Prognostic Value of Human Kallikrein 10 Expression in Epithelial Ovarian Carcinoma

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ABSTRACT

Purpose: **Human kallikrein 10 (hK10; also known as the
normal epithelial cell-specific 1 gene and protein) is a se-
creted serine protease, which belongs to the human kal-
likrein family. It has been reported that hK10 is down-
regulated in breast and prostate cancer cell lines and that it
may function as a tumor suppressor. Recently, we developed
a highly sensitive and specific immunoassay for hK10 and
found that this protein is abundantly expressed in ovarian
tissue. In this study, we measured quantitatively hK10 levels
in ovarian cancer cytosolic extracts and evaluated the prog-
nostic value of this biomarker in ovarian cancer.**

Experimental Design: **Specimens from eight normal
ovarian tissues, eight ovarian tissues with benign disease,
and 182 ovarian tumors were investigated.**

Results: **hK10 concentration in ovarian tumor cytosols
ranged from 0 to 84 ng/mg of total protein, with a median of
2.6. This median was highly elevated in comparison with
normal and benign ovarian tissues ($P < 0.001$). A cutoff of
1.35 ng/mg was selected to categorize tumors as hK10 high
and hK10 low. With χ^2 test and Fisher's exact test, high
concentration hK10 was found to be associated with ad-
vanced disease stage, serous histological type, suboptimal
debulking, and large residual tumor (>1 cm; all $P < 0.05$).
hK10 status was additionally correlated with clinical out-
come, including progression-free (PFS) and overall survival
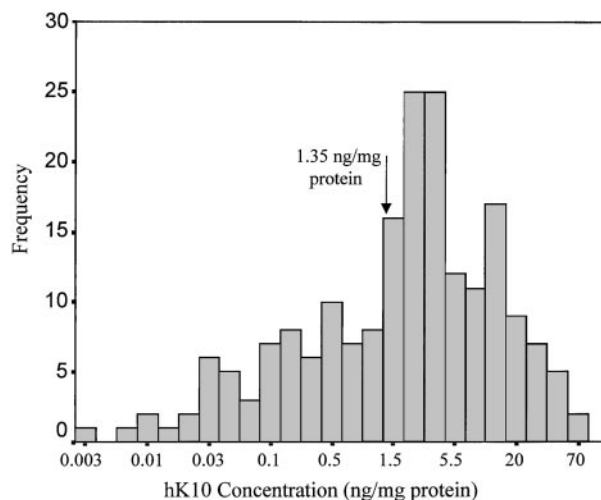(OS) using the Cox model. In univariate analysis, we found**

Fig. 1 Frequency distribution of hK10 concentration in ovarian tumor
cytosols. The optimal cutoff value, 1.35 ng/mg of total protein (33rd
percentile), was used to classify tumors as hK10-high and hK10-low.

**that patients with hK10 high tumors were more likely to die
and relapse, in comparison with patients with hK10 low
tumors (hazards ratios for PFS and OS were 1.93 and 2.42,
respectively; $P < 0.05$). Although this correlation disap-
peared after the entire patient population was subjected to
multivariate analysis, it remained significant in the sub-
group of patients with stage III/IV ovarian cancer (hazards
ratios for PFS and OS were 1.98 and 2.12, respectively;
$P < 0.05$).**

Conclusions: **Our results indicate that hK10 is a new,
independent, unfavorable prognostic marker, especially for
late-stage ovarian cancer.**

INTRODUCTION

hK10,[2] also known as normal epithelial cell-specific 1
protein, is a serine protease, which is secreted by breast and
other epithelial cells (1). hK10 is encoded by a gene designated
as *KLK10* (2), which maps on chromosome 19q13.3–4, spans
about 5.5 kb of genomic sequence, and contains 5 coding exons
and 1 untranslated exon (3). *KLK10* is expressed in many
tissues, including ovary, breast, prostate, colon, and testis, with
the highest expression found in ovary. The physiological func-
tion of hK10 is still not clear, but it has been suggested that
hK10 is a tumor suppressor, based on its down-regulation in
breast and prostate cancer cell lines (1). This hypothesis is

[2] The abbreviations used are: hK10, human kallikrein 10; PFS, progres-
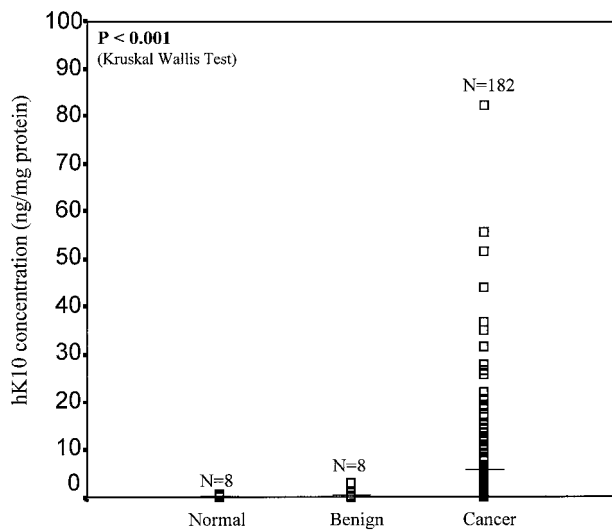sion-free survival; OS, overall survival; HR, hazards ratio.

Fig. 2 Comparison of hK10 concentration in extracts from normal ovarian tissues (*Normal*), ovarian tissues with benign disease (*Benign*), and ovarian cancer (*Cancer*). Numbers of patients in each group (*N*) measured are indicated. *Horizontal bars,* mean hK10 concentration. Kruskal-Wallis test showed hK10 concentration was significantly elevated in the ovarian tumor cytosols ($P < 0.001$).
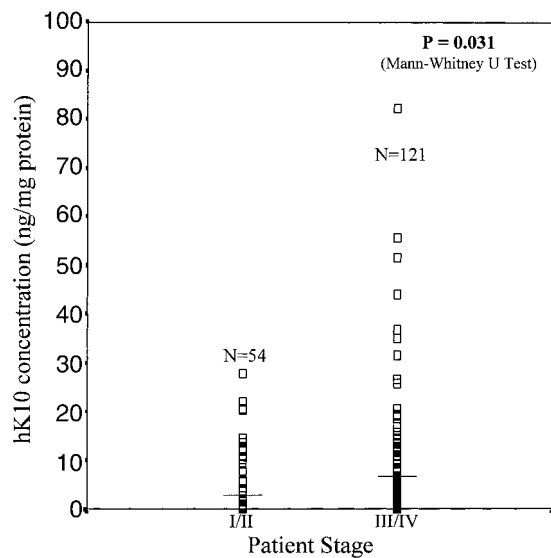

Fig. 3 Distribution of hK10 concentration in extracts from stage I/II and stage III/IV ovarian cancer patients. Patient number in each group (*N*) is shown. *Horizontal bars,* mean value of hK10 concentration. Mann-Whitney test indicated hK10 concentration was significantly elevated in patients with stage III/IV ovarian cancer ($P < 0.05$).

additionally supported by its ability to suppress tumor formation in nude mice (4).

hK10 is a new member of the human kallikrein family, comprised of 15 serine proteases, including hK1 (pancreatic/renal kallikrein), hK2 (glandular kallikrein 2), hK3 (prostate-specific antigen), hK4, and so on, up to hK15 (5). These serine proteases participate in various physiological processes, includ-

Table 1 Relationship between hK10 status and other variables in 182 ovarian cancer patients

Variable	Patients	No. of patients (%) hK10-low	hK10-high	P
Stage				
I	42	21 (50.0)	21 (50.0)	
II	13	2 (15.4)	11 (84.6)	0.015[a]
III	107	30 (28.0)	77 (72.0)	
IV	13	2 (15.4)	11 (84.6)	
x[b]	7			
Grade				
G1	21	10 (47.6)	11 (52.4)	
G2	29	10 (34.5)	19 (65.5)	0.22[a]
G3	115	33 (28.7)	82 (71.3)	
x	17			
Histotype				
Serous	81	12 (14.8)	69 (85.2)	
Undifferentiated	27	9 (33.3)	18 (66.7)	
Endometrioid	30	14 (46.7)	16 (53.3)	
Mucinous	*11*	*4 (36.4)*	*7 (63.6)*	<0.001[c]
Clear cell	*13*	*8 (61.5)*	*5 (38.5)*	
Mullerian	*11*	*7 (63.6)*	*4 (36.4)*	
Others	7	4 (57.1)	2 (42.9)	
x	2			
Residual tumor (cm)				
0	76	32 (42.1)	44 (57.9)	
1–2	25	6 (24.0)	19 (76.0)	0.005[a]
>2	64	11 (17.2)	53 (82.8)	
x	17			
Debulking success				
OD[d]	86	35 (40.7)	51 (59.3)	0.004[c]
SO[d]	81	16 (19.8)	65 (80.2)	
x	15			
Menopause				
Pre/peri	52	15 (28.8)	37 (71.2)	0.48[c]
Post	130	46 (35.4)	84 (64.6)	
Response to CTX[e]				
NC/PD	18	4 (22.2)	14 (77.8)	0.58[c]
CR/PR	138	43 (31.2)	95 (68.8)	
NE	26			

[a] χ^2 test.
[b] x, status unknown.
[c] Fisher's exact test.
[d] OD, optimal debulking (0–1 cm); SO, suboptimal debulking (>1 cm).
[e] CTX, chemotherapy; NC, no change; PD, progressive disease; CR, complete response; PR, partial response; NE, not evaluated.

ing release of vasoactive kinins, cleavage of seminogelins, activation of other enzymes, and so forth (5, 6). Some kallikreins are valuable disease biomarkers. The most well-known kallikrein is prostate-specific antigen, which is widely used as a biomarker for prostate cancer (7). Recently, other kallikreins have emerged as new biomarkers for various diseases (5). For example, hK2 is now being investigated as a new prostate cancer biomarker (8). hK6 was found to have value in the diagnosis of Alzheimer's disease (9) and hK4, hK5, and hK8 have been examined as ovarian cancer prognostic markers (10–12). Although the expression of hK10 has been reported to be down-regulated in breast and prostate cancer cell lines, and hK10 appears to function as a tumor suppressor (1, 4), its role in the pathogenesis of human disease has not been studied. To investigate the potential value of hK10 as a disease biomarker, we have recently developed a highly sensitive and specific

Table 2 Univariate and multivariate analysis of hK10 expression with PFS and OS

Variable	PFS			OS		
	HR[a]	95% CI[b]	P	HR[a]	95% CI[b]	P
	Univariate analysis					
hK10						
Low	1.00			1.00		
High	1.93	1.12–3.33	0.017	2.42	1.19–4.93	0.014
As a continuous variable	1.013	0.99–1.030	0.15	1.015	1.00–1.033	0.040
Stage of disease (ordinal)	2.82	2.07–3.84	<0.001	3.33	2.22–5.0	<0.001
Grading (ordinal)	2.20	1.59–3.03	<0.001	2.33	1.53–3.55	<0.001
Residual tumor (ordinal)	1.23	1.20–1.35	<0.001	1.33	1.22–1.41	<0.001
Histologic type[c]	0.68	0.46–1.01	0.059	0.74	0.46–1.21	0.23
Age	1.01	0.99–1.03	0.19	1.02	0.99–1.03	0.12
	Multivariate analysis					
hK10						
Low	1.00			1.00		
High	1.15	0.64–2.06	0.62	1.21	0.58–2.50	0.60
As a continuous variable	1.02	0.99–1.04	0.11	1.02	0.99–1.04	0.19
Stage of disease (ordinal)	1.78	1.21–2.62	0.003	1.78	1.11–2.85	0.016
Grading (ordinal)	1.27	0.86–1.87	0.22	1.31	0.79–2.17	0.29
Residual tumor (ordinal)	1.17	1.08–1.26	<0.001	1.22	1.11–1.34	<0.001
Histologic type[c]	0.97	0.61–1.54	0.91	1.11	0.65–1.90	0.69
Age	1.01	0.99–1.04	0.14	1.01	0.98–1.04	0.23

[a] HR estimated from Cox proportional hazards regression model.
[b] Confidence interval of the estimated HR.
[c] Endometrial, undifferentiated, and others *vs.* serous.

immunoassay for hK10 (13). With this assay, we were able to quantify hK10 protein in many tissues and biological fluids. We found that hK10 is abundantly expressed in the epithelial cells of ovarian tissue (13). The aim of this study is to investigate whether hK10 protein levels in ovarian tumor extracts correlate with clinical outcomes.

PATIENTS AND METHODS

Ovarian Cancer Patients. One hundred eighty-two patients with primary ovarian cancer were included in this study. These patients underwent surgery for ovarian cancer at the Department of Gynecology, University of Turin. Ages of these patients ranged from 25 to 82 with a median of 59 years. Clinical and pathological information were documented at the time of surgery, including stage, grade, histological types, residual tumor, debulking success, menopausal status, and response to chemotherapy. The staging of the tumors was according to the International Federation of Gynecologists and Obstetricians criteria. The classification of the histological types was based on the WHO and International Federation of Gynecologists and Obstetricians recommendations. Most of the tumors (81) included in this study were of serous papillary histological type, whereas the remaining tumors were endometrioid (30), undifferentiated (27), mucinous (11), clear cell (13), mullerian (12), sarcoma (2), other nonepithelial (4), and unknown (2). The size of the residual tumors ranged from 0 to 9 cm, with a median of 1 cm.

Follow-up information (median follow-up period of 62 months) includes survival status (alive or deceased) and disease status (disease-free or recurrence) and was available for 163 patients. Among these patients, 58 died and 85 relapsed.

Preparation of the Cytosolic Extracts. Tumor tissues were frozen in liquid nitrogen immediately after surgery and stored at −80°C until extraction. Frozen tissue (200 mg) was first pulverized on dry ice to a fine powder. One ml of extraction buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 5 mM EDTA, 10 g/liter of NP-40 surfactant, 1 mM phenylmethylsulfonyl fluoride, 1 g/liter of aprotinin, and 1 g/liter of leupeptin] was then added to the tissue powders and incubated on ice for 30 min with repeated shaking and vortexing every 10 min. Finally, the mixtures were centrifuged at 14,000 rpm at 4°C for 30 min, and the supernatants (cytosolic extracts) were collected. All of the tissue cytosolic extracts were stored at −80°C until they were analyzed. Protein concentration of the cytosolic extracts was determined with the bicinchoninic acid method with albumin as standard (Pierce Chemical Co., Rockford, IL).

Measurement of hK10 in Ovarian Cytosolic Extracts. The concentration of hK10 in the cytosolic extracts was quantified with a highly sensitive and specific noncompetitive immunoassay for hK10. This assay was described and evaluated in detail elsewhere (13). The assay incorporates both mouse and rabbit anti-hK10 antisera. In brief, mouse anti-hK10 polyclonal antiserum was captured with sheep antimouse IgG, Fc fragment-specific antibody (Jackson Immunoresearch, West Grove, PA) on 96-well polystyrene microtiter plates. Cytosolic extracts (25 μl) and 100 μl of BSA (60 g/liter) were then added into each well, incubated for 1 h with gentle shaking, and washed. Rabbit anti-hK10 polyclonal antiserum was subsequently applied, incubated, and washed. Finally, alkaline phosphatase-conjugated goat antirabbit IgG (Jackson Immunoresearch) was added, incubated, and washed as before. To detect the signal, time-resolved fluorometry was used. The detection range of this assay
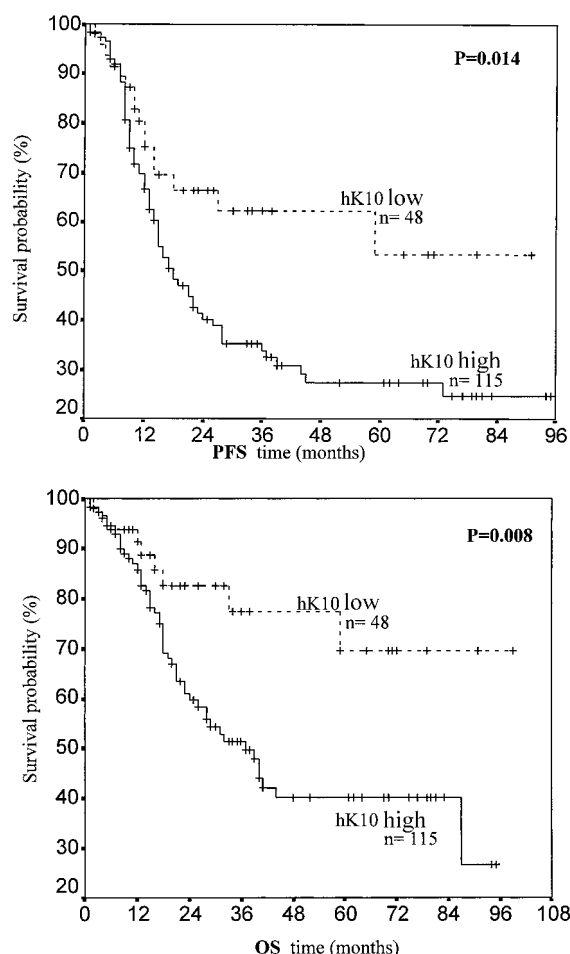
Fig. 4 Kaplan-Meier survival curves. *Top,* PFS; *Bottom,* OS. The patient number in each group (*n*) and *P*s are indicated. The lower PFS and OS rates in hK10-high patients are statistically significant.

is 0.05–20 μg/liter. All of the tumor extracts were measured in duplicate, and hK10 concentrations in μg/liter were converted to ng of hK10/mg of total protein to adjust for the amount of tumor tissue extracted.

Localization of hK10 in Ovarian Tumor Specimens by Immunohistochemistry. A rabbit polyclonal antibody was raised against hK10 full-size recombinant protein produced in yeast cells (13). Immunohistochemical staining for hK10 was performed according to a standard immunoperoxidase method. Briefly, paraffin-embedded tissue sections (4 μm) were fixed and dewaxed. Endogenous peroxidase activity was blocked with 3% aqueous hydrogen peroxide for 15 min. Sections were then treated with 0.4% pepsin at pH 2.0 for 5 min at 42°C and blocked with 20% protein blocker (Signet Labs) for 10 min. The primary antibody was then added at 1:400 dilution for 1 h at room temperature. After washing, we added biotinylated secondary antibody (Signet), diluted 4-fold in antibody dilution buffer (DAKO). We then added the streptavidin horseradish peroxidase complex for 30 min at room temperature. Detection was achieved with amino ethyl carbazole for 5–10 min. After

counterstaining with hematoxylin, the slides were mounted with coverslips.

Statistical Analysis. Statistical analysis was performed with SPSS software (SPSS Inc., Richmond, CA). To analyze data, patients were divided into different groups according to clinical and pathological parameters. Because the distribution of hK10 protein concentration in the ovarian tumor cytosols was not Gaussian, the differences between groups were determined by the nonparametric Mann-Whitney U test, and the analysis of differences among more than two groups was performed with the Kruskal-Wallis test in which hK10 was considered as a continuous variable. hK10 values were also classified into two categories (hK10-high and hK10-low groups), and their relationships to various clinicopathological variables were analyzed with the χ^2 test and the Fisher's exact test (where applicable). The impact of hK10 on patient survival (PFS and OS) was assessed with the HR (a relative risk for relapse or death) that was calculated with the univariate and multivariate Cox proportional hazards regression model (14). In the multivariate analysis, the clinical and pathological variables that may affect survival, including stage of disease, tumor grade, residual tumor, histological type, and age were adjusted. Kaplan-Meier PFS and OS curves (15) were constructed to demonstrate the survival differences between the hK10-high and hK10-low patients. The log rank test (16) was used to examine the significance of the differences among the survival curves. Furthermore, the patients were divided into different subgroups based on disease stage, tumor grade, and debulking success. The survival analysis was then repeated separately for each subgroup of patients.

RESULTS

Distribution of hK10 Concentration in Ovarian Tumor Cytosols. hK10 concentration in ovarian tumor cytosols from 182 patients ranged from 0 to 84 ng/mg of total protein. The mean was 7.1 ng/mg with a median of 2.6 ng/mg. The frequency distribution curve is shown in Fig. 1. hK10 concentration was highly elevated in ovarian tumor cytosols compared with the cytosols prepared from normal ovarian tissues or tissues with benign ovarian disease (Fig. 2). The mean, SE, and range of values, were: normal ovarian tissues, 0.27 ± 0.06, 0–0.62; benign ovarian disease, 0.61 ± 0.33, 0–3; ovarian cancer, 7.1 ± 0.7, 0–84. The optimal hK10 cutoff value for additional analyses was selected by the χ^2 test based on the ability of this value to predict the OS of the study population. A value of 1.35 ng/mg of total protein was found to be the optimal cutoff ($\chi^2 = 6.3$; $P = 0.012$) and represents the 33rd percentile. Tumors were then dichotomously categorized as hK10-high and hK10-low (Fig. 1).

Relationships between hK10 Status and Other Clinicopathological Variables. The distributions of various clinicopathological variables among hK10-high and hK10-low patients are summarized in Table 1. The relationships between hK10 and these variables were examined with χ^2 test and Fisher's exact test. No relationship was observed between hK10 status and tumor grade, menopause status, and response to chemotherapy. However, hK10-high patients more frequently had advanced disease stage (stage II-IV), serous histological type, larger residual tumor (>1 cm), and suboptimal debulking (all $P < 0.05$). With Mann-Whitney U test, it was also demonstrated that hK10

Table 3 Cox proportional hazard regression analysis for subgroups of patients

Variable	PFS			OS		
	HR[a]	95% CI[b]	P	HR[a]	95% CI[b]	P
Stage III/IV						
HK10 unadjusted	2.07	1.13–3.78	0.018	2.35	1.11–5.01	0.026
HK10 adjusted[c]	1.98	1.07–3.68	0.031	2.12	0.96–4.71	0.057
Stage I/II						
HK10 unadjusted	0.69	0.14–3.28	0.64	1.73	0.17–16.9	0.63
HK10 adjusted[c]	1.14	0.23–5.46	0.86	2.43	0.24–24.7	0.45
Tumor grade III						
HK10 unadjusted	1.91	0.93–3.16	0.083	2.26	1.01–5.08	0.047
HK10 adjusted[d]	0.94	0.48–1.81	0.85	1.09	0.48–2.45	0.83
Tumor grade I/II						
HK10 unadjusted	3.85	1.12–13.2	0.031	3.58	0.80–16.1	0.094
HK10 adjusted[d]	2.01	0.52–7.68	0.32	2.58	0.52–12.8	0.24
Suboptimal Debulking						
HK10 unadjusted	1.71	0.74–3.07	0.25	1.61	0.71–3.63	0.26
HK10 adjusted[e]	1.34	0.63–2.81	0.44	1.16	0.49–2.70	0.72
Optimal Debulking						
HK10 unadjusted	0.74	0.25–2.17	0.59	1.36	0.25–7.48	0.71
HK10 adjusted[e]	0.72	0.22–2.28	0.58	1.26	0.20–7.78	0.79

[a] HR estimated from Cox proportional hazards regression model.
[b] Confidence interval of the estimated HR.
[c] Multivariate models were adjusted for tumor grade, residual tumor, histologic type, and age.
[d] Multivariate models were adjusted for stage of disease, residual tumor, histologic type, and age.
[e] Multivariate models were adjusted for stage of disease, tumor grade, histologic type, and age.
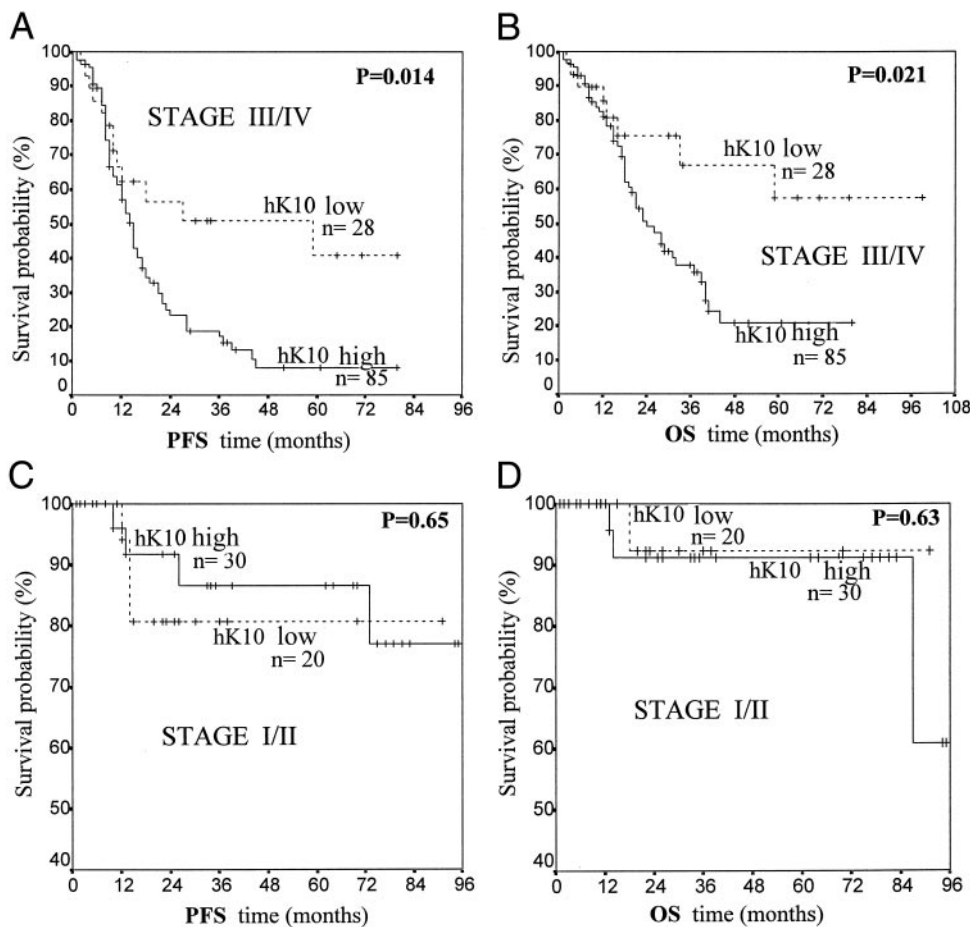

Fig. 5 PFS and OS of patients with hK10-high and hK10-low ovarian tumors stratified by the tumor stage. The patient number in each group (*n*) and *P*s are indicated. Among patients with stage III/IV disease, hK10-high was associated with lower PFS and OS rates. This association was not observed in patients with stage I/II disease.
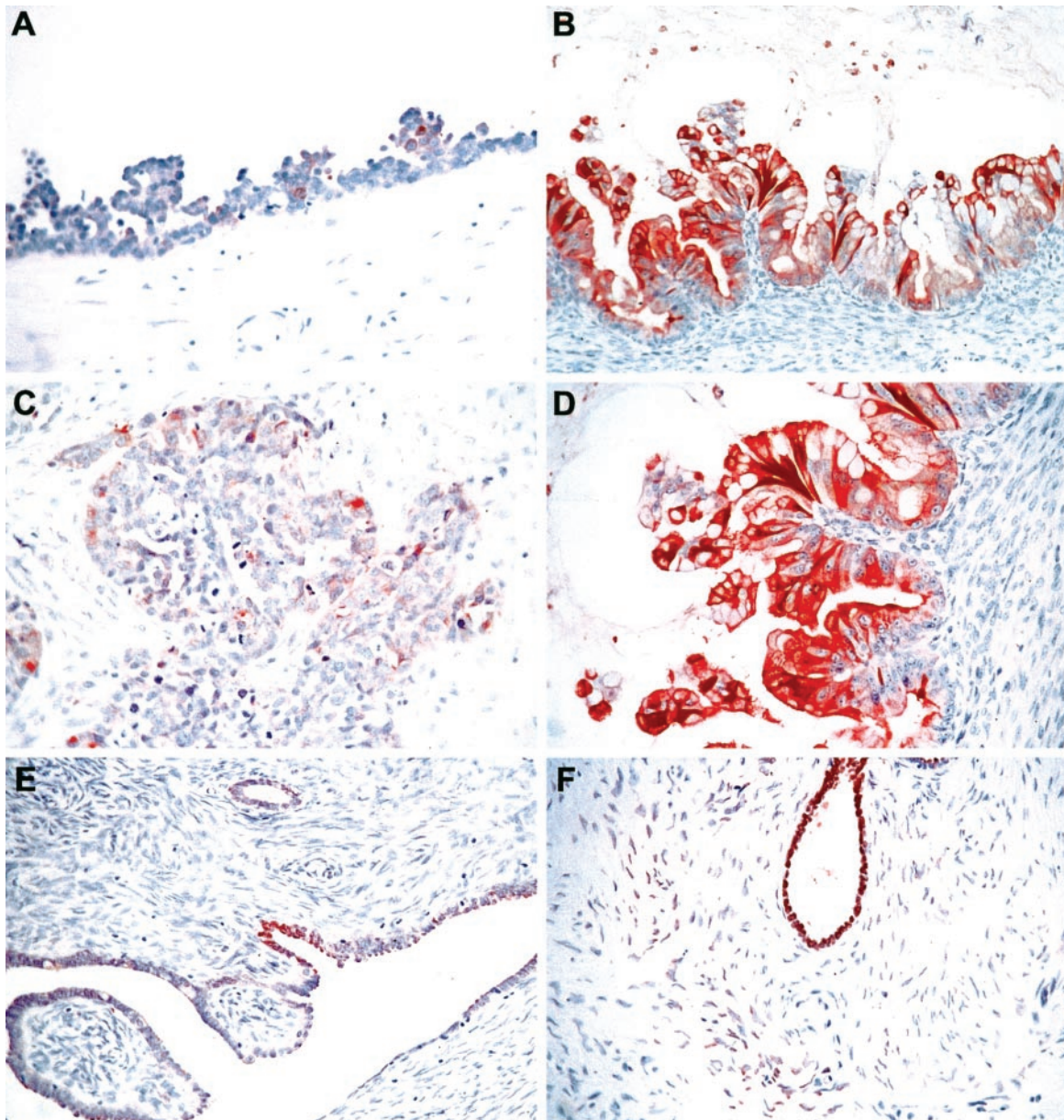
Fig. 6 Immunohistochemical localization of hK10 in ovarian tumor tissues. *A* and *C,* intracytoplasmic staining in epithelial cells in papillary lining (*A*) and invasive component of an invasive papillary serous carcinoma (×400). *B* and *D,* intracytoplasmic staining in epithelial cells of a borderline mucinous tumor (×400). *E* and *F,* intracytoplasmic staining in epithelial component of a serous cystadenofibroma (*E*) and in both epithelial and stromal cells (*F*) of the same tumor (×400).

concentration was significantly higher in cytosols from stage III/IV ovarian cancer than those from stage I/II, indicating that high hK10 concentration is associated with advanced disease stage (Fig. 3).

Univariate and Multivariate Survival Analysis. The results of survival analysis are presented in Table 2. In univariate analysis, hK10-high patients had significantly increased risk for relapse (HR = 1.93) and death (HR = 2.42; $P < 0.05$). When hK10 was considered as a continuous variable, a similar result was also observed. Kaplan-Meier survival curves demonstrated survival differences between hK10-high and hK10-low

patients. As Fig. 4 shows, the probabilities for PFS and OS are lower in hK10-high patients than in hK10-low patients. In multivariate analysis, PFS and OS in hK10-high patients were no different from the survival rates of hK10-low patients (Table 2). Stage of disease, grade, and residual tumor size were identified to have prognostic significance in univariate analysis; however, in multivariate analysis, only stage and residual tumor remained significant.

Univariate and Multivariate Survival Analysis in Subgroups of Patients. The patients were divided into different subgroups based on disease stage, tumor grade, and debulking

success. Univariate and multivariate survival analyses were then performed. The results are shown in Table 3. Univariate analysis has shown that among patients who have stage III/IV disease, hK10-high patients are ~2-fold more likely to relapse and die than hK10-low patients. This survival difference remained significant even after the data were subjected to multivariate analysis. However, this was not observed among patients who have stage I/II disease. hK10 status has no effect on relapse and survival between subgroups of patients who have different tumor grade or debulking success. When Kaplan-Meier survival curves were constructed, they showed similar results (Fig. 5).

Immunohistochemical Localization of hK10 in Ovarian Tumors. In Fig. 6 we present the immunohistochemical localization of hK10 protein in a few representative specimens from ovarian cancer patients.

DISCUSSION

In this study, we found that hK10 protein is expressed in the epithelial cells of the ovary and that its expression is dramatically elevated in cancerous *versus* normal tissues. This elevation was more strongly associated with late disease stage, serous histological type, suboptimal debulking, and large residual tumor. For patients who have stage III/IV ovarian cancer, hK10 overexpression is an independent prognostic indicator, which correlates with poor PFS and OS.

Ovarian cancer is the most lethal gynecological malignancy (17). This is attributable to the fact that at early stage, this disease is occult and asymptomatic. By the time diagnosis is made, more than half of the patients have stage III/IV disease. Furthermore, this disease has a tendency to relapse (18). Some improvement in the OS has been observed in the past decade because of better therapeutic strategies (19). The clinical outcome of ovarian cancer varies from patient to patient. Biomarkers that can predict disease outcome could help tailor different therapeutic strategies to meet individual needs. Our results indicate that hK10 is one such prognostic biomarker. Combined with other prognostic indicators, this new biomarker may contribute to patient subclassification for the purpose of individualizing more effective treatments to such subgroups.

Advanced disease stage, serous histological type, and large residual tumor are known indicators for aggressiveness and poor outcome in ovarian cancer (20). In our study, we also observed the same association (Table 2) and have shown that hK10 expression correlates with these clinicopathological features. The biological rationale underlying the overexpression of hK10 and its association with aggressiveness in ovarian cancer is not clear. It is known that the aggressiveness of a tumor largely depends on its ability to invade adjacent tissues and to metastasize to distant sites. During the process of cancer invasion and migration, natural barriers such as interstitial connective tissues and basement membranes have to be degraded. Proteases are widely believed to be involved in these processes (21, 22). Therefore, the amount of proteases released by the primary tumor may reflect the ability of a tumor to spread. Overexpression of a number of other proteases has been reported to be associated with poor outcome in many cancers, such as urokinase plasminogen activator (23, 24), cathepsin D (25), and matrix metalloproteinase (26). In stage III/IV ovarian cancer,

the tumor cells have already spread beyond the ovaries. We speculate that hK10 may participate in a cascade reaction, which catalyzes the breakdown of extracellular barriers, and, thus, overexpression of hK10 may facilitate ovarian tumor migration. However, this hypothesis needs experimental verification.

Early detection of ovarian cancer is hampered by the lack of a highly sensitive and specific biomarker. Currently, CA 125 is one widely used serum marker for ovarian cancer, but it is not specific or sensitive enough for diagnosis (27–29). Other, newly introduced serum markers (30) such as inhibin (31, 32) and OVX1 (33) have shown some promise but have not gained wide acceptance. Recently, we found that serum hK10 concentration (34) are significantly higher in ovarian cancer patients compared with normal individuals. Overexpression of hK10 in ovarian tumors may account for its elevation in serum. If this correlation is established, serum hK10 concentration may also have prognostic value.

In summary, this is the first report describing that hK10 is an independent prognostic biomarker for late-stage ovarian cancer. additional basic and clinical studies are warranted to help understand the role of hK10 in ovarian cancer pathogenesis and progression.

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