Higher Human Kallikrein Gene 4 (*KLK4*) Expression Indicates Poor Prognosis of Ovarian Cancer Patients

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ABSTRACT

Purpose: ***Kallikrein*** **gene 4 (*KLK4*, also known as prostase/*KLK-L1*), located on chromosome 19q13.4, is one of the newly discovered members of the human *KLK*-like gene family. This gene is up-regulated by androgens in the LNCaP prostatic carcinoma cell line and by androgens and progestins in the BT-474 breast cancer cell line. On the basis of its apparent association with hormonally regulated tissues, we have undertaken to examine the prognostic value of *KLK4* expression in 147 malignant ovarian tissues.**

Experimental Design: **Tumors were pulverized, total RNA was extracted, and cDNA was prepared by reverse transcription. *KLK4* was amplified by PCR using gene-specific primers, and its identity was verified by sequencing. Ovarian tissues were then classified as *KLK4*-positive or -negative, based on ethidium bromide visualization of the PCR product on agarose gels.**

Results: ***KLK4*** **was found to be expressed in 69 (55%) of 147 of ovarian cancer samples. We found a strong positive association between *KLK4* expression and tumor grade ($P = 0.02$) and clinical stage ($P < 0.001$). Univariate survival analysis revealed that patients with ovarian tumors positive for *KLK4* expression had an increased risk for relapse and death ($P = 0.003$ and 0.001, respectively). Whereas knowledge of *KLK4* status did not significantly increase the prognostic power of the multivariate models, additional analyses did determine that *KLK4* was an independent unfavorable prognostic factor in patients with grade 1 and 2 tumors.**

Conclusions: **Our findings indicate that *KLK4* expression is associated with more aggressive forms of ovarian cancer.**

INTRODUCTION

Among women, ovarian cancer is the sixth most common malignancy worldwide and causes more deaths than any other cancer of the female reproductive system. Overall survival for epithelial ovarian cancer patients is still very poor (1). This is attributable not only to the intrinsic aggressiveness of this disease but also to the fact that most patients are diagnosed in advanced stages. Up to this time, there is no method available to detect early-stage disease. Therefore, because survival rate could be theoretically improved (2), there is great interest in the identification of new markers that could aid in early detection and facilitate biological characterization of the disease. Although the etiology and the molecular mechanisms of ovarian cancer are poorly understood, there is some evidence indicating that the aberrant expression of serine proteases correlates positively with invasiveness and metastatic potential of ovarian cancer cells (3–5). Indeed, proteases are implicated in cancer progression because they can confer on tumor cells the ability to degrade the extracellular matrix, thereby aiding the spread of cancer cells (6–10).

The *KLK4*[2] gene, also known as prostase/*KLK-L1*, was first discovered independently by two groups using subtractive hybridization (11), or the positional candidate gene approach (12). This gene shares 38% identity with and 52% similarity to the *PSA* gene (2). On the basis of Northern blot analysis, the expression of this gene was determined to be restricted to the male prostate (11); however, subsequent reverse transcription-PCR studies demonstrated the presence of *KLK4* mRNA not only in the prostate but also at lower amounts in testicular, mammary, adrenal, brain, uterine and thyroid tissues (12). The expression of KLK4 has been shown to be up-regulated by androgens in the prostate cancer-derived cell line LNCaP (11), whereas in the breast carcinoma cell line BT-474, it is up-regulated by both androgens and progestins (12). A putative androgen response element (ARE) has been postulated in the promoter of *KLK4* gene (13), but its functionality is still to be confirmed experimentally. On the basis of the open reading frame predicted from the *KLK4* cDNA sequence, the *KLK4* mRNA is translated into a 254-amino acid prepropeptide with the first 26 NH_2-terminal amino acids as the signal peptide (11–13); this suggests that hK4 is a secreted protein.

Although the precise role of androgens in the ovary is not known, there is some evidence associating androgens with ovarian malignancy. Androgens are present in plasma at higher

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[2] The abbreviations used are: KLK, kallikrein; PSA, prostate specific antigen; KLK-L, KLK-like.

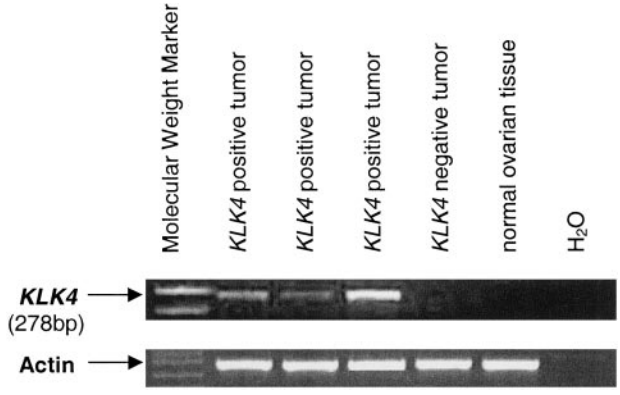

Fig. 1 *KLK4* expression in cancerous and normal ovarian tissues and their corresponding actin profiles. The length of the *KLK4* PCR product is 278 bp and that of the actin is 810 bp.

concentrations at any one time of the menstrual cycle in comparison to estrogen levels (14). Androgens are the principal sex steroids of the fluid in growing follicles (15); the presence of androgen receptors in ovarian epithelial cells suggests that the cells can respond to androgens (16). Epidemiological evidence also supports a relationship between the risk of ovarian cancer and androgens (17). This is especially the case in women suffering from polycystic ovary syndrome (18, 19) known to have elevated concentrations of circulating androgens.

Preliminary work by our group indicated that *KLK4* expression in normal ovarian tissue was very low or undetectable. Subsequently, we found significant expression in a subset of ovarian cancer tissue extracts. On the basis of this, we have undertaken to examine whether *KLK4* expression in ovarian cancer has any prognostic significance.

MATERIALS AND METHODS

Ovarian Cancer Specimens and Patients. One hundred forty-seven consecutive patients with epithelial ovarian carcinoma were included in this study, with ages ranging from 25 to 80 (median, 58 years). Patients underwent surgery and treatment for primary ovarian carcinoma at the Department of Obstetrics and Gynecology, Gynecological Oncology Unit, University of Turin, Turin, Italy, between July 1991 and April 1999. Follow-up information (median follow-up period, 48 months) was available for 139 patients, among whom 84 (54%) have relapsed and 59 (38%) have died. Of the 145 ovarian adenocarcinomas for which histological diagnoses was available, 64 (44%) were serous papillary, 26 (18%) endometrioid, 23 (16%) undifferentiated, 12 (8%) clear cell, 11 (8%) mucinous, and 9 (6%) Mullerian. Because of the relatively small sample size, the clear cell, mucinous, and Mullerian types were combined into one category termed "others." Classification of histological types followed the WHO criteria (20). 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 >70% tumor cells to be selected for storage until analysis. Samples were shipped at −80°C. Patients with disease at all four of the clinical stages (I-IV) were included in this study, in which clinical staging was determined according to the International Federation of Gynecology and Obstetrics system (21). Grading information was available for 143 patients: 10 (7%) of them had low potential malignancies (LPM); 17 (12%) had grade 1; 24 (17%) had grade 2; and 92 (64%) had grade 3 ovarian carcinoma. Grading was established for each ovarian tumor according to the criteria of Day *et al*. (22). All of the patients were treated with postoperative platinum-based chemotherapy. The first-line chemotherapy regiments included cisplatin in 82 (56%) patients, carboplatin in 44 (30%), cyclophosphamide in 60 (41%), doxorubicin in 10 (7%), epirubicin in 18 (12%), paclitaxel in 23 (16%), and methotrexate in 2 (1%) patients. 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 <50% or an increase of <25% in the product of the diameters of all measurable lesions, and an increase of at least 25% was defined

Table 1 Relationship between *KLK4* status and other variables in 147 patients with primary ovarian cancer

Variable	Patients	No. of patients (%)		*P*
		KLK4 negative	*KLK4* positive	
Stage				
I	28	22 (78.6)	6 (21.4)	
II	10	8 (80.0)	2 (20.0)	0.001[b]
III	93	43 (46.2)	50 (53.8)	
IV	11	2 (18.2)	9 (81.8)	
X[a]	5			
Grade				
GB	10	9 (90.1)	1 (10.0)	
G1	17	12 (70.6)	5 (29.4)	0.024[b]
G2	24	10 (41.7)	14 (58.3)	
G3	92	45 (48.9)	47 (51.1)	
x	4			
Histotype				
Serous	64	30 (46.9)	34 (53.1)	
Endometrioid	26	17 (65.4)	9 (34.6)	0.439[b]
Undifferentiated	23	12 (52.2)	11 (47.8)	
Others	32	18 (56.3)	14 (43.8)	
x	2			
Residual tumor (cm)				
0	57	42 (73.7)	15 (26.3)	
1–2	27	9 (33.3)	18 (66.7)	<0.001[b]
>2	56	24 (42.9)	32 (57.1)	
x	7			
Menopause				
Pre/peri	48	24 (50.0)	24 (50.0)	0.366[c]
Post	99	54 (54.5)	45 (45.5)	
Response to CTX[d]				
NC/PD	16	5 (31.3)	11 (68.8)	0.047[c]
CR/PR	121	69 (57.0)	52 (43.0)	
NE	10			

[a] x, status unknown.
[b] χ^2 test.
[c] Fisher's exact test.
[d] CTX, chemotherapy; CR, complete response; PR, partial response; NC, no change; PD, progressive disease; NE, not evaluated.

Table 2 Univariate and multivariate analysis of *KLK4* expression in relation to progression-free and overall survival

Variable	Progression-free survival			Overall survival		
	HR[a]	95% CI[b]	*P*	HR[a]	95% CI[b]	*P*
			Univariate analysis			
KLK4						
Negative	1.00			1.00		
Positive	1.95	1.26–3.02	0.0026	2.45	1.43–4.22	0.0012
Disease stage (ordinal)	2.96	2.08–4.21	<0.001	3.52	2.25–5.53	<0.001
Grading (ordinal)	2.26	1.61–3.21	<0.001	2.55	1.60–4.07	<0.001
Residual tumor (ordinal)	1.86	1.56–2.21	<0.001	2.03	1.63–2.53	<0.001
Histologic type[c]	1.01	0.89–1.13	0.92	1.09	0.95–1.25	0.21
Age	1.01	0.99–1.03	0.18	1.01	0.99–1.04	0.20
			Multivariate analysis			
KLK4						
Negative	1.00			1.00		
Positive	1.17	0.73–1.87	0.51	1.32	0.73–2.37	0.34
Disease stage (ordinal)	1.81	1.21–2.71	0.004	1.99	1.18–3.36	0.009
Grading (ordinal)	1.52	1.02–2.27	0.04	1.54	0.89–2.65	0.17
Residual tumor (ordinal)	1.46	1.19–1.79	<0.001	1.62	1.25–2.11	<0.001
Histologic type[c]	1.02	0.90–1.15	0.74	1.14	0.99–1.31	0.062
Age	1.01	0.98–1.03	0.44	1.01	0.97–1.03	0.88

[a] Hazard ratio (HR) estimated from Cox proportional hazard regression model.
[b] Confidence interval of the estimated HR.
[c] Serous *versus* all other types (endometrioid, undifferentiated, and others).

as progressive disease. Investigations were performed in accordance with the Helsinki declaration and were approved by the Institute of Obstetrics and Gynecology, Turin, Italy.

Preparation of Total RNA. Tumor tissue (50–100 mg) was pulverized on dry ice, followed by total RNA extraction using the TRIzol method (Life Technologies, Inc., Gaithersburg, MD). After measurement of total RNA concentration by spectrophotometry, 4 μg of total RNA from each tissue was used to carry out first-strand cDNA synthesis using the SuperScript preamplification system, as prescribed by the manufacturer (Life Technologies, Inc.). To test the success of cDNA synthesis, 1 μl of the reverse transcription product was amplified using PCR with primers specific for actin. Product was visualized on a 2% agarose gel stained with ethidium bromide.

Assessment of *KLK4* Expression. One μl of first-strand cDNA product was amplified using HotSta Taq DNA polymerase in PCR reactions, as prescribed by the manufacturer (Qiagen Inc, Mississauga, Ontario, Canada). Primers used in this reaction (5′-GAATTCCTTCCGCAGGATGT-3′ and (5′-TGACCCGCTGTACCACCCCA-3′) were specific for KLK4. PCR reactions were carried out in an Eppendorf thermocycler (enzyme activation at 95°C for 15 min; 40 cycles of denaturation at 94°C for 30 s; annealing at 68°C for 1 min; extension at 72°C for 30 s; final extension at 72°C for 10 min) and the 278-bp KLK4 product was visualized with ethidium bromide on 2% agarose gels. Gels were photographed under UV light with Speedlight Gel Documentation System (Lightools Research, Encinitas, CA), using ×20 magnification. Images were scored by two independent observers for presence (positive) or absence (negative) of a *KLK4* PCR-generated band. Each assay was performed twice to ensure reproducibility of data.

Statistical Analysis. *KLK4* status of ovarian tissues was compared with the distribution of clinical stage, histological grade, histological type, and residual tumor size using the contingency table and the χ^2 test. Comparison of *KLK4* expression profile of tumors to menopausal status (post *versus* pre/peri) and response to chemotherapy was performed using Fisher's exact test. The relationship between progression-free or overall survival and each of the parameters examined in this study was established using the hazards ratio (risk of relapse and death) and its corresponding 95% confidence interval, calculated univariately with the Cox proportional regression model (23). The multivariate form of this model, adjusted for patient age, clinical stage, tumor grade, residual tumor size and histological type, was also used to assess *KLK4* status of the ovarian tumors.

In addition to the survival analyses in which patients were considered as a whole group, the same analyses were performed on two subgroups containing patients with grade 1–2 tumors and grade 3 tumors. Kaplan-Meier survival curves (for relapse-free and overall survival) were constructed to demonstrate differential survival of patients with *KLK4*-positive and *KLK4*-negative ovarian tumors (24). The significance of the differences between the survival curves was established using the log-rank test (25).

RESULTS

Relationship between *KLK4* Expression and Other Parameters. Of the 147 patients included in this study, 69 (47%) were positive for *KLK4* expression in their ovarian tissue. Examples of *KLK4*-positive and -negative ovarian tumors, as well as of normal ovarian tissue, are shown in Fig. 1. Table 1 depicts the distribution of *KLK4* expression (positive or negative) in ovarian tumor tissues in relation to clinical stage, histological grade, histotype, size of residual tumor, menopausal status, and response to chemotherapy. A significantly higher percentage of ovarian tumors from patients with advanced stage ($P = 0.001$)

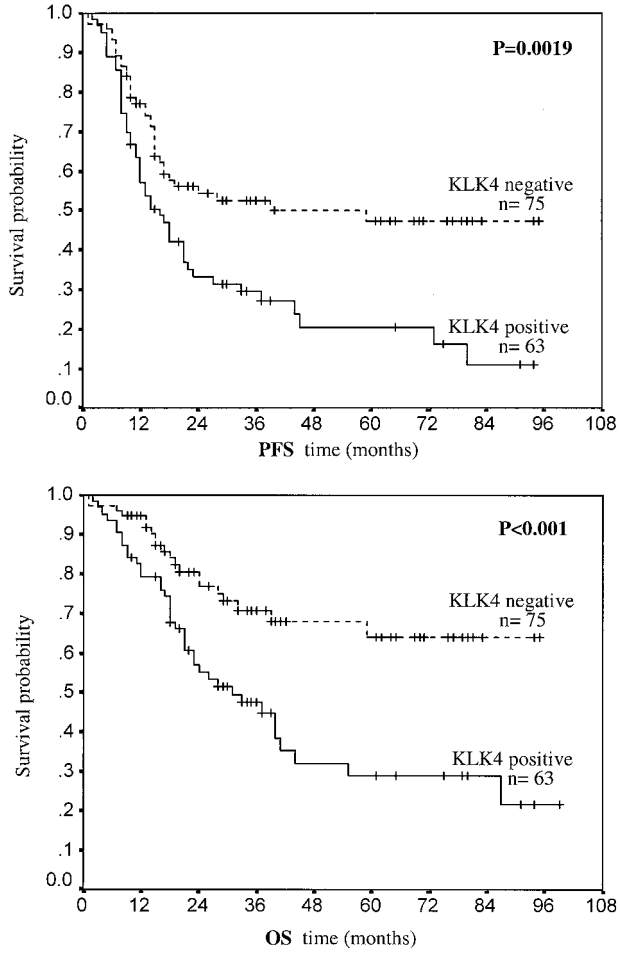

Fig. 2 Kaplan-Meier survival curves for patients with *KLK4*-positive and *KLK4*-negative tumors. *Top*, progression-free survival; *bottom*, overall survival.

and higher tumor grade ($P = 0.024$) and more residual tumor ($P < 0.001$) expressed *KLK4*, in comparison with patients with early stage, lower grade, and no residual tumor (Table 1). Between the two groups of patients (*i.e.*, *KLK4*-positive and *KLK4*-negative), there was no significant difference in relation to tumor histotype and menopausal status. Analysis of *KLK4* expression in relation to response to chemotherapy revealed that *KLK4* positivity was associated with lack of response ($P = 0.047$).

Univariate and Multivariate Survival Analysis of All of the Patients. Of the 147 patients included in this study, follow-up information was available for 139 of them, with a median follow-up of 48 months. The overall survival was 58% (80 patients), and 60% (84 patients) have relapsed. Cox regression models were developed to estimate the prognostic power of *KLK4*. As seen in Table 2, the relative risk of relapse and death was significantly higher in *KLK4*-expressing ovarian tumors in comparison with ovarian cancer patients not expressing *KLK4*. Validation of the obtained data are provided by the hazards ratios, indicating the expected increase in relative risk of relapse and death in terms of clinical stage, histological grade, and size of residual tumor. The Kaplan-Meier survival curve (Fig. 2) further demonstrates that *KLK4* expression is associated with reduced progression-free and overall survival periods. When ovarian cancer patients are considered as one group, in a multivariate Cox regression model, the expression of *KLK4* did not increase the prognostic power provided by stage, grade, and residual tumor size (Table 2). The same multivariate Cox regression model shows that clinical stage, histological grade, and residual tumor size are all independent indicators of poor prognosis, with the size of residual tumor being the strongest predictor of poor outcome, followed by clinical stage (Table 2). As expected, neither the age of patient nor the histological type of ovarian tumor are significant predictors of relapse-free survival and death.

Univariate and Multivariate Survival Analysis—Grade 1 and 2 *versus* Grade 3 Subgroups. Because grade 1 and 2 tumors are substantially different from grade 3 tumors in terms

Table 3 Associations between *KLK4* and progression-free survival and overall survival stratified by tumor grade

	Progression-free survival			Overall survival		
Variable	HR[a]	95% CI[b]	*P*	HR[a]	95% CI[b]	*P*
Tumor grade 1–2						
Univariate analysis						
KLK4-negative	1.00			1.00		
KLK4-positive	2.58	1.14–5.75	0.021	3.17	1.07–9.39	0.036
Multivariate analysis[c]						
KLK4-negative	1.00			1.00		
KLK4-positive	2.52	1.09–5.79	0.029	3.43	1.06–11.2	0.04
Tumor grade 3						
Univariate analysis						
KLK4-negative	1.00			1.00		
KLK4-positive	1.52	0.92–2.52	0.099	1.97	1.08–3.61	0.026
Multivariate analysis[c]						
KLK4-negative	1.00			1.00		
KLK4-positive	1.02	0.59–1.76	0.93	1.08	0.55–2.10	0.81

[a] Hazard ratio (HR) 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.

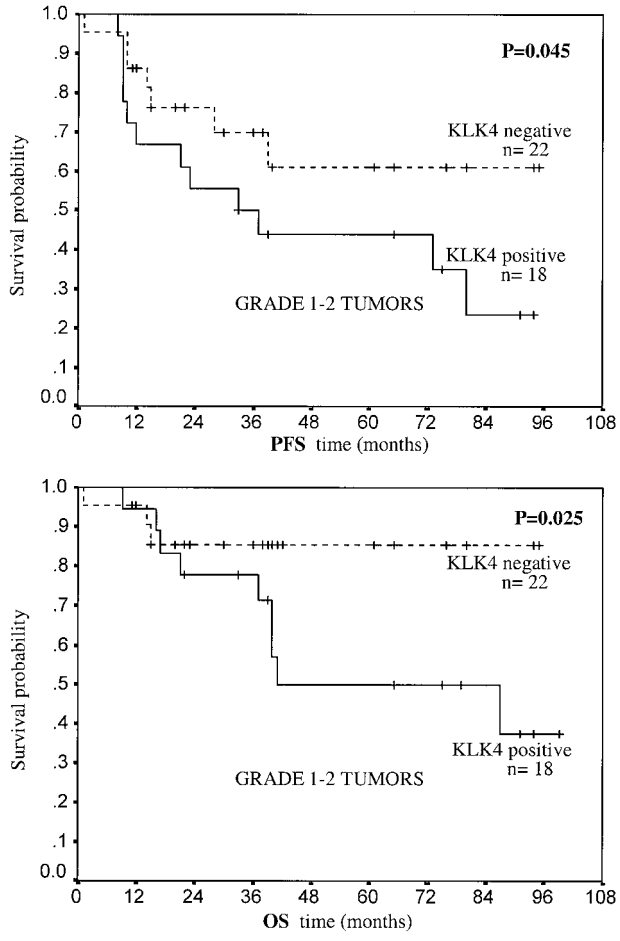

Fig. 3 Kaplan-Meier survival curves for patients with *KLK4*-positive and *KLK4*-negative, grade 1 and 2 tumors. *Top*, progression-free survival; *bottom*, overall survival.

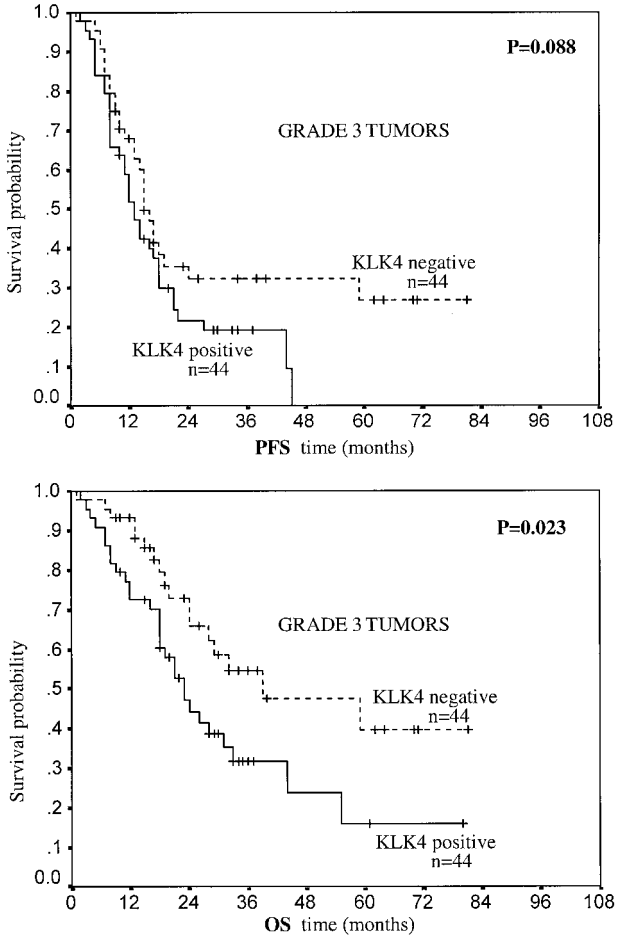

Fig. 4 Kaplan-Meier survival curves for patients with *KLK4* positive and *KLK4* negative, grade 3 tumors. *Top*, progression-free survival; *bottom*, overall survival.

of their prognostic outcome, the effect of *KLK4* expression was also analyzed separately in these two groups. Positivity for *KLK4* expression in grade 1 and 2 tumors indicates a 2.5-fold increase in the relative risk of relapse, and a >3-fold increase in the relative risk of death in both univariate and multivariate Cox regression analyses (Table 3). This is also demonstrated by the Kaplan-Meier curves, by which patients with *KLK4*-positive tumors have less favorable progression-free and overall survival than patients with *KLK*-positive tumors (Fig. 3). The results from multivariate analysis suggest that *KLK4* is an independent, unfavorable prognostic indicator for progression-free and overall survival in patients with grade 1 or 2 ovarian carcinoma (Table 3). However, in grade 3 tumors, *KLK4* expression had a significant impact only on overall survival (Fig. 4).

DISCUSSION

Ovarian carcinoma is the fourth most frequent cause of cancer deaths among North American women, after lung, breast, and colorectal cancer. Approximately 80–90% of ovarian cancers are of epithelial origin (1). Whereas overall survival increased from 38% in the mid 1980s to 47% by the mid 1990s, the number of deaths continues to increase (2). Because of the fact that in most cases this malignancy remains asymptomatic up to the late stages of the disease (26), long response to postsurgical treatment remains poor. Early diagnosis and subsequent treatment would much improve today's survival rate.

The involvement of proteases in the progression of ovarian and other types of cancer is well documented (8–10). Of special interest are the metalloproteinases that have been shown to degrade connective tissue, a process necessary for tumor cell invasion (27, 28). In addition, the urokinase-type plasminogen activator and its inhibitors have been shown to have prognostic significance in ovarian cancer patients (29–33). Other proteinases, including cathepsin D and several collagenases also appear to have similar prognostic value (34–38).

KLKs are a subfamily of serine proteases, and some of its members have already been implicated in having aberrant expression in ovarian cancer. For example, overexpression of human glandular hK4 and PSA has been noted in a case of cystic ovarian carcinoma (39). In another study, PSA was demonstrated to be expressed in primary ovarian carcinoma (40). In fact, it seems that many of the recently discovered *KLKs* also

appear to be involved in several types of cancer (41). Of particular interest are two of these new *KLKs*, the stratum corneum chymotryptic enzyme (*KLK7*) and neuropsin (*KLK8*); both are overexpressed in ovarian tumors and are suspected to facilitate metastasis of malignant cells into the peritoneal cavity (3, 42). Similarly, protease M (zyme/neurosin; KLK6) is strongly expressed in ovarian tissues and cells lines, although its prognostic value in ovarian cancer is yet unknown (43).

KLK4 appears to be a potential new candidate biomarker of ovarian cancer for several reasons. First, *KLK4* shares a high degree of similarity with other *KLKs* that are connected to ovarian cancer. Second, it is known that ovarian cancer tissues are androgen-receptor positive (44), and it seems that androgens play a role in the growth of ovarian tumor cells, at least *in vitro* (45). Consequently, we have reasoned that the monitoring of the expression of the androgen-regulated serine protease *KLK4* may yield prognostically significant results.

Our data demonstrate that *KLK4* is more frequently expressed in advanced (late-stage) cancers that cannot be completely debulked, and in tumors of higher grade (Table 1). Because other proteases were also found to be unfavorable prognostic markers in ovarian cancer (8–10, 29–38), we speculate that *KLK4*, a secreted serine protease, may also act through the degradation of the extracellular matrix, thus facilitating tumor cell spread. Alternatively, *KLK4* may be part of an enzymatic cascade pathway that involves enzyme activation followed by proteolysis.

KLK4 expression correlates with increased risk of relapse and death only in univariate analysis, indicating that it is not an independent prognostic factor; rather, it is a parameter associated with late-stage and more aggressive ovarian tumors (Table 2). However, when we examined only a subset of patients with lower grade tumors (grade 1 and 2), the expression of *KLK4* did predict relapse and death in the multivariate Cox regression model (Table 3; Fig. 3). Thus, *KLK4* is an independent prognostic indicator of poor outcome only in lower-grade tumors. Although this study could by no means demonstrate a cause-and-effect relationship between *KLK4* expression and tumor aggressiveness, it is tempting to speculate that *KLK4* confers a more aggressive phenotype to lower-grade tumors, possibly by facilitating early metastasis.

In summary, our study has shown for the first time that *KLK4* is more frequently expressed in high-grade and advanced-stage ovarian carcinomas. *KLK4* expression is an independent indicator of poor prognosis in patients with grade 1 and 2 ovarian tumors. It will be interesting to examine whether *KLK4* is an additional proteolytic enzyme involved in the metastatic process of ovarian carcinoma.

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