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Quantitative Expression of the Human Kallikrein Gene 9 (*KLK9*) in Ovarian Cancer: A New Independent and Favorable Prognostic Marker

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ABSTRACT

Many members of the human kallikrein gene family were found to be differentially expressed in various malignancies and some are useful cancer diagnostic/prognostic markers. *KLK9* is a newly discovered human kallikrein gene that is expressed in several tissues including thymus, testis, spinal cord, salivary gland, ovary, and skin. Like other kallikreins, the *KLK9* gene was found to be regulated by steroid hormones in cancer cell lines. Our purpose is to examine whether quantitative analysis of *KLK9* expression has prognostic value in ovarian cancer. We studied the expression of *KLK9* by quantitative reverse transcription-PCR in 168 consecutive ovarian tumors of different stages, grades, and histological types, and correlated the expression with clinicopathological parameters, response to chemotherapy, and patients' survival. We found that *KLK9* expression was significantly higher in patients with early disease stages (I or II; $P = 0.044$) and in patients with optimal debulking ($P = 0.019$). Kaplan-Meier survival curves demonstrated that patients with *KLK9*-positive tumors have substantially longer progression-free and overall survival ($P < 0.001$ and $P = 0.016$, respectively). When the Cox proportional hazard regression analysis was applied to subgroups of patients, *KLK9* expression was found to be a significant predictor of progression-free survival in the subgroup of patients with low-grade tumors [hazard ratio (HR), 0.13; $P = 0.0015$], early stage (HR, 0.099; $P = 0.031$); and those with optimal debulking (HR, 0.26; $P = 0.012$). After adjusting for other known prognostic variables, *KLK9* retained its independent prognostic value in all of these subgroups of patients. A negative correlation was found between the expression levels of CA125 and *KLK9* (r_s, 0.350; $P = 0.002$). Our results indicate that *KLK9* is under steroid hormone regulation in ovarian and breast cancer cell lines. Immmunohistochemically, human kallikrein protein (hK9) was localized in the cytoplasm, but not in the nuclei, of the epithelial cells of ovarian cancer tissues. We conclude that *KLK9* is a potential new independent favorable prognostic marker for early stage, low-grade, optimally debulked ovarian cancer patients.

INTRODUCTION

Ovarian cancer represents a great clinical challenge in gynecological oncology. Because most patients are asymptomatic until the disease has metastasized, two-thirds of the patients receive advanced-disease diagnoses (1). In the United States, ~23,000 new cases of ovarian cancer and ~14,000 deaths from the disease were expected for the year 2000 (2), giving it the highest mortality rate of all gynecological malignancies.

Currently, the only tumor marker that has a well-defined and validated role in the management of ovarian cancer is CA125. Serum CA125 has been evaluated in the screening for ovarian cancer, differentiation between benign and malignant ovarian masses, and prognosis (3–6). However, it does not yet have a clear place in diagnosis, prognosis, or in making treatment decisions (7, 8). In addition to ovarian cancer, high levels of CA125 were found in 1% of the normal population, 6% of patients with benign disease, and 28% of patients with nongynecological malignancies (9).

Many potential new serum markers have been evaluated, either alone or in combination with CA125, including CA15-3, CA19-9, OVX1, lysophosphatidic acid (LPA), and carcinoembryonic antigen (CEA; Refs. 7, 10, 11). These new markers do not have a well-defined contribution at present, and only the combination of CA125 with untrasonography yields the highest available sensitivity and specificity (8).

Kallikreins are serine proteases with diverse physiological functions. We, and others, have recently identified 12 new members of the KLK[2] gene family on chromosome 19q13.3-q13.4 (12–21). Several groups have shown that many KLK genes are differentially expressed in various malignancies (reviewed in Ref. 22). PSA is the best marker for prostate cancer (23). hK2 (encoded by the *KLK2* gene) is a useful marker for certain subgroups of patients (24–27). *KLK10* [(normal epithelial cell-specific gene 1 (*NES1*)] was found to be a tumor suppressor gene (28). The human stratum corneum chymotryptic enzyme (*HSCCE*) has been shown to be expressed at abnormally high levels in ovarian cancer (29), and *KLK5* is a poor prognostic marker for ovarian cancer (30). Two new kallikrein proteins, hK6 and hK10, appear to be novel serological markers of ovarian carcinoma (31, 32).

KLK9 (formerly known as *KLK-L3*) is a newly identified member of the KLK gene family (14, 33), expressed in many tissues including cerebellum, spinal cord, testis, prostate, ovary, and skin. *KLK9* was also found to be under steroid hormonal regulation in cancer cell lines (14). Interestingly, *KLK8* [tumor-associated differentially expressed gene-14 (*TADG-14*)/neuropsin] and *KLK10*, the two genes flanking *KLK9*, were found to be differentially expressed in ovarian cancer (34–36). In addition, a very closely localized gene, *KLK6*, is also differentially expressed in primary ovarian tumors (19, 31). We thus hypothesized that *KLK9* may be another member of this group of genes that are differentially expressed in ovarian cancer, and that it may represent a novel diagnostic and/or prognostic marker.

MATERIALS AND METHODS

Study Population. Included in this study were tumor specimens from 168 consecutive patients undergoing surgical treatment for epithelial ovarian carcinoma at the Department of Gynecology, Gynecological Oncology Unit at the University of Turin, Turin, Italy. Selection criteria included confirmation of diagnosis by histopathology. No patient received any treatment before surgery.

Patient ages ranged from 25 to 82 years, with a median of 59 years. The sizes of residual tumors after surgery ranged from 0 to 9 cm, with a median of 2 cm. Follow-up information (median follow-up period, 62 months) was available from 166 patients, among whom 91 (55%) had relapsed and 56 (34%) had died. With

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[2] The abbreviations used are: KLK, human kallikrein (gene); hK, human kallikrein (protein); PSA, prostate-specific antigen; PFS, progression-free survival; OS, overall survival; HR, hazard ratio.

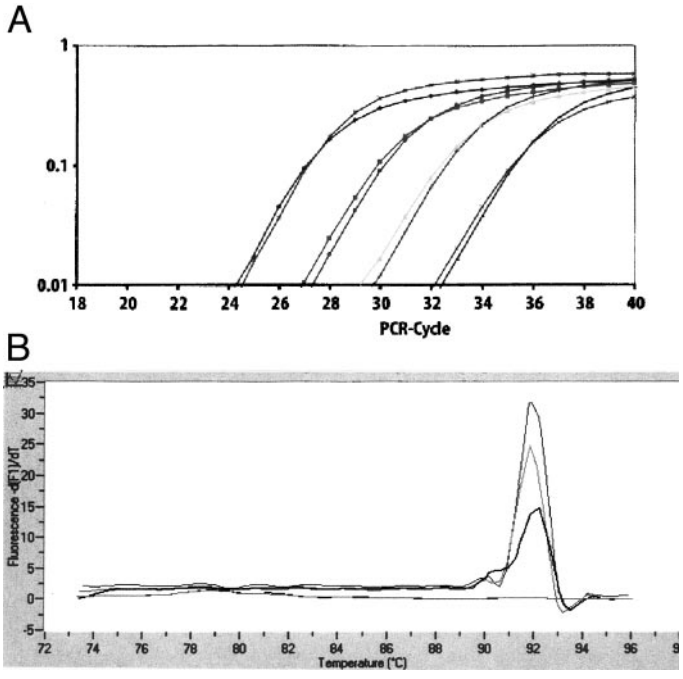

Fig. 1. Quantification of *KLK9* gene expression by real-time PCR. *A*, a logarithmic plot of fluorescence signal (*Y axis*) *versus* cycle number (*X-axis*). Serial dilutions of a total RNA preparation from ovarian tissue were made and an arbitrary copy number was assigned to each sample, according to the dilution factor. Each sample was analyzed in duplicate. *B*, a representative graph of the melting curve of the serial dilutions of the standard cDNA. The specific product melts at 92°C. This product was also run on agarose gel and sequenced to confirm the specificity of amplification.

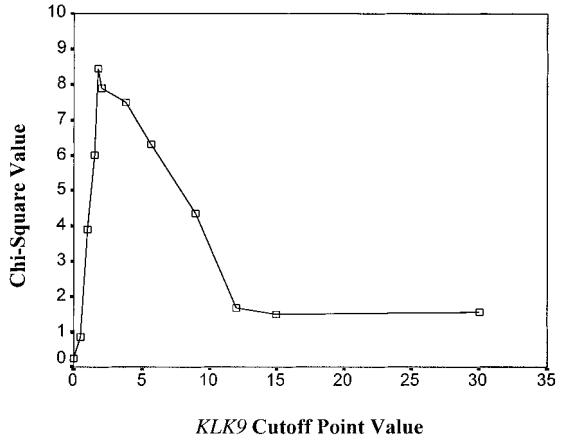

Fig. 2. Determination of the optimal cutoff point value for *KLK9* expression. For details, see text.

respect to histological type, 82 tumors were serous papillary, 31 were endometrioid, 27 were undifferentiated, 13 were mucinous, and 14 were clear cell. The size of the residual tumors ranged from 0 to 9 cm, with a median of 1.0 cm.

Classification of histological types followed the WHO criteria (37). All of the patients were staged according to the International Federation of Gynecology and Obstetrics staging system (38). Grading information was available for 167 patients; 59 (35%) had grade 1 or 2 and 108 (65%) had grade 3 ovarian carcinoma. Grading was established for each ovarian tumor according to the criteria of Day *et al.* (39). All of the patients were treated with postoperative platinum-based regimen chemotherapy. The first-line chemotherapy regimens included cisplatin in 94 (56%) patients, carboplatin in 50 (30%), cyclophosphamide in 69 (41%), doxorubicin in 12 (7%), epirubicin in 20 (12%), paclitaxel in 27 (16%), and methotrexate in 2 (1%). Grade 1 and stage I patients received no further treatment. Response to chemotherapy was assessed as follows: complete response was defined as a resolution of all evidence of disease for at least 1 month; a decrease (lasting at least 1 month) of at least 50% in the diameters of all measurable lesions without the development of new lesions was termed partial response. Stable disease was defined as a decrease of <25% in the product of the diameters of all measurable lesions, an increase of [mteq]25% was termed as a progressive disease. Investigations were performed in accordance with the Helsinki declaration and were approved by the Institute of Obstetrics and Gynecology, Turin, Italy. Tumor specimens were snap-frozen in liquid nitrogen immediately after surgery. Histological examination, performed during intrasurgery frozen-section analysis, allowed representative portions of each tumor containing >80% tumor cells to be selected for storage until analysis.

Total RNA Extraction and cDNA Synthesis. Samples were shipped and stored at −80°C. They were then minced with a scalpel, on dry ice, and transferred immediately to 2-ml polypropylene tubes. They were then homogenized, and total RNA was extracted using Trizol reagent (Life Technologies, Inc., Gaithersburg, MD) following the manufacturer's instructions. The concentration and purity of RNA were determined spectrophotometrically. Total RNA (2 μg) was reverse transcribed into first-strand cDNA using the Superscript preamplification system (Life Technologies, Inc.). The final volume was 20 μl.

Quantitative Real-Time Reverse Transcription-PCR Analysis. On the basis of the published genomic sequence of *KLK9* (GenBank accession no. AF135026), two gene-specific primers were designed (L2–3: 5′-CAA GAC CCC CCT GGA TGT GG-3′ and 5L2: 5′-AGT TTT CAG AGT CCG TCT CGG-3′). These primers spanned more than two exons to avoid contamination by genomic DNA.

Real-time monitoring of PCR reactions was performed using the LightCycler system (Roche Molecular Systems, Indianapolis, IN) and the SYBR Green I dye, which binds preferentially to double-stranded DNA. Fluorescence signals, which were proportional to the concentration of the PCR product, were measured at the end of each cycle and immediately displayed on a computer screen, permitting real-time monitoring of the PCR reaction (40). The reaction

Table 1 *Relationship between KLK9 status and other variables in 168 ovarian cancer patients*

Variable	Patients	No. of patients (%)		*P*
		KLK9 negative	*KLK9* positive	
Stage				
I/II	48	21 (43.7)	27 (56.3)	0.044[a]
III/IV	119	71 (59.7)	48 (40.3)	
x[b]	1			
Grade				
G1/G2	59	32 (54.2)	27 (45.7)	0.49[a]
G3	108	60 (55.6)	48 (44.4)	
x	1			
Histotype				
Serous	82	46 (56.1)	36 (43.9)	
Endometrioid	31	15 (48.4)	16 (51.6)	
Mucinous	13	9 (69.2)	4 (30.8)	0.13[c]
Clear cell	14	11 (78.6)	3 (21.4)	
Undifferentiated	27	11 (40.7)	16 (59.3)	
x	1			
Residual tumor (cm)				
0	69	30 (43.5)	39 (56.5)	
1–2	31	18 (58.1)	13 (41.9)	0.038[c]
>2	66	43 (65.2)	23 (34.8)	
x	2			
Debulking success[d]				
OD	82	37 (45.1)	45 (54.9)	0.019[a]
SO	84	54 (64.3)	30 (35.7)	
x	2			
Menopause				
Pre/peri	54	34 (62.9)	20 (37.1)	0.11[a]
Post	114	59 (51.7)	55 (48.2)	
Response to CTX[e]				
CR/PR	144	78 (54.2)	66 (45.8)	0.60[a]
NC/PD	16	10 (62.5)	6 (37.5)	
NE	8			

[a] Fisher's exact test.
[b] x, status unknown.
[c] χ^2 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.

Table 2 *Univariate and Multivariate Analysis of KLK9 with regard to PFS and OS*

Variable	PFS			OS		
	HR[a]	95% CI[b]	*P*	HR[a]	95% CI[b]	*P*
Univariate analysis						
KLK9						
Negative	1.00			1.00		
Positive	0.45	0.28–0.71	<0.001	0.49	0.28–0.89	0.019
As a continuous variable	0.99	0.98–1.00	0.21	0.97	0.95–1.00	0.087
Stage of disease (ordinal)	2.79	2.04–3.81	<0.001	3.16	2.06–4.83	<0.001
Grading (ordinal)	2.43	1.71–3.44	<0.001	2.66	1.65–4.31	<0.001
Residual tumor (ordinal)	1.27	1.19–1.34	<0.001	1.32	1.22–1.42	<0.001
Histological type[c]	0.88	0.77–1.01	0.067	0.91	0.76–1.07	0.26
Age	1.01	0.99–1.03	0.14	1.02	0.99–1.04	0.11
Multivariate analysis						
KLK9						
Negative	1.00			1.00		
Positive	0.58	0.36–0.93	0.025	0.71	0.31–1.12	0.28
As a continuous variable	0.99	0.98–1.00	0.11	0.98	0.95–1.00	0.085
Stage of disease (ordinal)	1.69	1.18–2.42	0.004	1.91	1.15–3.14	0.011
Grading (ordinal)	1.46	0.97–2.21	0.064	1.49	0.84–2.63	0.16
Residual tumor (ordinal)	1.13	1.06–1.22	<0.001	1.15	1.06–1.23	<0.001
Histological type[c]	1.01	0.87–1.17	0.84	1.09	0.91–1.31	0.31
Age	1.02	1.00–1.043	0.039	1.02	0.99–1.04	0.057

[a] Estimated from Cox proportional hazard regression model.
[b] Confidence interval of the estimated HR.
[c] Endometrioid, mucinous, clear cell, and undifferentiated *versus* serus.

was characterized by the point during cycling, when amplification of PCR products are first detected, rather than the amount of PCR product accumulated after a fixed number of cycles. The higher the starting quantity of the template, the earlier a significant increase in fluorescence was observed (41). The threshold cycle was defined as the fractional cycle number at which fluorescence passes a fixed threshold above baseline (42).

Endogenous Control. For each sample, the amount of the target and of an endogenous control (*β actin*, a housekeeping gene) were determined using a calibration curve (see "Calibration Curves" below). The amount of the target molecule was then divided by the amount of the endogenous reference, to obtain a normalized target value (41).

Calibration Curves. Separate calibration (standard) curves for actin and *KLK9* were constructed using serial dilutions of total cDNA from healthy human ovarian tissue (purchased from Clontech, Palo Alto, CA), as described previously (41, 43). The standard curve calibrators were included in each run. The LightCycler software automatically calculated the standard curve by plotting the starting dilution of each standard sample *versus* the threshold cycle, and the sample concentrations were then calculated accordingly (Fig. 1). Standards for both *KLK9* and *actin* RNAs were defined to contain an arbitrary starting concentration, and serial dilutions (with concentrations defined according to the dilution factor) were used to construct the standard curve.

PCR Amplification. The PCR reaction was carried out on the LightCycler system. For each run, a master mixture was prepared on ice, containing 1 μl of cDNA, 2 μl of LC DNA Master SYBR Green I mix, 50 ng of primers, and 1.2 μl of 25 mM $MgCl_2$. The final volume was adjusted with H_20 to 20 μl. After the reaction mixture was loaded into a glass capillary tube, the cycling conditions were carried out as follows: initial denaturation at 94°C for 10 min, followed by 45 cycles of denaturation at 94°C for 0 s, annealing at 55°C for 10 s, and extension at 72°C for 30 s. The temperature transition rate was set at 20°C per second. Fluorescent product was measured by a single acquisition mode at 86°C after each cycle.

Melting Curve. For distinguishing specific from nonspecific products and primer dimers, a melting curve was obtained after amplification by holding the temperature at 70°C for 30 s, followed by a gradual increase in temperature to 99°C at a rate of 0.1°C/s, with the signal acquisition mode set at step, as described previously (Ref. 44; Fig. 1). To verify the melting curve results, representative samples of the PCR products were run on 1.5% agarose gels, purified, and cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The inserts were sequenced using vector-specific primers, with an automated DNA sequencer.

Statistical Analysis. Associations between clinicopathological parameters such as stage, grade, histotype, and residual tumor, and *KLK9* expression were analyzed by the χ^2 test or the Fisher's exact test, when appropriate. For survival analysis, two different end points, cancer relapse (either local recurrence or distant metastasis) and death, were used to calculate PFS and OS, respectively. PFS was defined as the time interval between the date of surgery and the date of identification of recurrent or metastatic disease. OS was defined as the time interval between the date of surgery and the date of death.

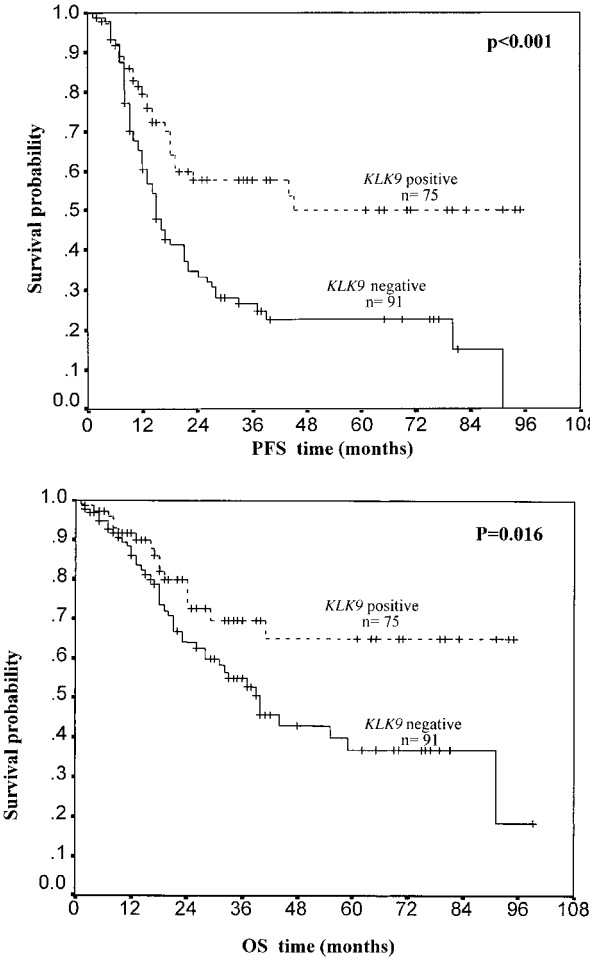

Fig. 3. Kaplan-Meier survival curves for patients with *KLK9*-positive and -negative ovarian tumors. *n*, number of samples.

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*
Tumor grade 1–2						
KLK9 unadjusted	0.13	0.039–0.46	0.0015	0.20	0.044–0.91	0.038
KLK9 adjusted[c]	0.19	0.049–0.72	0.016	0.25	0.045–0.89	0.042
Tumor grade 3						
KLK9 unadjusted	0.60	0.36–0.99	0.045	0.68	0.35–1.30	0.24
KLK9 adjusted[c]	0.65	0.38–1.12	0.12	0.81	0.40–1.65	0.58
Stage I–II						
KLK9 unadjusted	0.099	0.012–0.81	0.031	0.24	0.014–3.89	0.31
KLK9 adjusted[d]	0.097	0.010–0.96	0.046	0.57	0.15–2.041	0.38
Stage III						
KLK9 unadjusted	0.64	0.40–1.02	0.062	0.71	0.40–1.27	0.25
KLK9 adjusted[d]	0.74	0.45–1.22	0.24	0.83	0.43–1.59	0.58
Optimal debulking success						
KLK9 unadjusted	0.26	0.09–0.75	0.012	0.93	0.18–4.61	0.92
KLK9 adjusted[e]	0.27	0.09–0.78	0.015	0.91	0.16–5.12	0.91
Suboptimal debulking success						
KLK9 unadjusted	0.83	0.50–1.39	0.49	0.87	0.45–1.65	0.67
KLK9 adjusted[e]	0.68	0.39–1.19	0.19	0.67	0.34–1.32	0.25

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

The Cox univariate and multivariate proportional hazard regression model (45) was used to evaluate the HR (relative risk of relapse or death in the *KLK9*-positive group). In the multivariate analysis, the models were adjusted for *KLK9* expression, clinical stage, histological grade, residual tumor, and age.

Kaplan-Meier survival curves (46) were constructed for *KLK9*-positive and *KLK9*-negative patients. For further analysis, patients were divided into two groups, either by the tumor grade (grade 1–2 *versus* grade 3), tumor stage (stage I-II *versus* stage III-IV), or by the success of debulking (optimal *versus* suboptimal debulking group). In each category, survival rates (disease-free survival and OS) were compared between *KLK9*-positive and *KLK9*-negative groups. The differences between the survival curves between groups were tested for statistical significance by the log-rank test (47).

Immunohistochemistry. Rabbit polyclonal antibody was raised against hK9 peptide sequence: N_2H-CPHPGFNKDLSANDHN-$CONH_2$ according to standard procedures. Immunohistochemical staining for hK9 was performed according to a standard immmunoperoxidase 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

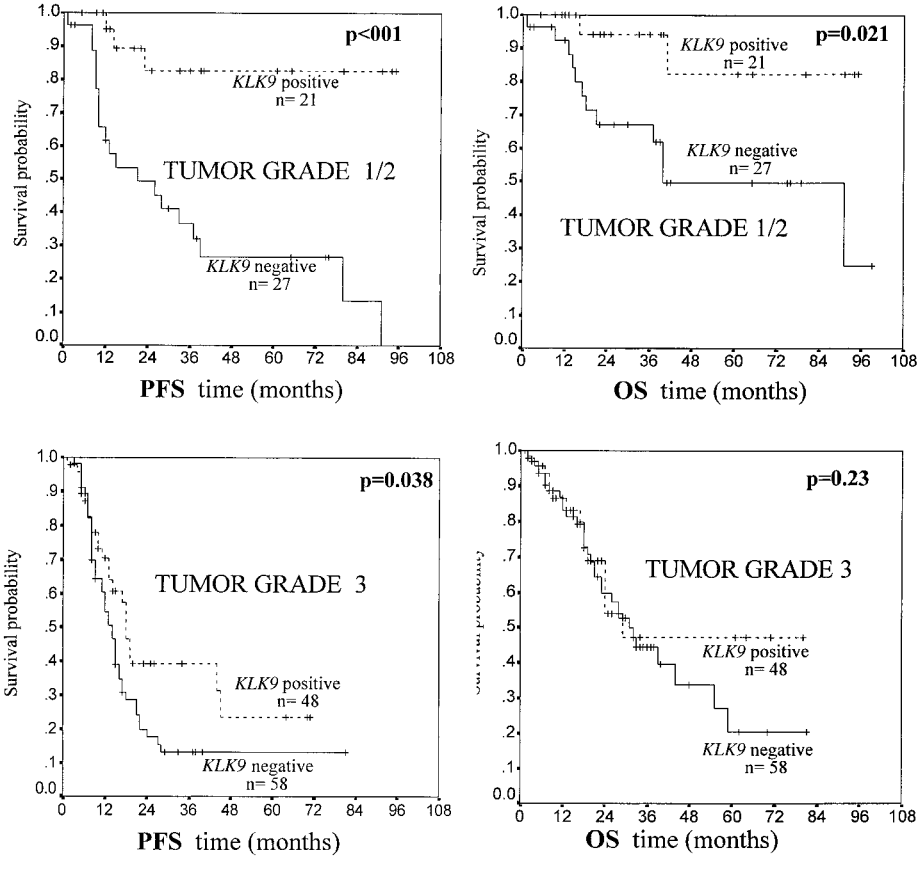

Fig. 4. Kaplan-Meier survival curves for patients with *KLK9*-positive and -negative tumors, stratified by tumor grade. *n*, number of samples.

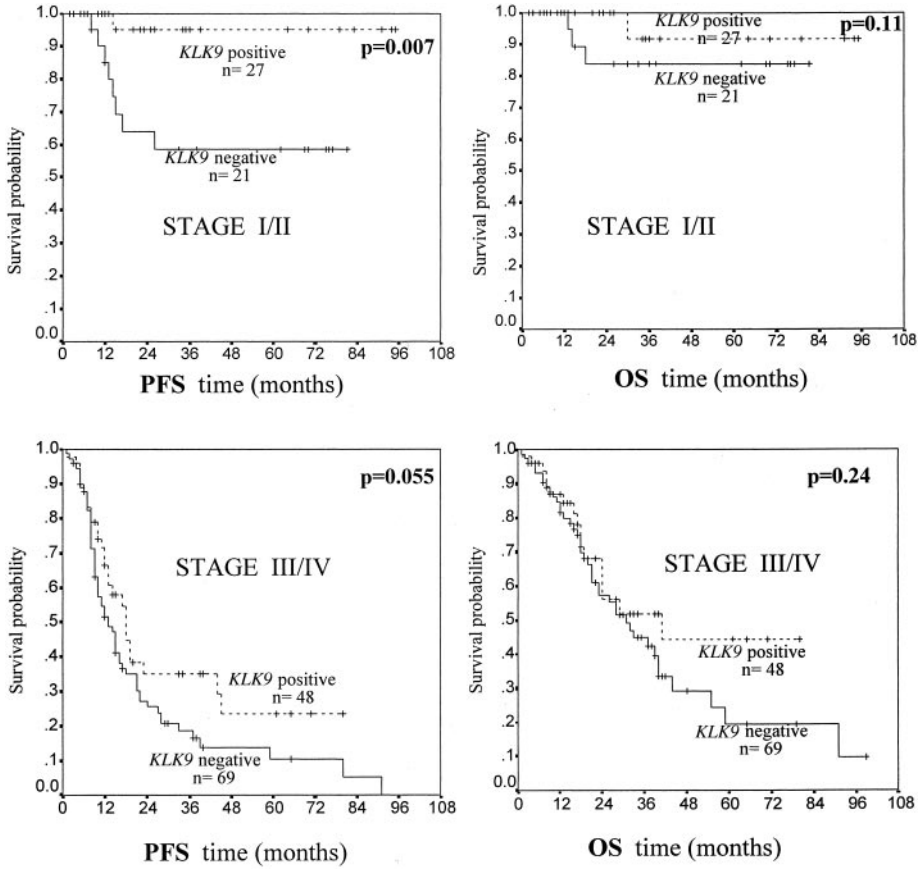

Fig. 5. Kaplan-Meier survival curves for patients with *KLK9*-positive and -negative tumors, stratified by tumor stage. *n*, number of samples.

with 20% protein blocker (Signet Labs) for 10 min. The primary antibody was then added at 1:6000 dilution for 1 h at room temperature. After washing, biotinylated antirabbit antibody (Signet Labs) was added, diluted 4-fold in antibody dilution buffer (Dako). After incubation and washing, streptavidin-tagged horseradish peroxidase was added for 30 min at room temperature. After washing, detection was achieved with amino ethyl carbazol (AEC) for 5–10 min. The slides were then counterstained with hematoxylin and then mounted with coverslips.

Cell Lines and Hormonal Stimulation Experiments. The epithelial ovarian cancer cell line BG-1 and breast cancer cell lines BT-474 and T-47D, and MCF-7 line were purchased from the American Type Culture Collection (ATCC), Manassas, VA. Cells were cultured in RPMI media (Life Technologies, Inc.) supplemented with glutamine (200 mmol/liter), bovine insulin (10 mg/liter), fetal bovine serum (10%), antibiotics, and antimycotics, in plastic flasks, to near confluency. The cells were then aliquoted into 24-well tissue culture plates and cultured to 50% confluency. Twenty-four h before the experiments, the culture media were changed into Phenol Red-free media containing 10% charcoal-stripped fetal bovine serum. For stimulation experiments, various steroid hormones dissolved in 100% ethanol were added to the culture media at a final concentration of 10^{-8} mM. Cells stimulated with 100% ethanol were included as controls. The cells were cultured for 24 h, and then were harvested for mRNA extraction.

RESULTS

***KLK9* Expression and Relation to Other Variables.** First, an optimal cutoff value was defined by χ^2 analysis, based on the ability of *KLK9* values to predict the OS of the study population. As shown in Fig. 2, a value of 1.84 (this is a unitless ratio) was shown to be the optimal cutoff ($\chi^2 = 8.54$; $P = 0.003$). This cutoff (54th percentile) identifies 46% of patients as being *KLK9* positive.

Table 1 depicts the distribution of *KLK9* expression (positive or negative) in ovarian tumor tissues, in relation to clinical stage, grade, histological type, size of residual tumor, menopausal status, and chemotherapy response. *KLK9* expression was significantly higher in patients with early stages (I or II) compared with advanced stages (III or IV; $P = 0.044$) and in patients with optimal debulking ($P = 0.019$). Also, a slightly higher percentage of patients with positive *KLK9* expression was found to have grade 1 or 2 (46%) compared with grade 3 (44%); however, this difference was not statistically significant ($P = 0.49$). Also, *KLK9* expression was higher (although not statistically significant) in patients with complete or partial response to chemotherapy, compared with those with no response or with progression of the disease (46% *versus* 37%, respectively) and in patients with no residual tumors (56%) compared with those with residual tumors (38%). On the other hand, no significant associations were found between *KLK9* expression and different histological types, or menopausal status.

Survival Analysis. The strength of the associations between each individual predictor and PFS or OS are shown in the univariate analysis in Table 2. Stage of disease, histological grade, and residual tumor size showed a strong association with cancer relapse and death ($P < 0.001$). *KLK9* expression was also found to be a significant predictor of PFS and OS (HR of 0.45 and 0.49 and P of < 0.001 and 0.019, respectively). Kaplan-Meier survival curves (Fig. 3) also demonstrate that patients with *KLK9*-positive tumors have substantially longer PFS and OS ($P < 0.001$ and $P = 0.016$, respectively) compared with those who are *KLK9*-negative.

When all of the predictors were included in the Cox model (multivariate analysis, Table 2), the stage of disease and residual tumor size retained their prognostic significance. *KLK9* expression retained its prognostic significance for PFS, but not the OS, (HR of 0.58 and 0.71 and P of 0.025 and 0.28 for the PFS and OS, respectively).

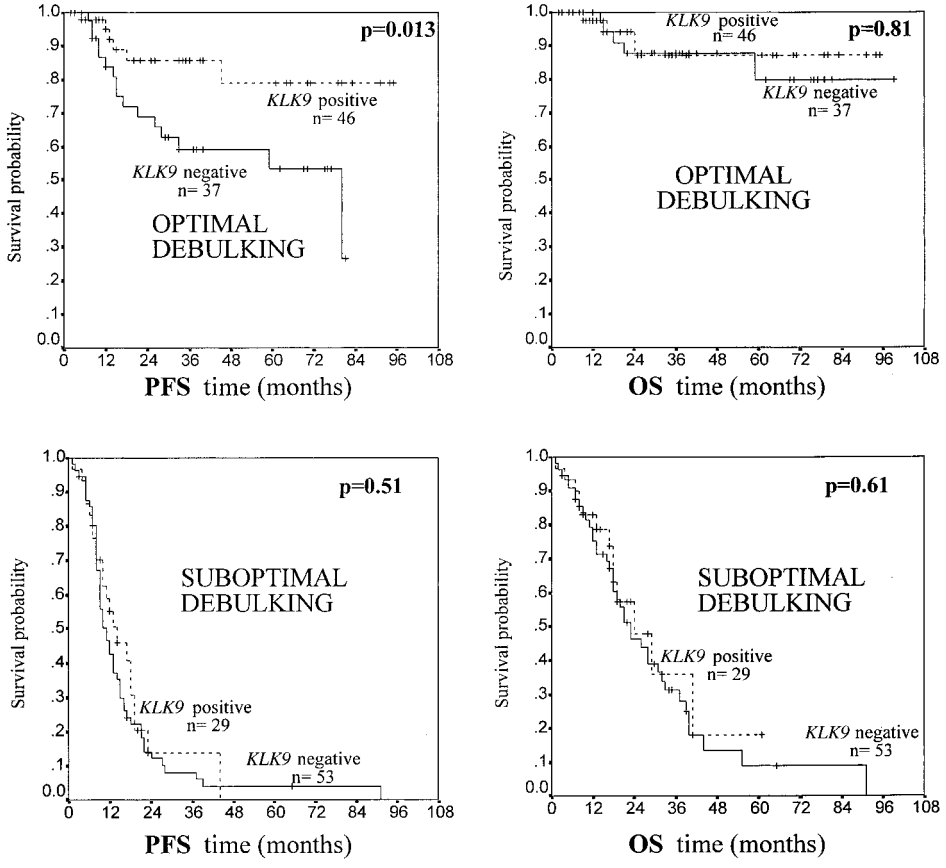

Fig. 6. Kaplan-Meier survival curves for patients with *KLK9* positive and negative tumors, stratified by the debulking success. *n*, number of samples.

When the Cox proportional hazard regression analysis was applied to subgroups of patients (Table 3), *KLK9* expression was found to be a significant predictor of PFS in the subgroup of patients with grade 1 or 2 (HR, 0.13; $P = 0.0015$; Table 3), Stage I or II (HR, 0.099; $P = 0.045$) and those with optimal debulking success (HR, 0.26; $P = 0.012$). After adjusting for other known prognostic variables, *KLK9* retained its independent prognostic value in all of these subgroups of patients. With respect to the OS, *KLK9* expression was a favorable prognostic marker for the subgroup of patients with grade 1 or 2 tumors and retained its independent prognostic value after adjusting for other known prognostic variables (adjusted HR, 0.20; $P = 0.038$; Table 3).

The same results were also demonstrated by the Kaplan-Meier curves, by which *KLK9* was found to be an independent favorable prognostic marker for PFS and OS ($P < 0.001$ and 0.016, respectively). Shown in Fig. 4 are the PFS and OS curves for cancer patients with histological grades 1–2 or 3. Patients with *KLK9*-positive tumors had substantially longer PFS and OS than did patients with *KLK9*-negative tumors ($P < 0.001$ and 0.021, respectively). These differences were not seen in patients with grade 3 tumors except in PFS. With respect to stage of the disease, *KLK9*-positive patients in stage I or II have a significantly better PFS ($P = 0.007$) but not OS (Fig. 5). Similarly, patients with *KLK9*-positive tumors who had undergone optimal debulking had a higher probability of PFS (but not OS) than did patients who had *KLK9*-negative tumors ($P = 0.013$; Fig. 6). No differences in the PFS or OS were observed when surgical debulking was suboptimal (Fig. 6).

A weak negative correlation was found between the expression levels of serum CA125 and *KLK9* mRNA levels (r_s, 0.350; $P = 0.002$; Fig. 7).

Immunohistochemical Localization of hK9. As shown in Fig. 8, hK9 is seen in the cytoplasm but not in the nuclei of the epithelial cells of normal ovarian and ovarian cancer tissues, which confirms the epithelial origin of the protein and is consistent with previous reports indicating that it is a secreted protein. These results are consistent with previous results for other kallikrein proteins which were also localized in the cytoplasm of epithelial cells.

Hormonal Regulation of hK9. We studied *KLK9* expression in BG-1 epithelial ovarian cell line and the BT-474, T47-D, and MCF-7 breast cancer cell lines. *KLK9* was found to be up-regulated by steroid hormones, particularly estrogens and progestins. Higher expression levels were obtained 48 h after hormonal stimulation (data not shown). No significant changes in *KLK9* level was seen in the receptor-negative BT-20 cell lines.

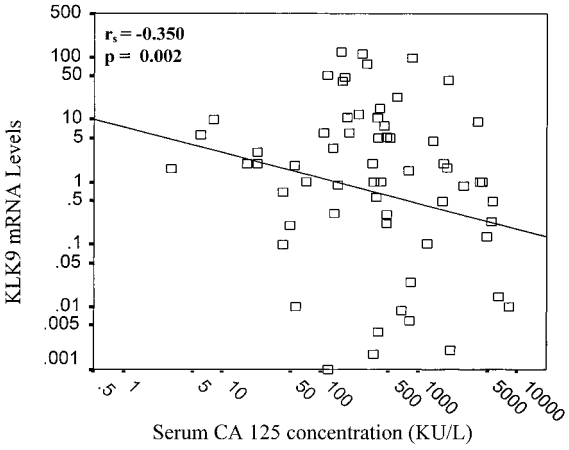

Fig. 7. Correlation between serum CA125 and tumor levels of *KLK9*. r_s, Spearman correlation coefficient.

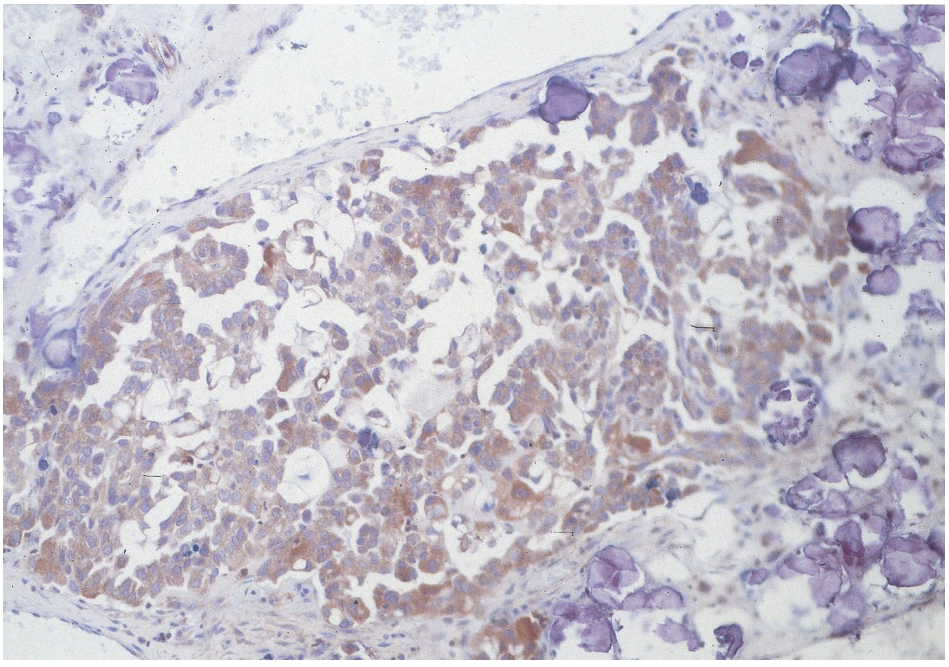

Fig. 8. Immunohistochemical localization of hK9 protein in a serous ovarian carcinoma. Moderate cytoplasmic positivity in tumor cells with no nuclear staining and negative stroma.

DISCUSSION

Population screening is a milestone for improving ovarian cancer prognosis. CA125 has limitations as a single marker, because its levels are elevated in only about one-half of women with stage I ovarian cancer. The development of new biomarkers for ovarian cancer may help to improve the diagnostic/prognostic power of CA125 (10–11). Although newly identified markers for ovarian cancer may also not be sufficient alone, the development of a panel of markers that can be used together, in multiparametric strategies, may be one solution (48). Jacobs *et al.* (49) recently reported the first study with annual multimodal screening for 3 years. *KLK9*, along with a few other newly identified kallikreins, may be good candidates for this application (31, 32).

A recent study suggested that CA125 could be used for prediction of optimal primary tumor cytoreduction, but only in stage III tumors (8). Because *KLK9* expression levels are significantly different in patients with optimal and suboptimal cytoreduction, and in patients with early and late stages of the disease (Table 3 and Figs. 5 and 6), it might also be tested for such applications. In addition, the role of CA125 in follow-up and prediction of prognosis is uncertain (7). *KLK9*, being a favorable prognostic factor (Fig. 3), may find applicability in this regard.

Our findings indicate that *KLK9* is a favorable prognostic factor in ovarian cancer. Interestingly, additional data from other groups and our laboratory indicate that four other kallikrein genes (*KLK6*, *KLK7*, *KLK8*, and *KLK10*) are all differentially expressed in ovarian cancer (19, 29, 31, 34, 35, 50), and, with the exception of KLK8, all of the genes are found to have higher expression levels in advanced and more aggressive cancer. In view of this data, it will be interesting to examine simultaneously the expression of many kallikreins in ovarian cancer and to determine their function in this tissue.

The mechanism by which *KLK9* and other kallikreins might be involved in the pathogenesis or progression of ovarian cancer is not known. We speculate that the enzymatic activity of these serine proteases might initiate or terminate certain biological events, *e.g.*, the onset of angiogenesis, activation or inactivation of growth factors, receptors, cytokines, and so forth. A recent report provided evidence that another closely related kallikrein, hK3 (PSA), has antiangiogenic activity, and that this activity may be related to its action as a serine protease (51). This study suggested also that other members of the kallikrein multigene family should be evaluated for potential antiangiogenic action. Other studies suggested that PSA inhibits the growth of MCF-7 breast cancer cell lines and prolongs the doubling time of PC-3 prostate cancer cell lines (52, 53).

To explore the mechanism by which *KLK9* is down-regulated in advanced ovarian cancer, we examined the effect of steroid hormones on *KLK9* expression in different ovarian and breast cancer cell lines. Our results indicate that *KLK9* is up-regulated by steroid hormones, primarily progesterone and estrogen. Our data show also that *KLK9* is a favorable prognostic factor for ovarian cancer. Ovarian cancer is one of the endocrine-related malignancies (54), and oral contraceptive pill administration decreases the risk of ovarian cancer (1). Furthermore, the growth of ovarian carcinoma cell lines is sensitive to estrogen (55). Progesterone promotes cell differentiation and apoptosis, and it has been shown to inhibit DNA synthesis and cell division (56). Recent studies supported the favorable prognostic value of progesterone receptor and its level of expression in ovarian cancer, and indicated that progesterone receptor-negative status is more abundant in grade 3 ovarian tumors (54, 57). Taken together, these data allow us to hypothesize that *KLK9* is a candidate downstream target through which progestins and estrogens are involved in the pathogenesis of ovarian cancer.

KLK9 expression levels are negatively correlated with serum CA125 concentration (Fig. 7), in agreement with previous studies showing that higher CA125 levels are associated with poor prognosis in ovarian cancer (58). High CA125 expression levels were associated with serous and endometrioid tumors (58). Here, we found equal levels of *KLK9* expression in serous and nonserous tumors (45 *versus* 42%; $P = 0.39$; Table 1). This can be used for assessing prognosis, in the subgroup of patients with nonserous ovarian cancer, in which CA125 is not usually informative.

In conclusion, we here report for the first time that higher *KLK9* expression has favorable prognostic value in ovarian cancer. These data add to the growing recent literature, which suggests that many other members of the same gene family (notably *KLK6*, *KLK7*, *KLK8*, and *KLK10*) also have prognostic value in ovarian cancer. It is conceivable

that all of these kallikreins participate in a common pathway that is activated during ovarian cancer initiation and progression.

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