Prognostic Value of the Human Kallikrein Gene 15 Expression in Ovarian Cancer

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Purpose: ***KLK15*** **is a newly cloned human kallikrein gene. Many kallikreins were found to be differentially expressed in ovarian cancer. Like other kallikreins,** ***KLK15*** **is regulated by steroid hormones in cancer cell lines.** ***KLK15*** **is upregulated mainly by androgens and to a lesser extent by progestins. The purpose of this study was to examine the prognostic value of** ***KLK15*** **in ovarian cancer tissues.**

Materials and Methods: **We studied** ***KLK15*** **expression by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) in 168 consecutive patients with epithelial ovarian cancer. Ten patients with benign ovarian tumors were also included in the study. An optimal cutoff point equal to the 50th percentile was defined based on the ability of** ***KLK15*** **to predict progression-free survival and overall survival of the study population.**

Results: ***KLK15*** **expression levels were significantly higher in cancerous tissues compared with benign tumors. Kaplan-Meier survival curves showed that** ***KLK15*** **overexpression is a significant predictor of reduced progression-free survival (PFS;** ***P*** **< .001) and overall survival (OS;** ***P*** **< .009). Univariate and multivariate analyses indicate that** ***KLK15*** **is an independent prognostic factor for PFS and OS. A weak positive correlation was found between** ***KLK15*** **expression and serum CA-125 levels.**

Conclusion: ***KLK15*** **expression, as assessed by quantitative RT-PCR, is an independent marker of unfavorable prognosis for ovarian cancer.**

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EPITHELIAL OVARIAN cancer is the most lethal of all gynecologic malignancies. The only validated marker for ovarian cancer management is CA-125, which can be detected in the serum of more than 80% of women with ovarian carcinomas.[1] However, CA-125 is robust only in following response to treatment or progression of the disease and not as a diagnostic or prognostic marker.[2] Thus, there is an urgent need for additional diagnostic and prognostic markers for this disease.

Several other putative markers have been sought to compensate for the limitations of CA-125, including inhibin[3] and prostasin.[4] These novel markers may by used in conjunction with CA-125, thereby enhancing the overall diagnostic and prognostic capability.[5] A novel approach for finding new tumor markers is the analysis of gene expression profiles in normal and neoplastic ovarian tissues, which has recently identified several candidate molecular markers of epithelial ovarian cancer[6]; yet, the value of these markers has not been validated.

Kallikreins are a subgroup of the serine protease family of proteolytic enzymes.[7] The human kallikrein gene family comprises 15 genes, clustered together in a small region of approximately 400 kb on chromosome 19q13.4.[7-9] In the past few years, several groups have shown that many members of the human kallikrein gene family are related to ovarian cancer. Underwood et al[10] and Magklara et al[11] have shown that *KLK8* (also known as neuropsin, TADG14) is differentially expressed in ovarian cancer, *KLK7* is upregulated in patients with ovarian cancer,[12] and *KLK4* and *KLK5* are indicators of poor prognosis of ovarian cancer.[13-15] More recently, *KLK9* has been shown to be a marker of favorable prognosis.[16] In addition, two kallikrein proteins, hK6 and hK10, have been shown to be putative serum biomarkers for ovarian cancer diagnosis.[17-19]

KLK15 (encoding for hK15, a protein also called prostinogen) is the most recently cloned member of the human kallikrein gene family.[20,21] It is formed by five coding exons and encodes for a serine protease of a predicted molecular weight of approximately 28 kd. *KLK15* shares a high degree of structural similarity with *KLK3* (also known as prostate-specific antigen) and other kallikreins. Similar to *KLK3*, but unlike other trypsin-like serine proteases, *KLK15* does not have an aspartate residue in the substrate-binding pocket, suggesting a chymotrypsin-like substrate specificity. We have previously shown preliminarily that *KLK15* is upregulated at the mRNA level in prostate cancer.[20] A recent report indicated that hK15 can readily activate the precursor of prostate-specific antigen by cleaving an amino terminal peptide bond.[21] In addition, we have also shown that *KLK15* is under steroid hormone regulation, possibly through the androgen receptor (AR; unpublished data).

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Given the aforementioned associations of kallikreins with cancer, their potential applicability as cancer biomarkers,[22,23] and the fact that many proteases are known to be mediators of tumor progression, we postulated that *KLK15* may also be implicated in ovarian cancer prognosis. This investigation examines this hypothesis.

MATERIALS AND METHODS

Study Population

In this study we included tumor specimens from 168 consecutive patients undergoing surgical treatment for epithelial ovarian carcinoma at the Department of Gynecology, Gynecological Oncology Unit, University of Turin, Turin, Italy. All tumor specimens were confirmed by histopathologic examination. No patient received any treatment before surgery.

Patient age ranged from 25 to 89 years, with a median age of 59 years. Residual tumor sizes after surgery ranged from 0 to 9 cm, with a median of 2.0 cm. With respect to histologic type, 76 tumors were serous papillary, 28 were endometrioid, 28 were undifferentiated, 17 were mucinous, and 15 were clear cell. We also included 10 benign ovarian tissues from women whose median age was 52 years. Classification of histologic types followed the World Health Organization criteria.[24] All patients were staged according to the International Federation of Gynecology and Obstetrics staging system.[25] Grading information was available for 162 patients; 54 (33%) had grade 1 or 2, whereas 108 (67%) had grade 3 ovarian carcinoma. Grading was established for each ovarian tumor according to the criteria of Day et al.[26] All patients were treated with postoperative platinum-based chemotherapy. The first-line chemotherapy regimens included cisplatin in 95 patients (56%), carboplatin in 50 patients (30%), cyclophosphamide in 69 patients (41%), doxorubicin in 12 patients (7%), epirubicin in 20 patients (12%), paclitaxel in 27 patients (16%), and methotrexate in two patients (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, and 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 less than 25% in the product of the diameters of all measurable lesions. Progressive disease was defined as an increase of at least 25%. In patients with no clinically measurable disease, response to chemotherapy was assessed by serial measurements of serum CA-125. Responders (partial or complete) experienced a decrease in their CA-125 level by more than 50% after two cycles of chemotherapy.

Investigations were performed in accordance with the Helsinki Declaration and was approved by the Institute of Obstetrics and Gynecology, Turin. Tumor specimens were snap-frozen in liquid nitrogen immediately after surgery. Histologic examination, performed during intrasurgery frozen-section analysis, allowed representative portions of each tumor containing more than 80% of tumor cells to be selected for storage until analysis. Serum CA-125 values before operation were available for 67 patients.

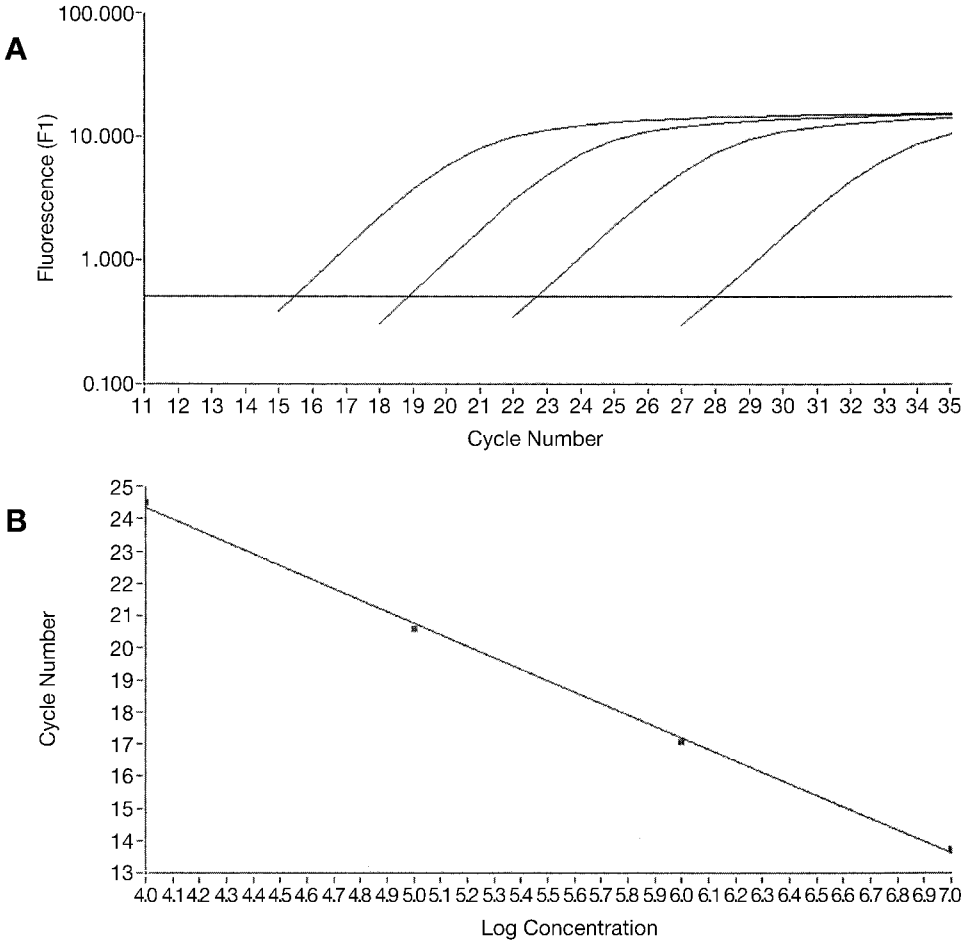

Fig 1. Quantification of *KLK15* gene expression by real-time polymerase chain reaction. (A) A logarithmic plot of fluorescence signal versus cycle number. (B) A representative calibration curve for *KLK15* mRNA quantification. Curves were obtained with serially diluted (10-fold) plasmid containing *KLK15* cDNA.

Table 1. Descriptive Statistics of *KLK15* Expression in Cancer and Benign Tissues

	KLK15 Expression (arbitrary units)			Percentiles				
	Mean	SE	Range	10	25	50 (median)	75	90
Cancer tissues (n = 168)	328	64	0.00-5330	0.00	0.025	1.00	38	1179
Benign tissues (n = 10)	0.08	0.04	0.00-0.20	0.00	0.008	0.056	0.16	0.19

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 (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. The concentration and purity of RNA were determined spectrophotometrically. We reverse-transcribed 2 μg of total RNA into first-strand cDNA using the Superscript preamplification system (Gibco BRL). The final volume was 20 μL.

Quantitative Real-Time Polymerase Chain Reaction (PCR) and Continuous Monitoring of PCR Products

On the basis of the published genomic sequence of *KLK15* (GenBank [National Institutes of Health, Bethesda, MD] accession no. AF242195), two gene-specific primers were designed (15-F3: 5′ TGT GGC TTC TCC TCA CTC TC 3′ and 15-R3 5′AGG CTC GTT GTG GGA CAC 3′). These primers spanned more than two exons to avoid contamination by genomic DNA. Real-time monitoring of PCR reaction was performed on the LightCycler system (Roche Molecular Systems, Indianapolis, IN) and the SYBR Green I dye (Roche, Nutley, NJ), which binds preferentially to double-stranded DNA. Fluorescence signals are proportional to the concentration of the product and are measured at the end of each cycle rather than after a fixed number of cycles. The higher the starting quantity of the template, the earlier the threshold cycle, which is defined as the fractional cycle number at which fluorescence passes a fixed threshold above baseline, will be attained.[27] For each sample, the amount of *KLK15* and an endogenous control (beta actin, a housekeeping gene) were determined using a calibration curve. The amount of *KLK15* was then divided by the amount of the endogenous reference, to obtain a normalized *KLK15* value.

Standard Curve Construction

The full-length mRNA sequence of the *KLK15* gene was amplified by PCR using gene-specific primers, and the PCR product was cloned into a TOPO TA cloning vector (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Plasmids were purified using Mini-prep kit (Qiagen Inc, Valencia, CA). Different standard curves for actin and *KLK15* were constructed using serial dilutions of the plasmid, as described elsewhere.[27] These standards were included in each run. An example is given in Figure 1. The reliability of the *KLK15* assay was determined by evaluating within- and between-run precision. In all cases, the coefficients of variation were less than 10%.

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 1 mix, 50 ng of primers, and 1.2 μL of 25 mmol/L $MgCl_2$. The final volume was adjusted to 20 μL with water. After the reaction mixture was loaded into the glass capillary tube, the cycling conditions were carried out as follows: initial denaturation at 94°C for 10 minutes, followed by 45 cycles of denaturation at 94°C for 0 seconds, annealing at 63°C for 5 seconds, and extension at 72°C for 30 seconds. The temperature transition rate was set at 20°C per second. Fluorescent product was measured by a single acquisition mode at 88°C after each cycle. A melting curve was then performed by holding the temperature at 70°C for 30 seconds, followed by a gradual increase in temperature to 98°C at a rate of 0.2°C per second, with the signal acquisition mode set at step. To verify the melting curve results, representative samples of the PCR products were purified and sequenced.

Statistical Analysis

First, an optimal cutoff value was defined by χ^2 analysis, based on the ability of *KLK15* values to predict the progression-free survival (PFS) and overall survival (OS) of the study population. This cutoff (1.0 arbitrary units; 50th percentile) identifies 50% of patients as being *KLK15*-positive.

Associations between clinicopathologic parameters, such as stage, grade, histotype, and residual tumor, and *KLK15* 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.

The Cox univariate and multivariate proportional hazards regression model[28] was used to evaluate the hazard ratio (relative risk of relapse or death in the *KLK15*-positive group). In the multivariate analysis, the models were adjusted for *KLK15* expression, clinical stage, histologic grade, residual tumor, and age.

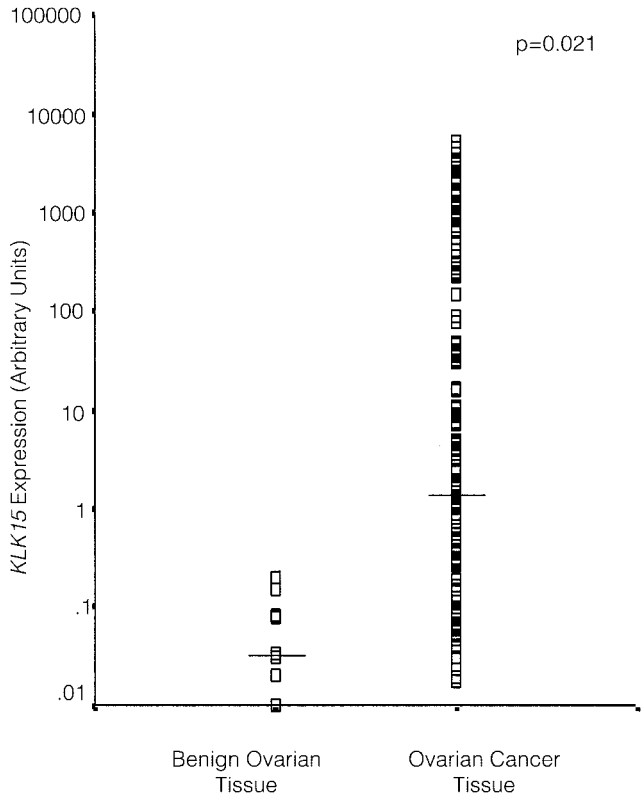

Fig 2. Distribution of *KLK15* expression levels in cancerous and benign ovarian tissues. Horizontal lines show median values (*P* = .021 by Mann-Whitney *U* test).

Table 2. Relationship Between *KLK15* Status and Other Variables in 168 Ovarian Cancer Patients

Variable	No. of Patients	*KLK15*-Negative*		*KLK15*-Positive		*P*
		No. of Patients	%	No. of Patients	%	
Stage						
I/II	42	22	52.4	20	47.6	.72†
III	120	58	48.3	62	51.7	
X	6					
Grade						
G1/G2	54	29	53.7	25	46.3	.51†
G3	108	51	47.2	57	52.8	
X	6					
Histotype						
Serous	76	38	50.0	38	50.0	
Endometrioid	28	12	42.9	16	57.1	
Mucinous	17	7	41.2	10	58.8	.57‡
Clear cell	15	6	40.0	9	60.0	
Undifferentiated	28	17	60.7	11	39.3	
X	4					
Residual tumor						
0 cm	68	38	55.9	30	44.1	
1-2 cm	28	12	42.9	16	57.1	.31‡
>2 cm	66	29	43.9	37	56.1	
X	6					
Debulking success						
SO	82	33	40.2	49	59.8	.028†
OD	80	46	57.5	34	42.5	
X	6					
Menopause						
Pre/peri	57	26	45.6	31	54.4	.26†
Post	111	58	52.2	53	47.8	
Response to CTX						
NC/PD	17	5	29.4	12	70.6	.076†
CR/PR	141	75	53.2	66	46.8	
NE	10					

Abbreviations: OD, optimal debulking (0 to 1 cm); SO, suboptimal debulking (> 1 cm); CTX, chemotherapy; NC, no change; PD, progressive disease; CR, complete response; PR, partial response; NE, not evaluated; X, status unknown.

*Cutoff was equal to 50th percentile.

†Fisher's exact test.

‡χ^2 test.

Kaplan-Meier survival curves[29] were constructed for *KLK15*-positive and *KLK15*-negative patients. For further analysis, patients were divided into two groups either by the tumor grade (grade 1 to 2 *v* grade 3), tumor stage (stage I/II *v* stage III/IV), or by the success of debulking (optimal *v* suboptimal debulking group). In each category, survival rates (DFS and OS) were compared between *KLK15*-positive and *KLK15*-negative groups. The differences between the group survival curves were tested for statistical significance by the log-rank test.[30]

RESULTS

KLK15 *Expression in Benign and Cancerous Ovarian Tissues*

Table 1 shows the mean and median *KLK15* expression levels in benign and malignant ovarian tumors. Expression levels were found to be much higher in cancerous tissues (mean, 328 arbitrary units; median, 1.0 arbitrary units) compared with benign ovarian tumors (mean, 0.077 arbitrary units; median, 0.056 arbitrary units). The distribution of *KLK15* expression in ovarian cancer and benign tissues is depicted in Figure 2. The differences between medians of the two groups were highly significant ($P = .021$ by Mann-Whitney *U* test).

KLK15 *Expression in Relation to Other Variables*

As shown in Table 2, no significant associations were found between *KLK15* expression and other clinical variables, except for a weakly significant difference between patients with optimal and suboptimal debulking.

Survival Analysis

Of the 168 patients included in this study, follow-up information was available for 162 patients (median follow-up period, 67 months), among whom 96 (59%) had experienced relapse, and 61 (38%) died.

Kaplan-Meier survival curves demonstrated that patients with *KLK15*-positive tumors have substantially lower PFS ($P < .001$) and OS ($P = .009$; Fig 3) compared with those who are *KLK15*-negative. The strength of the associations between each individual prognostic factor, and PFS or OS are shown in the univariate analysis in Table 3. Stage of disease, histologic grade, and residual tumor size showed strong associations with cancer relapse and death ($P < .001$). *KLK15* expression

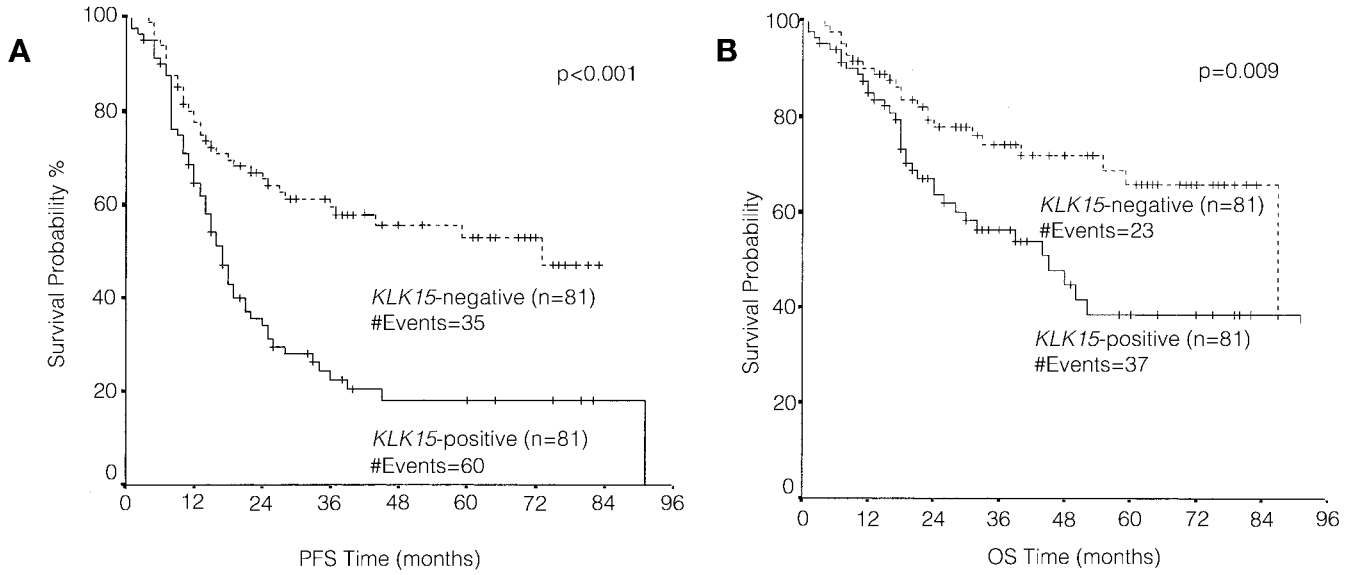

Fig 3. Kaplan-Meier survival curves for (A) progression-free survival (PFS) and (B) overall survival (OS) in patients with *KLK15*-positive and *KLK15*-negative ovarian tumors.

was also found to be a significant predictor of lower PFS and OS (hazard ratios of 2.33 and 1.96, respectively, and *P* values of < .001 and .012, respectively).

When all the confounders were included in the Cox model (multivariate analysis, Table 3), only residual tumor size and grade, in addition to *KLK15*, retained their prognostic significance. *KLK15* expression showed hazard ratios of 2.27 and 1.79 and *P* values of less than .001 and .039 for the PFS and OS, respectively. CA-125, as a continuous variable, was found to be an unfavorable prognostic indicator in the Cox univariate analysis for PFS ($P = .007$) but not for OS ($P = .14$). When *KLK15* was included in the Cox model, CA-125 did not retain its significance for PFS ($P = .13$ for CA-125 and $P = .001$ for *KLK15*) (data not shown).

As shown in Figure 4, a weak positive correlation was found between the expression levels of *KLK15* levels and presurgical serum CA-125 ($r_s = 0.37$; $P = .002$).

When Cox proportional hazard regression analysis was applied for subgroups of patients (Table 4), *KLK15* was found to be a significant predictor of reduced PFS, but not OS, in the

Table 3. Univariate and Multivariate Analysis of *KLK15* With Regard to Progression-Free and Overall Survival

Variable	Progression-Free Survival			Overall Survival		
	HR*	95% CI†	*P*	HR*	95% CI†	*P*
Univariate analysis						
KLK15 (n = 161)						
Negative	1.00	—	—	1.00	—	—
Positive	2.33	1.52 to 3.55	< .001	1.96	1.16 to 3.31	.012
As a continuous variable	1.001	0.99 to 1.003	.24	1.002	0.99 to 1.004	.16
Stage of disease (ordinal)	2.48	1.89 to 3.25	< .001	2.52	1.76 to 3.58	< .001
Grading (ordinal)	2.09	1.58 to 2.75	< .001	2.14	1.47 to 3.11	< .001
Residual tumor (ordinal)	1.27	1.21 to 1.33	< .001	1.30	1.21 to 1.40	< .001
Histologic type‡	1.46	0.99 to 2.14	.055	1.27	0.78 to 2.07	.32
Age	1.01	0.99 to 1.03	.12	1.01	0.99 to 1.03	.11
Multivariate analysis						
KLK15 (n = 144)						
Negative	1.00	—	—	1.00	—	—
Positive	2.27	1.46 to 3.54	< .001	1.79	1.03 to 3.13	.039
Stage of disease (ordinal)	1.35	0.95 to 1.91	.087	1.39	0.87 to 2.22	.16
Grading (ordinal)	2.03	1.35 to 3.05	< .001	1.92	1.07 to 3.43	.027
Residual tumor (ordinal)	1.16	1.08 to 1.25	< .001	1.21	1.10 to 1.33	< .001
Histologic type‡	1.03	0.67 to 1.58	.88	1.38	0.80 to 2.38	.24
Age	1.02	0.99 to 1.05	.087	1.03	1.00 to 1.05	.044

Abbreviations: HR, hazard ratio; CI, confidence interval.

*HR estimated from Cox proportional hazards regression model.

†CI of the estimated HR.

‡Serous versus others.

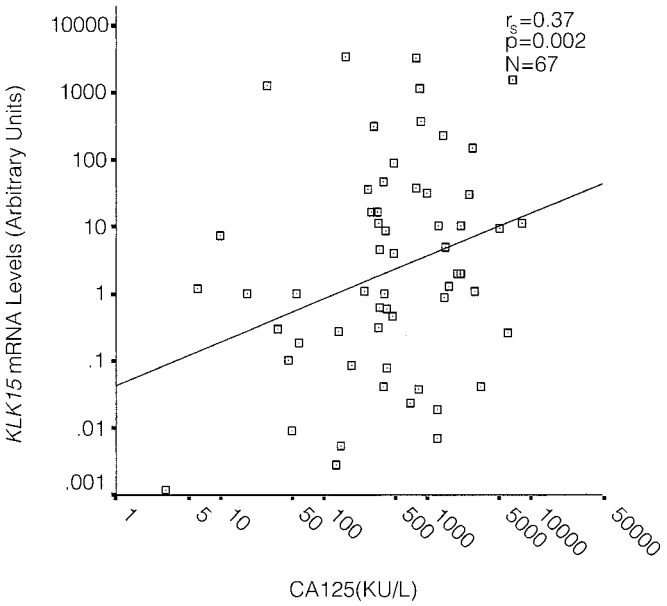

Fig 4. Correlation between serum CA-125 and tumor levels of *KLK15* expression. r_s, Spearman correlation coefficient.

subgroups of patients with grade 1 to 2 (hazard ratio, 5.35; $P = .004$), grade 3 (hazard ratio, 1.89; $P = .007$), stage I to II (hazard ratio, 7.1; $P = .014$), and stage III (hazard ratio, 1.93; $P = .004$). *KLK15* retained its prognostic significance after adjusting for other confounders. *KLK15* expression retained a highly statistically significant prognostic value for both PFS and OS in patients with optimal debulking, even after adjusting for all other confounders (Table 4).

DISCUSSION

Our results show that *KLK15* is an independent marker of unfavorable prognosis in ovarian cancer. *KLK15* is not the only kallikrein that has been found to be differentially regulated in ovarian cancer. We have recently reported that *KLK9* is a marker of favorable prognosis.[16] In addition, data from other groups and our laboratory indicate that multiple kallikrein genes (*KLK4* through *KLK10*) are differentially expressed in ovarian cancer.[11-14,17,31] It will be interesting to simultaneously examine the expression of all these kallikreins in ovarian cancer and to develop multiparametric models of prognosis.

We have recently shown that *KLK15* is a hormonally regulated gene.[20] *KLK15* is upregulated mainly by androgens and to a lesser extent by progestins (our data, submitted for publication). We also provided evidence suggesting that this regulation is possibly mediated through the AR. Appreciable evidence implicates androgens in the pathogenesis of ovarian cancer[32] and supports the existence of a physiologic interaction between androgens and the ovarian surface epithelium, as well as the possible role of this interaction in ovarian neoplasia.[33] Androgens stimulate growth of rodent ovarian epithelial cells in vivo, leading to benign ovarian neoplasms.[34] Furthermore, ovarian cancer patients have higher levels of circulating androgens before their diagnosis than women without cancer.[35] Additionally, the majority of ovarian cancers express AR,[36,37] and ovarian cancer cell growth is inhibited in vitro by antiandrogens.[38] Recent observations show a correlation between AR and susceptibility to ovarian cancer.[37] In this study, an optimal cutoff point equal to the 50th percentile was selected, based on the ability of *KLK15* to predict PFS and OS. It has been previously

Table 4. Cox Proportional Hazards Regression Analysis for Subgroups of Patients

	Progression-Free Survival			Overall Survival		
Variable	HR*	95% CI†	*P*	HR*	95% CI†	*P*
Tumor grade 1/2						
KLK15 unadjusted	5.35	1.73 to 16.55	.004	2.67	0.66 to 10.73	.16
KLK15 adjusted‡	4.12	1.16 to 14.55	.027	1.84	0.38 to 8.95	.44
Tumor grade 3						
KLK15 unadjusted	1.89	1.18 to 3.01	.007	1.68	0.95 to 2.99	.073
KLK15 adjusted‡	2.29	1.39 to 3.75	.001	1.85	1.00 to 3.39	.047
Stage I/II						
KLK15 unadjusted	7.10	1.47 to 34.21	.014	1.35	0.65 to 2.81	.33
KLK15 adjusted§	3.18	0.58 to 17.39	.18	1.59	0.43 to 5.81	.48
Stage III						
KLK15 unadjusted	1.93	1.24 to 3.02	.004	1.74	1.014 to 2.99	0.044
KLK15 adjusted§	2.11	1.31 to 3.38	.002	1.79	1.00 to 3.21	.048
Optimal debulking						
KLK15 unadjusted	3.71	1.57 to 8.72	.003	6.59	1.39 to 31.11	.017
KLK15 adjusted‖	3.52	1.48 to 8.35	.005	7.11	1.42 to 35.66	.016
Suboptimal debulking						
KLK15 unadjusted	1.52	0.93 to 2.48	.09	1.21	0.67 to 2.14	0.52
KLK15 adjusted‖	2.03	1.17 to 3.51	.01	1.53	0.82 to 2.87	.17

Abbreviations: HR, hazard ratio; CI, confidence interval.

*HR estimated from Cox proportional hazards regression model.

†CI of the estimated HR.

‡Multivariate models were adjusted for stage of disease, residual tumor, histologic type, and age.

§Multivariate models were adjusted for tumor grade, residual tumor, histologic type, and age.

¶Multivariate models were adjusted for stage of disease, tumor grade, histologic type, and age.

pointed out that this approach may overestimate the marker's prognostic value.[39] In this study, however, the prognostic value of KLK15 is further supported by the statistically significant differences between ovarian cancer and benign tissues between patients with optimal versus suboptimal debulking and by the positive correlation between the expression levels of *KLK15* and presurgical serum CA-125.

It is now widely accepted that no single biomarker will produce all the necessary information for diagnosis, prognosis, and development of treatment strategies for patients with ovarian cancer. Instead, research is now focusing on generating a panel of ovarian cancer biomarkers. An artificial network approach for combining and interpreting information from a group of biomarkers will enable more accurate diagnosis and prognosis; this method is currently underway and has already produced promising preliminary results.[40-42]

Our results show a weak positive correlation between *KLK15* expression and serum CA-125 levels (Fig 4). These results are consistent with previous reports showing that higher CA-125 levels are associated with poor prognosis in ovarian cancer.[43] On other hand, although high CA-125 expression levels are associated only with the serous histologic type,[43] no significant relationships were found between *KLK15* levels and any of the histologic types of ovarian carcinoma (Table 2), implicating a possible role of *KLK15* in monitoring nonserous ovarian cancer patients, whereas CA-125 is not usually informative. Serum assays for hK15 are required for such application.

In conclusion, we report for the first time that higher *KLK15* expression is an indicator of poor prognosis in ovarian cancer. These data would need validation with additional tumor sets. These data add to the growing recent literature suggesting that many other members of the kallikrein gene family have prognostic value in ovarian cancer. It is conceivable that all these enzymes may participate in a common pathway that is activated during ovarian cancer initiation and progression.

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