terms of long-term survival in this disease. This results primarily from the late detection of this cancer and from phenotypic and genotypic variation that make some tumors refractory to conventional treatment regimens. This variation leads to considerable effort directed toward the definition of the expression and the biologic and clinical roles of tumor-associated molecules with the aim of specifically targeting them. The vast majority of these studies, however, have focused on primary tumors rather than on metastatic cells, which are the almost universal cause of tumor-related death in ovarian cancer. Carcinoma cells in serous effusions are different biologically from their counterparts at primary and solid metastatic sites.[2] It therefore is important to study expression patterns at this site to define events in the tumor progression of ovarian carcinoma.

Tissue kallikreins (KLKs) are a family of serine proteases, currently consisting of 15 different members in humans, all encoded by a single gene cluster located at chromosome region 19q13.4.[3,4] The 5 coding exons are highly conserved, as are the 3 residues forming the catalytic triad, which are encoded in the second, third, and fifth exons.[3,4] KLKs are expressed in a variety of benign and malignant tissues and body fluids, but abundant expression is highly tissue-restricted. The latter fact is related to the regulation of KLK expression by androgens and estrogens, resulting in high expression of KLKs in tissues that are under endocrine regulation and tumors originating from those organs, such as prostate, ovary, and breast.[3,4] One member of the family, *KLK3*, also known as prostate-specific antigen, is used routinely for the diagnosis

and monitoring of prostate carcinoma.[3,4] The roles of KLKs are poorly defined at present, but current data suggest that they are involved in proteolysis of various molecules in body fluids, such as semen, and activation of other proteases.[3,4]

Protein or messenger RNA (mRNA) expression of many members of the KLK family, including *KLK5*, *KLK6*, *KLK7*, *KLK8*, *KLK9*, *KLK10*, *KLK11*, and *KLK14*, has been demonstrated in primary ovarian carcinoma.[5-15] These studies generally have shown up-regulation of KLKs in ovarian carcinoma compared with normal ovarian tissue and benign conditions and/or higher expression in the serum of patients diagnosed with ovarian carcinoma.[8,9,11,13-15] The presence of KLKs was demonstrated in ascites specimens from patients with ovarian carcinoma.[16,17] Correlation with clinicopathologic parameters known to be associated with poor prognosis or directly with poor relapse-free and overall survival was demonstrated for *KLK5*,[5] *KLK10*,[8,9] and *KLK6*,[10,11] whereas the opposite was true for *KLK8*,[6,16] *KLK9*,[7] and *KLK11*.[12]

KLK4 is a recently discovered member of the KLK family.[18-21] Although reported to be highly expressed in normal prostate and prostate cancer,[22,23] it also was found in some other tissues in lower levels.[19,21] *KLK4* is a unique member of the KLK family because it is predicted to encode an intracellular protein owing to lack of signal peptide coding sequences in its mRNA.[20] Like other members of the KLK family, the biologic function of *KLK4* is not known.

Recent studies have shown that *KLK4* is expressed in ovarian carcinoma[24,25] and that its expression predicts poor survival in this malignancy.[24] However, no studies are available to date regarding the expression of hK4 protein in ovarian carcinoma or in effusions.

The present study analyzed the expression of hK4 in a large number of effusions and solid tumors from patients diagnosed with advanced-stage epithelial ovarian carcinoma and investigated its potential role as predictor of disease outcome at both sites.

Materials and Methods

Effusion Specimens and Patient Data

The material consisted of 181 consecutive, fresh, nonfixed peritoneal and pleural effusions submitted to the Division of Cytology, Department of Pathology, Norwegian Radium Hospital, Oslo, Norway, during the period January 1998 to January 2003. Effusion specimens consisted of 140 peritoneal and 41 pleural effusions. Eighty-seven specimens were preoperative or intraoperative effusions, tapped before the administration of chemotherapy, and the remaining 94 specimens were postchemotherapy specimens, tapped at disease recurrence **Table 1**.

The 144 effusions were obtained from 131 patients with serous or clear cell ovarian carcinoma, 4 with serous carcinoma of the fallopian tube, and 9 with primary peritoneal carcinoma. The majority of recurrent specimens were peritoneal effusions in patients who previously had peritoneal effusions. Only 1 patient had both peritoneal and pleural effusions, and 1 other patient had 2 consecutive pleural effusions. Fresh, nonfixed specimens submitted to our laboratory arrived within minutes after tapping and were processed immediately. None of the studied effusions was fixed in alcohol or any other fixative to avoid alteration in antigenicity. Following centrifugation, cells were allocated for the preparation of cell blocks or suspended and frozen in equal volumes of RPMI + 20% dimethyl sulfoxide (total, 2 mL) at –70°C. Cell blocks were prepared using the thrombin clot method, with subsequent overnight fixation in buffered formalin and embedding in paraffin the following day, similar to the procedure for biopsy sections at our laboratory. Smears and cell-block sections from all specimens underwent morphologic evaluation by 3 experienced cytopathologists and were characterized further using immunocytochemical analysis with broad antibody panels against carcinoma and mesothelial epitopes, as previously detailed.[26-28]

All effusion specimens and relevant clinical data were obtained from the Department of Gynecologic Oncology, Norwegian Radium Hospital (Table 1). Informed consent was obtained according to national Norwegian and institutional guidelines.

Table 1
Clinicopathologic Data for 87 Prechemotherapy and 94 Postchemotherapy Effusion Specimens

Specimen Type	Prechemotherapy	Postchemotherapy
Tumor origin		
Ovary (n = 165)	82	83
PPC (n = 11)	4	7
Fallopian tube (n = 5)	1	4
Grade		
1 (n = 16)	9	7
2 (n = 52)	27	25
3 (n = 77)	30	47
Not graded* (n = 36)	21	15
FIGO stage		
I (n = 2)	2	0
II (n = 9)	3	6
III (n = 104)	42	62
IV (n = 66)	40	26
Residual disease		
<2 cm (n = 71)	30	41
>2 cm (n = 81)	40	41
Not graded (n = 29)	17	12
Age (y)		
30-40 (n = 3)	2	1
41-50 (n = 19)	8	11
51-60 (n = 57)	24	33
61-70 (n = 52)	25	27
71-80 (n = 45)	23	22
81-90 (n = 5)	5	0

FIGO, International Federation of Gynecology and Obstetrics; PPC, primary peritoneal carcinoma.
* Specimens from inoperable tumors or clear cell carcinomas.

DOI: 10.1309/PTBB5BPCKX8K9V69

Tumor Specimens

We studied 103 surgical specimens consisting of 43 primary tumors (localized to the ovary) and 60 metastatic lesions from the aforementioned patients **Table 2**. Biopsy specimens were available for 54 patients. Primary tumor alone was available for 15 cases, primary tumor and 1 or (more commonly) several metastases for 28 patients, and metastases alone for 11 patients. Specimens were obtained before the institution of chemotherapy. Tumors were placed rapidly in buffered formalin following their removal. Formalin-fixed, paraffin-embedded tissue blocks were obtained from archival material at the Department of Pathology, Norwegian Radium Hospital. All tissue specimens underwent microscopic confirmation of diagnosis, tumor type, and histologic grade, following established criteria.[29]

Table 2
Clinicopathologic Data for 103 Solid Serous Carcinoma Lesions

	No. of Lesions
Tumor site	
Ovary	43
Omentum	32
Peritoneum	11
Intestine	7
Other	10
Grade	
1	3
2	41
3	59
FIGO stage	
II	3
III	56
IV	44
Residual disease	
<2 cm	68
>2 cm	35

FIGO, International Federation of Gynecology and Obstetrics.

Production of the Anti-*KLK4* Antibody

An N-terminal *KLK4* peptide (QIINGEDCSPHSQPW) was used as an antigen to raise a rabbit polyclonal anti-hK4 antibody (MedProbe, Oslo, Norway). The antiserum was affinity-purified before use. Antibody specificity was verified by antigen competition assay in immunoblotting analysis. Briefly, protein extracts from the prostate cancer cell line LNCaP induced by R1881 and from Cos-7 cells transfected with *KLK4* were applied on Western blot. The hK4 antibody (1 μg/mL) was incubated with antigenic peptides (10 μg/mL) for 2 hours at room temperature before it was applied as primary antibody, in parallel with the untreated hK4 antibody. Both specific bands from endogenous (LNCaP-induced) and ectopically expressed (Cos-7–transfected) hK4 were competed by the antigenic peptides.

A similar competition assay has been performed in immunohistochemical experiments. Briefly, serial sections of a human prostatectomy sample were stained with the hK4 antiserum in the presence or absence of the antigenic peptide (10 μg/mL). The specific staining of hK4 in prostate luminal cells in normal glands and in prostate cancer glands was diminished significantly in the presence of the antigenic peptide (data not shown). In addition, the antiserum has been tested extensively using immunohistochemical analysis, immunoblotting, and immunofluorescence on prostate cancer specimens and cell lines.[30] Furthermore, the hK4 antiserum does not cross-react with other KLKs in prostate cancer cells (data not shown).

Immunohistochemical Analysis

Sections from paraffin-embedded blocks were deparaffinized by xylene and serial ethanol dilutions. Antigen retrieval was done by autoclaving at 121°C for 5 minutes in a 0.01-mol/L concentration of citrate buffer (pH 6.4). Sections were rinsed in tris(hydroxymethyl)aminomethane (Tris)-buffered saline (TBS)-polysorbate (0.05-mol/L concentration of Tris chloride, pH 7.5; 0.3-mol/L concentration of sodium chloride; 0.1% polysorbate 20) for 10 minutes, followed by blocking with 1% bovine serum albumin in TBS. Rabbit anti-hK4 antibody was applied at a concentration of 3 μg/mL in the blocking solution. Following incubation for 30 minutes at room temperature, sections were washed with TBS-polysorbate 3 times before incubation with an alkaline phosphatase–labeled polymer (30 minutes at room temperature). Slides then were rinsed again, followed by application of fast red substrate chromogen and counterstaining using hematoxylin (DakoCytomation, Glostrup, Denmark). Slides treated with preimmunized serum from the same rabbit were used as negative control slides; positive control samples consisted of normal prostate tissue. Negative control slides were consistently hK4– in all experiments.

Evaluation of Immunohistochemical Results

Staining in effusions was scored in carcinoma cells, and carcinoma and stromal cell expression was studied in solid tumors. Staining extent was scored on a scale of 0 to 4, corresponding to staining extents of 0%, 1% to 5%, 6% to 25%, 26% to 50%, and 51% to 100% of cells, respectively. At least 500 cells were scored, when present. Approximately 70% of cytologic specimens and 90% of biopsy specimens met this criterion. The lowest cancer cell count allowed was 100 cells.

Immunoblotting

Specimens chosen for immunoblotting were cases for which frozen protein lysates were available and that contained a predominant population of tumor cells. Protein concentrations were determined by using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Protein extracts were electrophoresed on 15% sodium dodecyl sulfate–polyacrylamide

DOI: 10.1309/PTBB5BPCKX8K9V69

gel electrophoresis. The proteins then were transferred to a polyvinylidene fluoride membrane (Bio-Rad), which was blocked with 5% milk for 2 hours, followed by incubation with the primary antibody at 4°C overnight. The ECL kit (Amersham Pharmacia, Little Chalfont, England) was used for detection according to the manufacturer's recommendations. The primary antibody used was the hK4 rabbit polyclonal antibody (1 µg/mL). The positive control sample consisted of the prostate cancer cell line LNCaP.

Statistical Analysis

Statistical analysis was performed with the SPSS-PC package (version 10.1, SPSS, Chicago, IL). Probability of less than .05 was considered statistically significant. Full clinical and pathologic data were available for all patients. Studies of the association between hK4 expression in effusions and solid tumors and clinicopathologic parameters were undertaken using the 2-sided χ^2 test. These consisted of analyses of the association between immunohistochemical results and effusion site, International Federation of Gynecology and Obstetrics (FIGO) stage, tumor grade, patient age, and chemotherapy status (pretreatment vs posttreatment specimen). Comparative analyses of staining results in tumor cells in effusions vs primary tumors and solid metastases were done using the Wilcoxon signed rank test. Univariate analyses of overall survival (OS) and disease-free survival (DFS) were done using the Kaplan-Meier method and log-rank test. Expression categories in these tests were clustered to permit inclusion of a sufficient number of cases in each category. All correlative analyses of effusion specimens with clinicopathologic parameters were done using a single value for every patient. For patients with more than 1 effusion, the registered value was the average score.

Results

Expression of hK4 in Effusions

hK4 is expressed widely in ovarian carcinoma cells in effusions. Expression of hK4 was detected in carcinoma cells in 144 (79.6%) of 181 effusions. In 31 patients with only 1 effusion, expression was negative in tumor cells. An additional patient had 2 hK4– specimens, and 4 patients had hK4+ and hK4– specimens. Of the effusions, 70 specimens expressed the protein in 51% or more of tumor cells **Table 3**, **Image 1A**, **Image 1B**, and **Image 1C**. Staining of mesothelial cells was seen in a few specimens (data not shown), but the dominating pattern in effusions was cancer-specific expression, a pattern that differed from the one observed in solid tumors (see the next section). hK4 protein expression was comparable in carcinoma cells in peritoneal (113/140 [80.7%]) and pleural (31/41 [76%]) effusions ($P > .05$). Expression was not associated with tumor grade, FIGO stage, patient age, extent of residual disease, or the administration of chemotherapy before tapping ($P > .05$; data not shown). Immunoblotting showed a band for hK4 at the predicted size (45 kd) in 31 of 32 specimens **Image 2**, in agreement with immunohistochemical analysis results.

Expression of hK4 in Solid Tumors

hK4 showed frequent but more limited expression in solid tumors. Expression in carcinoma cells in solid tumors was similarly frequent (85/103 [82.5%]) (Table 3) **Image 1D** and **Image 1E**, but most often was limited to 5% or fewer cells and was of weaker intensity. Stromal cell expression of hK4 was seen in 48 (46.6%) of 103 lesions (Table 3) **Image 1F** and **Image 1G**, but this did not correlate with hK4 expression in the tumor areas (data not shown). In Wilcoxon

Table 3
Kallikrein-4 Protein Expression Results (Staining Extent) Using Immunohistochemical Analysis in 181 Effusions and 103 Solid Tumors of Different Sites*

	Staining Extent (%)				
Site	**0**	**1-5**	**6-25**	**26-50**	**51-100**
Tumor cells, effusions					
Peritoneal (n = 140)	27 (19.3)	12 (8.6)	16 (11.4)	34 (24.3)	51 (36.4)
Pleural (n = 41)	10 (24)	4 (10)	2 (5)	6 (15)	19 (46)
Tumor cells, solid tumors					
Ovary (n = 43)	5 (12)	20 (47)	5 (12)	5 (12)	8 (19)
Omentum (n = 32)	5 (16)	10 (31)	4 (13)	8 (25)	5 (16)
Peritoneum (n = 11)	4 (37)	3 (27)	0 (0)	3 (27)	1 (9)
Intestine (n = 7)	3 (43)	3 (43)	0 (0)	1 (14)	0 (0)
Other (n = 10)	1 (10)	3 (30)	2 (20)	2 (20)	2 (20)
Stromal cells, solid tumors					
Ovary (n = 43)	17 (40)	12 (28)	10 (23)	4 (9)	0 (0)
Omentum (n = 32)	19 (59)	12 (38)	0 (0)	1 (3)	0 (0)
Peritoneum (n = 11)	8 (73)	1 (9)	0 (0)	2 (18)	0 (0)
Intestine (n = 7)	5 (71)	2 (29)	0 (5)	0 (0)	0 (0)
Other (n = 10)	6 (60)	1 (10)	3 (30)	0 (0)	0 (0)

* Data are given as number (percentage).

DOI: 10.1309/PTBB5BPCKX8K9V69

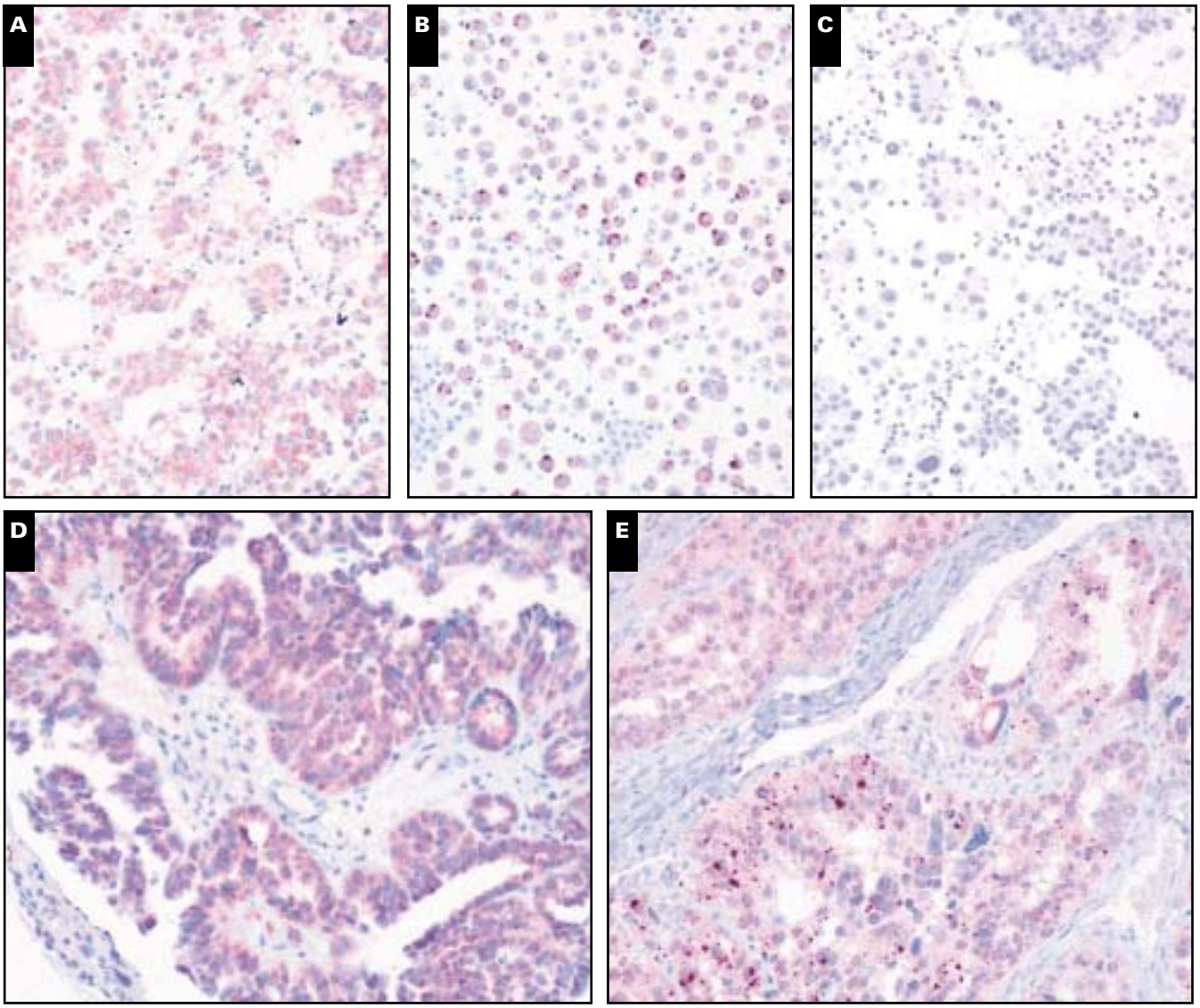

Image 1 Kallikrein 4 protein (hK4) expression in advanced-stage ovarian carcinoma. **A**, Expression of hK4 in a peritoneal effusion from a patient diagnosed with a stage IV serous ovarian carcinoma. All cancer cells are stained (×200). **B**, Poorly differentiated ovarian carcinoma metastatic to a pleural effusion. Cells show distinct staining for hK4 in a Golgi pattern. This patient had a protracted clinical course with an overall survival of 47 months (×200). **C**, An hK4– pleural effusion. This was exceptional (37/181 effusions) in our cohort (×200). **D** and **E**, hK4 expression in carcinoma cells in a primary carcinoma and a peritoneal metastasis from 1 patient. All carcinoma cells stained for hK4. This patient had a disease-free survival of 8 months and was alive with disease at the last follow-up, 17 months after diagnosis (**D**, ×200; **E**, ×200).

analysis, hK4 expression in carcinoma cells was significantly higher in tumors from patients with FIGO stage III than in patients with stage IV disease ($P = .004$, all lesions; $P = .012$, primary tumors) **Table 4**. No association was seen with patient age, histologic grade, the extent of residual disease, or chemotherapy status ($P > .05$; data not shown).

Tumor and Stromal Expression of hK4 in Different Sites

Tumor and stromal expression of hK4 was altered significantly at different anatomic sites in ovarian carcinoma. Wilcoxon analysis of effusions and corresponding solid tumors showed significantly elevated expression (higher percentage of stained cells) of hK4 in carcinoma cells in effusions compared with primary tumors ($P = .009$) and solid metastases ($P = .002$), as evidenced by average expression of 2.58, 1.79, and 1.75 in effusions, primary tumors, and solid metastases, respectively. Site-related differences for 28 patients with effusions, primary tumors, and metastases are shown in **Table 5**. Stromal cell expression (percentage of stained cells) of hK4 was significantly higher in primary tumors than in solid metastases (26/43 vs 22/60; $P = .019$).

DOI: 10.1309/PTBB5BPCKX8K9V69

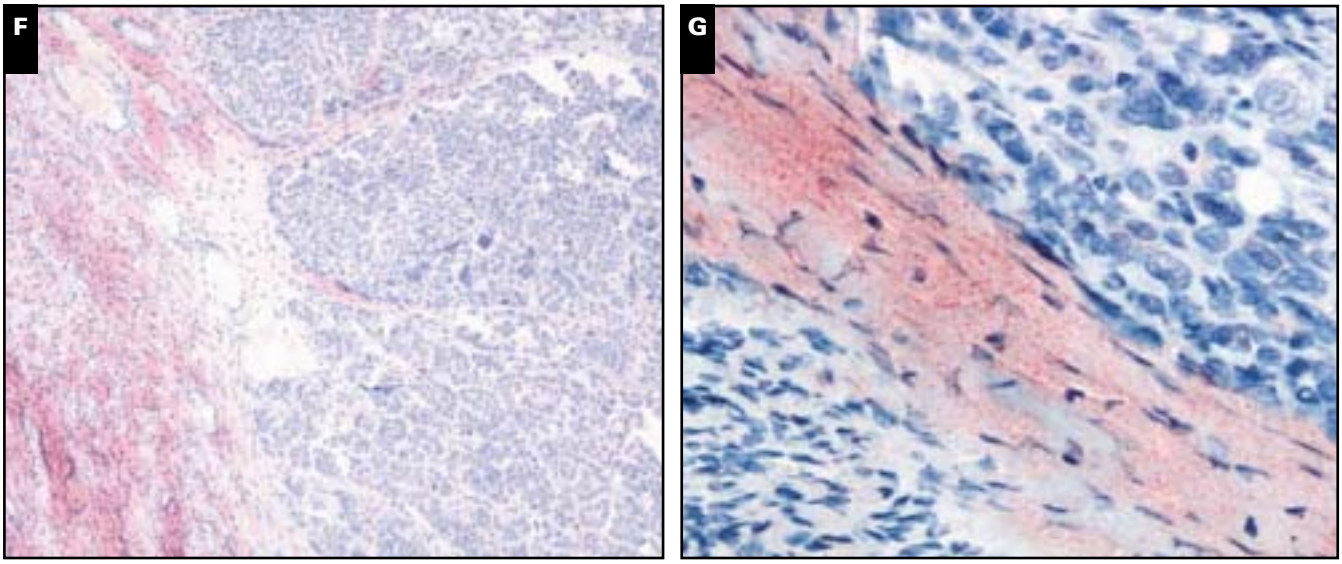

Image 1 **F** and **G**, Two primary carcinomas showing unequivocal staining in peritumoral stromal cells. Tumor cells are negative (**F**, ×100; **G**, ×400).

Tumor and Stromal Expression of hK4 and Survival

Tumor and stromal expression of hK4 did not correlate with survival in epithelial ovarian carcinoma. Survival data were available for 144 patients. DFS ranged from 0 to 53 months (mean, 7 months; median, 5 months). OS ranged from 1 to 84 months (mean, 24 months; median, 22 months). At the time of last follow-up, 90 patients had died of disease, 42 were alive with disease, and 12 had no evidence of disease.

In the Kaplan-Meier survival analysis of effusions, FIGO stage was the only clinicopathologic parameter that correlated significantly with survival ($P = .004$).

Expression of hK4 in more than 50% of tumor cells showed a trend for better OS, but this finding was not statistically significant ($P = .14$, 40 vs 32 months for peritoneal effusions; $P = .3$, 35 vs 23 months for pleural effusions) (data not shown). To evaluate whether the correlation with survival differed between prechemotherapy and postchemotherapy effusions, we performed separate survival analyses for the 2 groups. The analyses showed a similar trend of better OS for patients with prechemotherapy specimens with high expression of hK4 ($P = .16$, 37 vs 25 months), with no survival difference for the postchemotherapy group (data not shown). Expression of hK4 showed no correlation with DFS.

Analysis of solid tumors failed to show correlation with OS or DFS for tumor and stromal cells in primary and metastatic lesions.

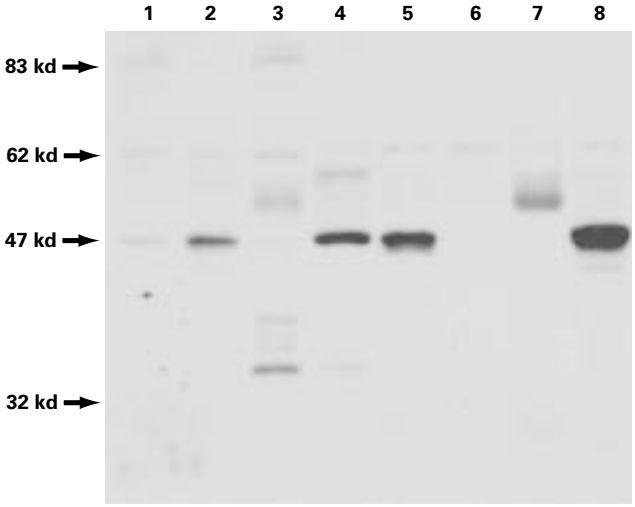

Image 2 Immunoblotting results using the hK4 (kallikrein 4 protein) antibody in 8 of 32 studied effusions (2 peritoneal, 6 pleural). Strong expression is seen in the specimens in lanes 2, 4, 5, and 8, with weaker expression in lanes 1, 3, and 6. The specimen in lane 7 had minimal expression in densitometric analysis but appeared negative in this exposure. Migration positions of the molecular weight (MW) markers are indicated by arrows to the left of the figure.

Table 4
Association Between Immunohistochemical Kallikrein-4 Protein Expression Results (Staining Extent) and FIGO Stage in 100 Solid Tumors*

	Staining Extent (%)				
FIGO Stage	**0**	**1-5**	**6-25**	**26-50**	**51-100**
III (n = 56)	10 (18)	14 (25)	8 (14)	10 (18)	14 (25)
IV (n = 44)	6 (14)	25 (57)	2 (5)	9 (20)	2 (5)

FIGO, International Federation of Gynecology and Obstetrics.
* $P = .004$. Three lesions from stage II tumors were excluded from this analysis.

DOI: 10.1309/PTBB5BPCKX8K9V69

Table 5
Site-Related Expression of hK4 for 28 Cases With Data on Effusion, Primary Tumor, and Metastasis Available for Comparative Analysis

	Staining Extent*		
Case No.	Effusion	Primary Tumor	Metastasis†
1	2	3	1
2	2	3	1
3	3	1	0
4	3	1	1
5	0	0	0
6	0	0	0
7	0	2	2
8	4	2	3
9	4	4	4
10	3	1	1
11	4	4	4
12	3	4	3
13	3	1	1
14	2	2	1
15	3	1	2
16	4	4	0
17	3	1	2
18	3	1	1
19	1	1	1
20	0	0	0
21	4	0	3
22	4	1	0
23	4	3	1
24	4	4	0
25	4	0	3
26	4	1	4
27	4	4	4
28	3	4	4

hK4, kallikrein 4 protein.
* Percentage of hK4+ tumor cells according to the following scale: 1, 0%; 1, 1%-5%; 2, 6%-25%; 3, 26%-50%; 4, 51%-100%.
† Average expression shown for cases with >1 solid metastasis.

Discussion

Dissemination and, therefore, tumor progression in ovarian carcinoma consists primarily of intraperitoneal spread, manifested as malignant effusions and solid metastases, frequently with secondary extension to the pleural cavity. Pleural effusion is, in fact, the most common manifestation of stage IV disease.[31] Because the overwhelming majority of patients with ovarian cancer develop effusions, understanding the biology of ovarian cancer inevitably involves the study of cancer cells at this site. With the exception of 2 reports of high KLK6 and KLK8 expression in supernatants of ascites,[16,17] studies of KLK expression in ovarian carcinoma have focused solely on primary tumors to date.

In the present study, we found hK4 protein expression in 79.6% and 82.5% of the effusions and corresponding solid tumors studied. The values for solid tumors are intermediate between those of Obiezu et al,[24] who reported mRNA expression in 47% of their specimens, and of Dong et al,[25] who reported expression in all serous carcinomas studied, although the latter study included only 11 specimens. As in the majority of studies by our group to date,[2] no significant differences were seen in hK4 expression between carcinoma cells in pleural and peritoneal effusions, supporting the similar biologic characteristics of these cells.

A finding of interest was the expression of hK4 in stromal cells of both primary and metastatic tumors, a finding that has not been reported to date for any of the KLKs. Davidson et al[32-39] have shown prominent expression of metastasis-associated molecules, including matrix metalloproteinases, angiogenic molecules, and transcription factors, in stromal cells in 2 cohorts of patients with ovarian carcinoma patients. The essential contribution of peritumoral fibroblasts to tumor progression has been reviewed.[40] Our data provide additional evidence supporting the dual (tumor and stromal cell) origin of proteases in ovarian carcinomas. The significantly higher expression in stromal cells of primary tumors compared with metastases suggests that any potential hK4-mediated proteolytic activity undertaken by cells of this compartment occurs in early tumor progression.

Carcinoma cells in effusion specimens showed significantly elevated hK4 expression compared with primary tumors and solid metastases, as reflected by staining of the majority of cancer cells in many specimens. This might reflect selection of *KLK4*+ cells in primary tumors for metastatic spread to serosal cavities or changes that are related to the microenvironment of this unique anatomic site and the biological roles of cancer cells there. The latter hypothesis would be the more plausible one in view of the down-regulated expression of hK4 in solid metastases. In fact, extensive site-related alterations in expression of proteases, adhesion molecules, and growth factors have been documented for this cohort (reviewed by Davidson et al[2]). One hypothesis related to hK4 expression would be that the absence of stromal production of this protease in effusions is one of the factors contributing to compensatory up-regulated expression in carcinoma cells at this site. The frequent expression of hK4 in our cohort supports the role of this molecule as a survival factor for ovarian carcinoma cells, in agreement with this hypothesis. Future work might determine whether the role of hK4 in carcinoma cells that have metastasized to the pleural and peritoneal cavities is related to proteolytic activity within the effusion itself or to invasion of the solid organs bordering these cavities.

The prognostic role of KLKs in primary ovarian carcinoma has been the subject of intensive research, with results showing these proteases to be predictors of both better and poorer outcome.[5-12,16] In the only study published to date regarding *KLK4* and survival in ovarian carcinoma, mRNA expression of this molecule was associated with high grade and advanced stage and with worse relapse-free survival and OS, and *KLK4* mRNA expression in grade I and II tumors independently predicted poor prognosis.[24] The results for our cohort do not support this earlier observation, because hK4

DOI: 10.1309/PTBB5BPCKX8K9V69

expression in effusions and solid tumors did not correlate with survival. In fact, hK4 expression in carcinoma cells was significantly higher in tumors from patients with FIGO stage III compared with stage IV disease.

There are several notable differences between our study and the previous report by Obiezu et al.[24] The most evident one is the fact that Obiezu et al[24] studied expression of *KLK4* mRNA, whereas we studied expression of hK4, 2 levels of expression that do not always correlate in terms of absolute expression and relationship to clinical parameters. Second, Obiezu et al[24] evaluated exclusively primary tumors, which consisted of epithelial carcinomas of all types, 10 borderline tumors, and 9 nonepithelial tumors of müllerian origin. Both borderline and müllerian tumors are biologically distinct from invasive epithelial ovarian carcinoma, and their inclusion in a cohort of invasive ovarian carcinomas creates a potential bias. Our study, on the other hand, included only invasive epithelial carcinomas, of which more than 95% were serous, and the analyses were performed for effusions or solid tumors of primary and metastatic sites. Finally, because Obiezu et al[24] studied *KLK4* expression using reverse transcriptase–polymerase chain reaction, it is not possible to determine which cell population(s) expressed *KLK4*.

There is up-regulated expression of hK4 in ovarian carcinoma cells in effusions compared with solid tumors, underscoring the unique characteristics of tumor cells at the former site. The ubiquitous expression of hK4 in ovarian cancer might explain the lack of prognostic value related to expression of this protease, irrespective of biopsy site, but its overexpression in effusions suggests a role in the biology of this malignant neoplasm.

From the Departments of [1]Pathology and [3]Gynecologic Oncology, the Norwegian Radium Hospital, and [2]Molecular Biosciences, University of Oslo, Oslo, Norway.

Address reprint requests to Dr Saatcioglu: Dept of Molecular Biosciences, University of Oslo, N-0316 Oslo, Norway.

** These authors contributed equally to the study.*

References

1. Jemal H, Murray T, Samuels A, et al. Cancer statistics, 2003. *CA Cancer J Clin*. 2003;53:5-26.
2. Davidson B, Risberg R, Reich R, et al. Effusion cytology in ovarian cancer: new molecular methods as aids to diagnosis and prognosis. *Clin Lab Med*. 2003;23:729-754.
3. Yousef GM, Diamandis EP. The new human kallikrein gene family: structure, function, and association to disease. *Endocrine Rev*. 2001;22:184-204.
4. Clements J, Hooper J, Dong Y, et al. The expanded human kallikrein gene family: genomic organisation, tissue-specific expression and potential functions. *Biol Chem*. 2001;382:5-14.
5. Kim H, Scorilas A, Katsaros D, et al. Human kallikrein gene 5 (*KLK5*) expression is an indicator of poor prognosis in ovarian cancer. *Br J Cancer*. 2001;84:643-650.
6. Magklara A, Scorilas A, Katsaros D, et al. The human *KLK8* (neuropsin/ovasin) gene: identification of two novel splice variants and its prognostic role in ovarian cancer. *Clin Cancer Res*. 2001;7:806-811.
7. Yousef GM, Kyriakopoulou LG, Scorilas A, et al. Quantitative expression of human kallikrein gene 5 (*KLK9*) in ovarian cancer: a new independent and favorable prognostic marker. *Cancer Res*. 2001;61:7811-7818.
8. Luo LY, Katsaros D, Scorilas A, et al. Prognostic value of human kallikrein 10 expression in epithelial ovarian carcinoma. *Clin Cancer Res*. 2001;7:2372-2379.
9. Luo LY, Katsaros D, Scorilas A, et al. The serum concentration of human kallikrein 10 represents a novel biomarker for ovarian cancer diagnosis and prognosis. *Cancer Res*. 2003;63:807-811.
10. Hoffman BR, Katsaros D, Scorilas A, et al. Immunofluorometric quantitation and histochemical localisation of kallikrein 6 protein in ovarian cancer tissue: a new unfavourable prognostic biomarker. *Br J Cancer*. 2002;87:763-771.
11. Diamandis EP, Scorilas A, Fracchioli S, et al. Human kallikrein 6 (hK6): a new potential serum biomarker for diagnosis and prognosis of ovarian carcinoma. *J Clin Oncol*. 2003;21:1035-1043.
12. Borgono CA, Fracchioli S, Yousef GM, et al. Favorable prognostic value of tissue human kallikrein 11 (hK11) in patients with ovarian carcinoma. *Int J Cancer*. 2003;106:605-610.
13. Shvartsman HS, Lu KH, Lee J, et al. Overexpression of kallikrein 10 in epithelial ovarian carcinomas. *Gynecol Oncol*. 2003;90:44-50.
14. Dong Y, Kaushal A, Brattsand M, et al. Differential splicing of *KLK5* and *KLK7* in epithelial ovarian cancer produces novel variants with potential as cancer biomarkers. *Clin Cancer Res*. 2003;9:1710-1720.
15. Yousef GM, Polymeris ME, Yacoub GM, et al. Parallel overexpression of seven kallikrein genes in ovarian cancer. *Cancer Res*. 2003;63:2223-2227.
16. Kishi T, Grass L, Soosaipillai A, et al. Human kallikrein 8, a novel biomarker for ovarian carcinoma. *Cancer Res*. 2003;63:2771-2774.
17. Hutchinson S, Luo LY, Yousef GM, et al. Purification of human kallikrein 6 from biological fluids and identification of its complex with α_1-antichymotrypsin. *Clin Chem*. 2003;49:746-751.
18. Nelson PS, Gan L, Fergusson C, et al. Molecular cloning and characterization of prostase, an androgen-regulated serine protease with prostate-restricted expression. *Proc Natl Acad Sci U S A*. 1999;96:3114-3119.
19. Yousef GM, Obiezu CV, Luo LY, et al. Prostase/*KLK-L1* is a new member of the human kallikrein gene family, is expressed in prostate and breast tissues, and is hormonally regulated. *Cancer Res*. 1999;59:4252-4256.
20. Korkmaz KS, Korkmaz CG, Pretlow TG, et al. Distinctly different gene structure of *KLK4*/*KLK-L1*/prostase/ARM1 compared with other members of the kallikrein family: intracellular localization, alternative cDNA forms, and regulation by multiple hormones. *DNA Cell Biol*. 2001;20:435-445.
21. Stephenson SA, Verity K, Ashworth LK, et al. Localization of a new prostate-specific antigen–related serine protease gene, *KLK4*, is evidence for an expanded human kallikrein gene family cluster on chromosome 19q13.3-13.4. *J Biol Chem*. 1999;274:23210-23214.

DOI: 10.1309/PTBB5BPCKX8K9V69

22. Obiezu CV, Soosaipillai A, Jung K, et al. Detection of human kallikrein 4 in healthy and cancerous prostatic tissues by immunofluorometry and immunohistochemistry. *Clin Chem*. 2002;48:1232-1240.
23. Day CH, Fanger GR, Retter MW, et al. Characterization of KLK4 expression and detection of KLK4-specific antibody in prostate cancer patient sera. *Oncogene*. 2002;21:7114-7120.
24. Obiezu CV, Scorilas A, Katsaros D, et al. Higher human kallikrein gene 4 (*KLK4*) expression indicates poor prognosis of ovarian cancer patients. *Clin Cancer Res*. 2001;7:2380-2386.
25. Dong Y, Kaushal A, Bui L, et al. Human kallikrein 4 (KLK4) is highly expressed in serous ovarian carcinomas. *Clin Cancer Res*. 2001;7:2363-2371.
26. Davidson B, Risberg B, Kristensen G, et al. Detection of cancer cells in effusions from patients diagnosed with gynecological malignancies: evaluation of five epithelial markers. *Virchows Arch*. 1999;435:43-49.
27. Davidson B, Berner A, Nesland JM, et al. Carbohydrate antigen expression in primary tumors, metastatic lesions, and serous effusions from patients diagnosed with epithelial ovarian carcinoma: evidence of up-regulated Tn and sialyl Tn antigens expression in effusions. *Hum Pathol*. 2000;31:1081-1087.
28. Davidson B, Nielsen S, Christensen J, et al. The role of desmin and N-cadherin in effusion cytology: a comparative study using established markers of mesothelial and epithelial cells. *Am J Surg Pathol*. 2001;25:1405-1412.
29. Young RH, Clement PB, Scully RE. Surface epithelial-stromal tumors. In: Sternberg SS, Antonioli DA, Carter D, et al, eds. *Diagnostic Surgical Pathology*. Vol 2. Philadelphia, PA: Lippincott Williams & Wilkins; 1999:2319-2382.
30. Xi Z, Klokk TI, Korkmaz K, et al. Kallikrein 4 is a predominantly nuclear protein and is overexpressed in prostate cancer. *Cancer Res*. 2004;64:2365-2370.
31. Akahira JI, Yoshikawa H, Shimizu Y, et al. Prognostic factors of stage IV epithelial ovarian cancer: a multicenter retrospective study. *Gynecol Oncol*. 2001;81:398-403.
32. Davidson B, Reich R, Berner A, et al. Ovarian carcinoma cells in serous effusions show altered MMP-2 and TIMP-2 mRNA levels. *Eur J Cancer*. 2001;37:2040-2049.
33. Davidson B, Reich R, Kopolovic J, et al. Interleukin-8 and vascular endothelial growth factor mRNA levels are down-regulated in ovarian carcinoma cells in serous effusions. *Clin Exp Metastasis*. 2002;19:135-144.
34. Davidson B, Goldberg I, Kopolovic J, et al. Expression of angiogenesis-related genes in ovarian carcinoma: a clinicopathologic study. *Clin Exp Metastasis*. 2000;18:501-507.
35. Davidson B, Reich R, Goldberg I, et al. Ets-1 mRNA expression is a novel marker of poor survival in ovarian carcinoma. *Clin Cancer Res*. 2001;7:551-557.
36. Davidson B, Risberg B, Goldberg I, et al. Ets-1 mRNA expression in effusions of serous ovarian carcinoma patients is a marker of poor outcome. *Am J Surg Pathol*. 2001;25:1493-1500.
37. Davidson B, Goldberg I, Berner A, et al. EMMPRIN (extracellular matrix metalloproteinase inducer) is a novel marker of poor outcome in serous ovarian carcinoma. *Clin Exp Metastasis*. 2003;20:161-169.
38. Davidson B, Goldberg I, Gotlieb WH, et al. High levels of MMP-2, MMP-9, MT1-MMP and TIMP-2 mRNA correlate with poor survival in ovarian carcinoma. *Clin Exp Metastasis*. 1999;17:799-808.
39. Davidson B, Goldberg I, Gotlieb WH, et al. PEA3 is the second Ets family transcription factor involved in tumor progression in ovarian carcinoma. *Clin Cancer Res*. 2003;9:1412-1419.
40. Elenbaas B, Weinberg RA. Heterotypic signaling between epithelial tumor cells and fibroblasts in carcinoma formation. *Exp Cell Res*. 2001;264:169-184.

DOI: 10.1309/PTBB5BPCKX8K9V69