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Unfavorable Prognostic Value of Human Kallikrein 7 Quantified by ELISA in Ovarian Cancer Cytosols

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Background: **Human tissue kallikrein 7 (gene, *KLK7*; protein, hK7) is a member of the kallikrein family of secreted serine proteases. Reports indicate that in ovarian cancer, *KLK7* is significantly up-regulated at the mRNA level. The aim of this study was to determine whether hK7, measured quantitatively by ELISA in ovarian cancer cytosols, is a prognostic biomarker for ovarian cancer.**

Methods: **We used a newly developed ELISA with 2 monoclonal antibodies to quantify hK7 production in 260 ovarian tumor cytosols and correlated these data with various clinicopathologic variables and patient outcomes [progression-free survival (PFS) and overall survival (OS)] over a median follow-up period of 52 months.**

Results: **Median (range) hK7 concentration in ovarian tumor cytosols was 2.84 (0–32.8) ng/mg of total protein. Compared with healthy and benign ovarian tissues and nonovarian tumors that metastasized to the ovary, malignant ovarian tumor cytosols highly overproduced hK7 ($P < 0.001$). We used the median value as the cutoff value to categorize tumors as hK7-positive and hK7-negative. Women with hK7-positive tumors most frequently had advanced-stage disease, higher tumor grade (G3), suboptimal debulking, and serous or undifferentiated histotype ($P < 0.001$). Univariate analysis showed that hK7 positivity was associated with significantly shorter PFS ($P = 0.01$) but not OS. Kaplan–Meier survival curves confirmed an increased risk of relapse in women with hK7-positive tumors ($P = 0.009$). In multivariate analysis, hK7 was not significantly associated with either PFS or OS.**

Conclusions: **hK7 is associated with other unfavorable characteristics of ovarian cancer, but it is not an independent prognosticator for ovarian cancer.**

Ovarian cancer is the most lethal gynecologic malignancy and the 4th leading cause of cancer-related deaths in women in industrialized countries *(1)*. The high mortality rate of ovarian cancer is due not only to the intrinsic aggressiveness of the disease but also to the difficulty of early detection. Early-stage ovarian cancer tends to evade current screening procedures because it typically presents with nonspecific symptoms. Therefore, at diagnosis, the majority of ovarian cancer cases have already progressed to advanced stages with distant metastases. Until more efficient screening or diagnostic strategies become available, identification of new prognostic biomarkers could contribute to the development of individualized and more effective treatment plans for ovarian cancer patients.

Prognostic markers correlate with disease progression and patient survival and are used to improve the accuracy of medical prediction and patient subclassification. Currently, the major prognostic determinant for ovarian cancer is the Fédération Internationale des Gynaecologistes et Obstetristes (FIGO)[5] stage. Patients with ovarian cancers diagnosed at FIGO stages I and II have 5-year survival rates of 80%–95% *(2)*, whereas those whose cancer is diagnosed at FIGO stages III and IV have significantly lower 5-year survival rates of 10%–30% *(3)*. Other conventional prognostic markers include clinicopathologic variables such as grade, tumor size, histotype,

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[5] Nonstandard abbreviations: FIGO, Fédération Internationale des Gynaecologistes et Obstetristes; hK, human tissue kallikrein protein; PFS, progression-free survival; OS, overall survival; HR, hazard ratio.

residual tumor after surgery, and patient age. It is important to note, however, that ovarian cancer is a highly heterogeneous disease. Consequently, cancers with similar clinicopathologic profiles may have different outcomes. Therefore, the discovery of biomarkers that can provide additional prognostic information is highly desirable.

Considerable effort has been expended on identifying novel markers that can be used to accurately predict ovarian cancer outcome. A variety of proteins, including proteases, such as trypsinogen-1, trypsinogen-2, and several matrix metalloproteinases; protease inhibitors, such as tumor-associated trypsin inhibitor; and extracellular matrix components have been implicated as potential ovarian cancer prognostic markers *(4–8)*. In addition, recent microarray analyses have revealed molecular markers as well as gene expression profiles that may have prognostic significance *(9–11)*. Among the newly identified prognostic markers for ovarian cancer are the human tissue kallikreins (hKs), a family of 15 trypsin and chymotrypsin-like secreted serine proteases encoded by genes localized in tandem on chromosome 19q13.4 *(12)*. Ample evidence suggests that members of the kallikrein family are differentially produced in several cancer types, particularly in hormone-dependent malignancies such as prostate, ovarian, breast, and testicular cancers *(13)*. The most intensively studied kallikrein, hK3, more commonly known as prostate-specific antigen, is a widely used biomarker for the detection and management of prostate cancer. For ovarian cancer, 12 kallikreins are differentially produced at either the mRNA or protein level *(13, 14)* and some have prognostic value.

hK7, also known as human stratum corneum chymotryptic enzyme, was first identified in human skin extracts *(15)*. It is produced in the keratinizing squamous epithelium and may be involved in the conversion of the interleukin-1β precursor to its active form *(16)* and in the process of desquamation *(17, 18)*. Aside from the skin, this serine protease is predominantly produced in the esophagus and kidney and is secreted into various bodily fluids, including malignant ascites from ovarian cancer patients *(19)*, where its presence suggests a potential relationship between hK7 and ovarian cancer. Further evidence has shown that human tissue kallikrein 7 (*KLK7*)[6] mRNA is significantly up-regulated in ovarian tumors *(20, 21)*. Collectively, these results raise the possibility that hK7 could be an ovarian cancer biomarker. To investigate this possibility, we measured hK7 protein in ovarian tumors and assessed its prognostic value.

Materials and Methods

TISSUE SAMPLE COLLECTION

We examined a total of 260 patients with ovarian cancer; 48 with benign ovarian conditions; 43 with nonovarian tumors that had metastasized to the ovary from the gastrointestinal tract, endometrium, uterus, or breast; and 34 apparently healthy women. The median age of the study participants was 58 years (range, 19–89 years). All tissues were collected between April 1988 and April 2003 at the Department of Gynecology, University of Turin, Turin, Italy. Specimen collection and processing protocols were identical for all participants. During surgery, histologic examination was performed on the ovarian tissues through intrasurgery frozen section analysis, which allowed representative tumor portions containing more than 80% tumor cells to be selected. The tumor specimens were snap-frozen in liquid nitrogen and stored at −80 °C until extraction.

Clinicopathologic information documented at the time of surgery included tumor stage, grade, histotype, residual tumor size, and debulking success. Tumors were staged according to FIGO criteria *(22)* and graded according to the protocol of Day et al. *(23)*. The classification of histotypes was based on the WHO and FIGO recommendations *(24)*. Patients with ovarian carcinoma at all clinical stages (I–IV) and grades (1–3) were represented in our study. Of the 260 ovarian tumors, the majority (110; 42%) were of serous papillary histotype, followed by endometrioid (46; 18%), undifferentiated (33; 13%), mucinous (20; 8%), clear cell (18; 7%), or other nonepithelial types (23; 9%) (Fig. 1).

After surgery, all patients were treated with platinum-based chemotherapy. The first-line chemotherapy regimens included cisplatin (for 56% of patients), carboplatin (30%), cyclophosphamide (41%), doxorubicin (7%), epirubicin (12%), paclitaxel (16%), and methotrexate (1%). To assess response to chemotherapy, we defined complete response as a resolution of all evidence of disease for at least 1 month; partial response was defined as a decrease (for at least 1 month) of at least half in the diameters of all measurable lesions without the development of new lesions; stable disease was defined as a decrease of <25% in the diameters of all measurable lesions; and progressive disease was defined as an increase of at least 25%. Patients with ovarian cancer were monitored for clinical response to chemotherapy and survival outcomes for a median duration of 52 months. Follow-up information was available for 232 patients, of whom 147 (63%) had relapsed and 117 (50%) had died.

All investigations were carried out in accordance with the ethical standards of the Helsiniki Declaration of 1975 (revised in 1983) and were approved by the Institute of Obstetrics and Gynecology (Turin, Italy) and the Institutional Review Board of Mount Sinai Hospital (Toronto, Ontario, Canada). Study participants gave signed, informed consent.

PREPARATION OF CYTOSOLIC EXTRACTS

Frozen tissue samples (20–100 mg) were homogenized in liquid nitrogen to a fine powder and added to 10 volumes of extraction buffer (50 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% NP-40 surfactant). The result-

[6] Human gene: *KLK*, human tissue kallikrein.

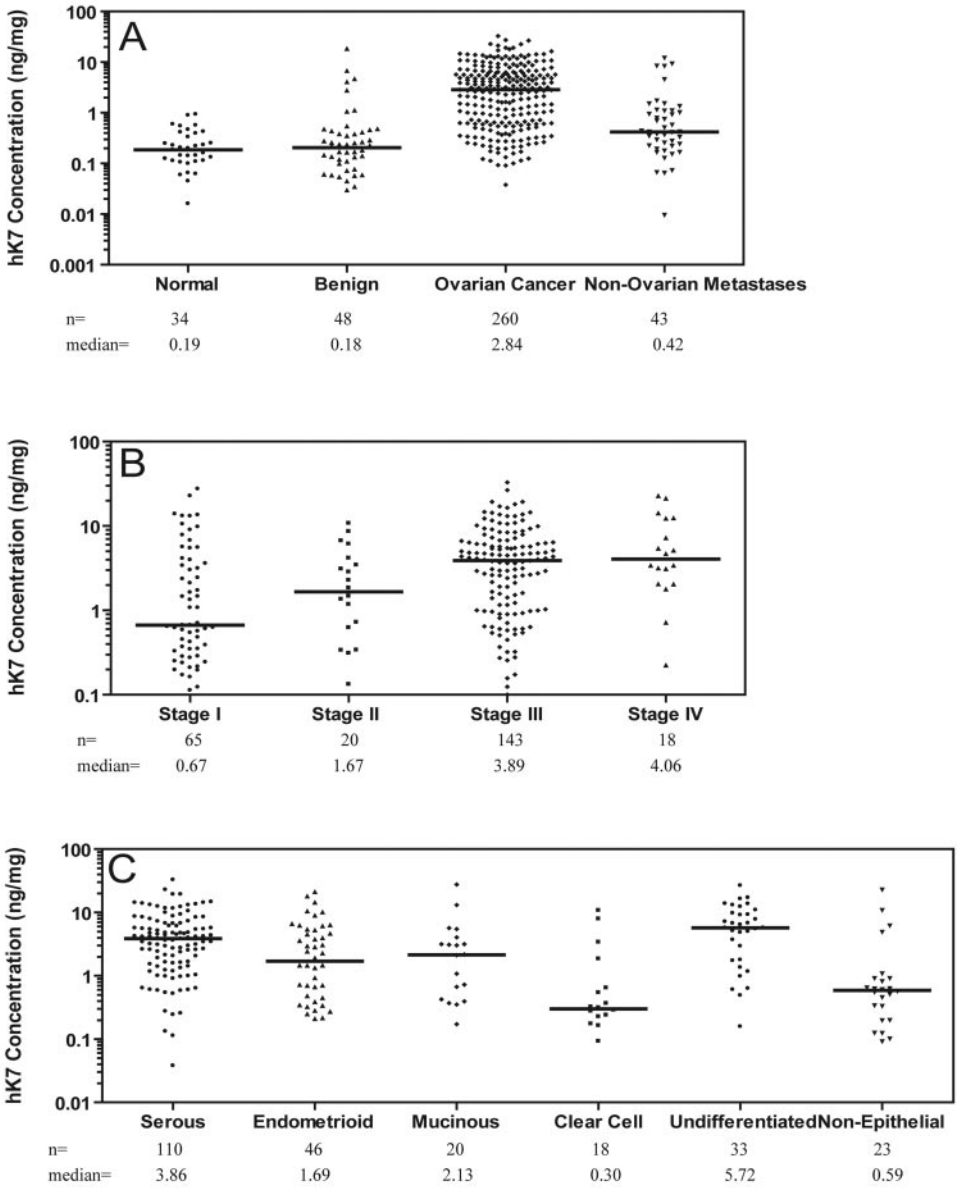

Fig. 1. Distribution of hK7 concentration in ovarian tissue cytosols.

(*A*), distribution of hK7 concentration in healthy women, women with benign ovarian conditions, women with ovarian cancer, and women with nonovarian cancers that metastasized to the ovary. (*B*), distribution of hK7 concentration in women with different stages of ovarian cancer. (*C*), distribution of hK7 concentration in various histotypes of ovarian cancer. *Horizontal lines* represent medians.

ing suspensions were incubated on ice for 30 min, with repeated vortex-mixing every 10 min. The mixtures were then centrifuged at 10 000*g* at 4 °C for 30 min. The supernatant (cytosolic extract) was collected and stored at −80 °C until further analysis. Total protein concentrations of the extracts were determined with the bicinchoninic acid method, with bovine serum albumin as standard (Pierce Chemical Co).

MEASUREMENT OF HK7 AND CA125 PROTEIN PRODUCTION IN OVARIAN CYTOSOLIC EXTRACTS

We measured the concentration of hK7 with a highly sensitive and specific sandwich-type immunoassay previously described and evaluated *(19)*. This assay uses 2 hK7-specific monoclonal mouse antibodies developed in-house and has a detection limit of 0.2 μg/L and a dynamic interval of 0–20 μg/L. White polystyrene microtiter plates were first coated with 100 μL of the coating antibody solution (5 mg/L anti-hK7 monoclonal antibody clone 73-1 in 50 mmol/L Tris-HCl buffer, pH 7.8) and incubated overnight at room temperature. The plates were then washed twice with the washing buffer (10 mmol/L Tri-HCl buffer, pH 7.4, 150 mmol/L NaCl, 0.5 mL/L Tween-20). After the washing step, we added hK7 calibrators or ovarian cytosolic extracts to the wells in duplicates (100 μL/well) after 2-fold dilution in buffer A

(50 mmol/L Tris-HCl buffer, pH 7.8, 60g/L bovine serum albumin, 1 g/L goat globulin, 0.2 g/L mouse globulin, 10 g/L bovine globulin, and 5 mL/L Tween 20). The plates were incubated for 2 h with gentle shaking and then washed. Subsequently, 100 μL of the biotinylated detection antibody solution (200 μg/L anti-hK7 monoclonal antibody clone 83-1 in buffer A) was applied. The plates were incubated for 1 h and washed. Then, 100 μL of alkaline phosphatase–conjugated streptavidin solution (Jackson Immunoresearch Laboratories) diluted 20 000-fold in 60 g/L bovine serum albumin was added to each well. The plates were incubated for 15 min and washed. Signal detection and data reduction were performed automatically with a time-resolved fluorometer, the CyberFluor 615 Immunoanalyzer (MDS Nordion), as described elsewhere *(25)*. The hK7 concentrations in nanograms per milliliter were converted to nanograms hK7 per milligram of total protein to adjust for the amount of tumor tissue extracted.

CA-125 concentrations (kU/mg) in extracts of ovarian tissue samples were measured with the Immulite 2000 assay (Diagnostic Products Corp.).

STATISTICAL ANALYSIS

Because the distribution of hK7 concentrations in the ovarian tumor cytosolic extracts was nongaussian, we used the nonparametric Mann–Whitney *U*-test to determine differences among the 4 types of samples. This test treated hK7 concentration in the tumor cytosolic extracts (ng/mg of total protein) as a continuous variable. We also assessed the association between hK7 and CA125 concentrations by calculating the Spearman rank correlation coefficient (r) and associated P values. Subsequently, we used the median (2.84 ng/mg) as the cutoff point to categorize the ovarian cancer cases as either hK7-positive or hK7-negative. We used the χ^2 test or the Fisher exact test, as appropriate, to analyze the relationship between hK7 production and various clinicopathologic variables.

For survival analysis, cancer relapse (local recurrence or distant metastasis) and death were used to calculate progression-free survival (PFS) and overall survival (OS), respectively. PFS was defined as the time interval between the first surgery and the identification of recurrence or metastatic disease. OS was defined as the time interval between the first surgery and death. The impact of hK7 on patient survival (PFS and OS) was assessed with the hazard ratio (HR), which was the relative risk of relapse or death in the hK7-positive group, calculated with the Cox univariate and multivariate proportional hazard regression models *(26)*. The multivaraiate models were adjusted for hK7 production in tumors and other clinicopathologic variables that may affect survival, including age, stage of disease, tumor grade, and histotype. Only patients for whom the status of all variables was known were included in the multivariate models. In addition, we constructed Kaplan–Meier PFS and OS curves *(27)* to demonstrate survival differences between the hK7-positive and hK7-negative patients. We used the log rank test *(28)* to test for statistical significance of the differences between the survival curves.

Results

DISTRIBUTION OF HK7 CONCENTRATION IN OVARIAN TISSUES

The median protein concentration of hK7 in healthy ovarian tissues was 0.19 (range, 0–0.94) ng/mg of total protein. hK7 production was also relatively low in benign ovarian tissues (median, 0.18; range, 0–18.5 ng/mg of total protein) and nonovarian tumors that metastasized to the ovary (median, 0.42; range, 0.01–12.1 ng/mg of total protein). In contrast, the median protein concentration of hK7 in the 260 ovarian tumor cytosolic extracts was 2.84 (range, 0.013–32.8) ng/mg of total protein (Table 1). We used this median for categorizing ovarian tumors as hK7-positive or hK7-negative.

RELATIONSHIPS BETWEEN HK7 STATUS AND OTHER CLINICOPATHOLOGIC VARIABLES

We categorized hK7-positive and hK7-negative patients according to various clinicopathologic variables, including tumor stage, grade, and histotype; debulking success; and response to chemotherapy (Table 2). We then used either the χ^2 test or Fisher exact test, as appropriate, to evaluate the statistical significance of the relationships between hK7 and these variables. Patients with hK7-positive ovarian tumors more frequently had late stage (stage III/IV) disease, higher tumor grades, suboptimal debulking, and serous and undifferentiated histotypes ($P < 0.001$; Fig. 1, B and C), but we observed no relationship between hK7 positivity and response to chemotherapy. The weak positive correlation between tissue CA125 and hK7 production in ovarian cancer (Spearman correlation, $r_s = 0.471$; $P < 0.001$) is shown in Fig. 2.

UNIVARIATE AND MULTIVARIATE SURVIVAL ANALYSIS

The association between hK7 protein production and patient survival is presented in Table 3. Univariate Cox regression analysis demonstrated that hK7-positive patients were at a greater risk of relapse (HR = 1.54; $P = 0.01$) but not death than were hK7-negative patients. Other variables, such as tumor histologic type, stage, and grade, but not age, had an even higher HR for both PFS and OS. In multivariate Cox regression analysis, when tumor stage was included in the model, the relationship between hK7 status and survival outcome was no longer significant. This was also true for histologic type, grade, and age. Kaplan–Meier survival curves (Fig. 3) further confirmed the above findings.

Discussion

Ovarian cancer is a highly lethal and heterogeneous disease. The optimal management of ovarian cancer can be enhanced by the use of prognostic markers that can accurately predict disease outcome. In the past decade,

Table 1. Tissue hK7 concentrations in 4 groups of patients.

Hk7, ng/mg	Mean (SE)	Median	Range	*P* value[a]
Normal (n = 34)	0.26 (0.04)	0.19	0.016–0.94	0.92[b]
Benign (n = 48)	0.98 (0.41)	0.18	0.016–18.5	<0.001[c]
Ovarian cancer (n = 260)	4.52 (0.33)	2.84	0.013–32.8	<0.001[d]
Nonovarian cancer metastatic to ovary (n = 43)	1.46 (0.42)	0.42	0.01–12.1	<0.001[e]

[a] Calculated by the Mann–Whitney test.
[b] Between normal and benign groups.
[c] Between normal and ovarian cancer groups.
[d] Between benign and ovarian cancer groups.
[e] Between ovarian cancer and nonovarian cancer metastatic to ovary.

numerous investigations have been conducted to identify determinants of ovarian cancer prognosis. Both traditional approaches and novel technologies such as microarrays and proteomics have been used. Of the plethora of potential prognostic markers, the most interesting are those that might have relevance to cancer initiation and metastasis.

A significant proportion of these tumor-associated markers are proteases of various catalytic types (e.g., serine, cysteine, metalloprotease) *(29)*. Proteases play a role in extracellular matrix degradation, which in turn facilitates tumor invasion and metastasis. Accumulating evidence suggests that the hKs, a family of serine proteases found in diverse tissues and biological fluids, have

Table 2. Relationship between tissue hK7 status and other variables in ovarian cancer patients.

		No. of patients, %		
Variable	Patients	hK7-negative[a]	hK7-positive	*P* value
Stage				<0.001[b]
I	65	45 (69.2)	20 (30.8)	
II	20	12 (60.0)	8 (40.0)	
III	143	59 (41.3)	84 (58.7)	
IV	18	5 (27.8)	13 (72.2)	
x[c]	14			
Grade				<0.001[b]
G1	58	37 (63.8)	21 (36.2)	
G2	45	31 (68.9)	14 (31.1)	
G3	139	50 (36.0)	89 (64.0)	
x[c]	18			
Histotype				<0.001[b]
Serous	110	44 (40.0)	66 (60.0)	
Endometrioid	46	26 (56.5)	20 (43.5)	
Mucinous	20	11 (55.0)	9 (45.0)	
Clear cell	18	15 (83.3)	3 (16.7)	
Undifferentiated	33	9 (27.3)	24 (72.7)	
Other nonepithelial	23	19 (82.6)	4 (17.4)	
x[c]	10			
Debulking success[d]				<0.001[e]
SD	103	36 (35.0)	67 (65.0)	
OD	140	83 (59.3)	57 (40.7)	
x[c]	17			
Response to CTX[f]				0.64[b]
NC/PD	19	8 (42.1)	11 (57.9)	
PR	41	19 (46.3)	22 (53.7)	
CR	180	93 (51.7)	87 (48.3)	
NE	20			

[a] Cutoff = 2.84 ng/mg (50th percentile).
[b] χ^2 test..
[c] Status unknown.
[d] OD, optimal debulking (0–1 cm); SO, suboptimal debulking (>1 cm).
[e] Fisher exact test.
[f] CTX, chemotherapy; NC, no change; PD, progressive disease; CR, complete response; PR, partial response; NE, not evaluated.

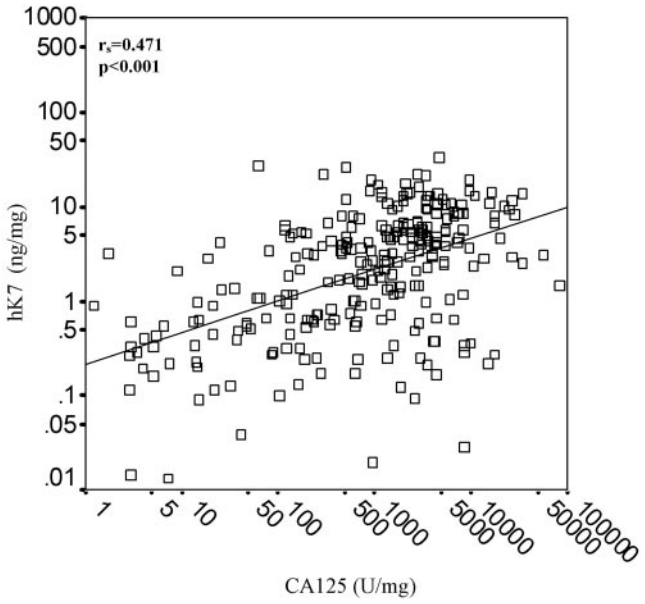

Fig. 2. Correlation between tissue CA125 and hK7 concentrations. r_s, Spearman correlation coefficient.

prognostic value in various cancer types. For ovarian cancer, 7 of the 15 hK members (hKs 5, 6, 7, 8, 10, 11, and 14) are overproduced in parallel at the mRNA level *(14)*, and at least 5 of these hKs are also up-regulated at the protein level and have prognostic importance *(13)*.

hK7 has been studied mostly for its involvement in the process of desquamation in the skin, but it has also been implicated in ovarian cancer. Three independent groups have found KLK7 mRNA to be significantly up-regulated in ovarian cancer *(14, 20, 21)*. The hK7 protein has also been detected in ascites fluid of ovarian cancer patients *(19)*. These findings suggest that hK7 may join other kallikrein members as a potential ovarian cancer biomarker.

We observed that hK7 protein was produced at relatively low concentrations in healthy and benign ovarian tissues, as well as in nonovarian tumors metastatic to this organ. In ovarian cancer, however, hK7 shows 10–15-fold up-regulation, and this increase correlates with cancer stage (Fig. 1B). Metastatic tumors to the ovary from primary gastrointestinal, endometrial, uterine, or breast cancer had low concentrations of hK7, similar to those in benign ovarian tissues. Thus, the phenomenon of hK7 up-regulation seems to be specific to ovarian cancer.

Multivariate Cox regression models adjusted for disease stage, tumor grade, histotype, and patient age indicated that hK7 production was no longer significantly associated with patient survival, a result that could be attributed to our finding that hK7 production correlates with disease stage (Fig. 1B). Kaplan–Meier survival curves showed a slight difference in OS between hK7-positive and hK7-negative patients, but this difference was not significant. Hence, compared with other hKs that bear prognostic importance for ovarian cancer, such as hK6 *(30)* and hK13 *(31)*, hK7 may not be a strong prognostic indictor. Rather, hK7 may be a surrogate marker of advanced stage disease.

Accumulating evidence suggests that combinations of markers can yield higher sensitivity and specificity than single markers *(32–34)*. Therefore, multiparametric strat-

Table 3. Univariate and multivariate analysis of tissue hK7 and other parameters with regard to ovarian cancer survival.

	PFS			OS		
Variable	**HR[a]**	**95% CI[b]**	***P* value**	**HR[a]**	**95% CI[b]**	***P* value**
Univariate analysis						
hK7 (n = 230)						
Negative	1.00			1.00		
Positive	1.54	1.11–2.14	0.01	1.16	0.81–1.67	0.42
Continuous logarithmic variable	1.32	1.01–1.73	0.04	1.14	0.85–1.53	0.37
Histologic type[l]	1.95	1.40–2.72	<0.001	1.58	1.10–2.28	0.014
Stage (ordinal)	2.62	2.08–3.31	<0.001	2.52	1.92–3.29	<0.001
Grading (ordinal)	1.90	1.50–2.41	<0.001	1.99	1.51–2.64	<0.001
Age (ordinal)	1.012	0.99–1.025	0.08	1.02	1.01–1.03	0.009
Multivariate analysis						
hK7 (n = 226)						
Negative	1.00			1.00		
Positive	1.02	0.72–1.44	0.89	0.71	0.48–1.05	0.09
Continuous logarithmic variable	0.88	0.64–1.18	0.39	1.74	0.53–1.04	0.09
Stage (ordinal)	2.29	1.75–3.00	<0.001	2.35	1.71–3.23	<0.001
Histologic type[c]	1.14	0.81–1.63	0.45	0.97	0.66–1.43	0.88
Grading (ordinal)	1.28	0.97–1.68	0.078	1.36	0.98–1.89	0.06
Age (ordinal)	0.99	0.72–1.44	0.89	1.01	0.99–1.027	0.28

[a] HR estimated from Cox proportional hazard regression model.
[b] Confidence interval of the estimated HR.
[c] Serous vs others.

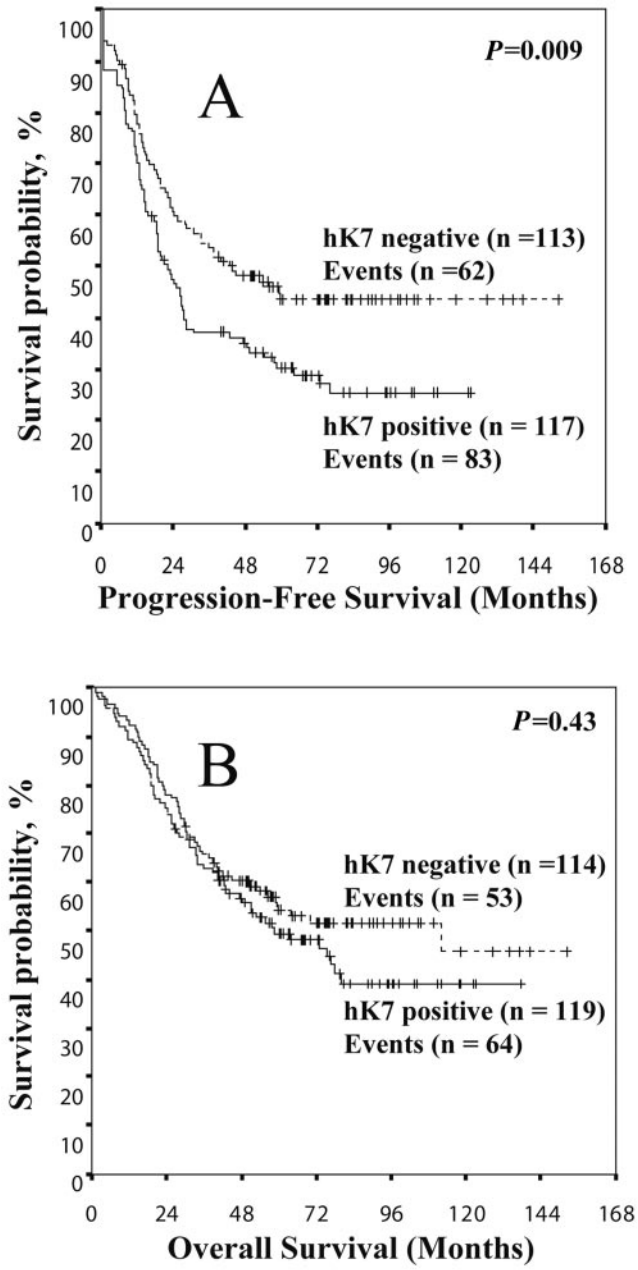

Fig. 3. Kaplan–Meier survival curves for PFS (*A*) and OS (*B*) in patients with hK7-positive and hK7-negative ovarian tumors.
n, number of samples.

egies could yield more informative and accurate medical predictions. To this end, future studies should examine the combined prognostic value of hK7 with other kallikreins, as well as nonkallikrein prognostic biomarkers, in ovarian cancer.

Future investigations should also examine the underlying biologic basis and functional importance of hK7 overproduction in ovarian cancer. Given that hK7 is a serine protease involved in desquamation by degrading intercellular cohesive structures at the skin surface *(17)*, it is reasonable to postulate that hK7 may also contribute to tumor cell invasion and metastasis by digesting extracellular matrix and/or adhesion molecules.

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