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Clinical Biochemistry 36 (2003) 135–143

CLINICAL BIOCHEMISTRY

Prognostic value of quantitatively assessed KLK7 expression in ovarian cancer

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Received 11 September 2002; received in revised form 26 November 2002; accepted 26 November 2002

Abstract

Background: Among females, ovarian cancer is the sixth most common malignancy. Women with ovarian cancer have poor overall survival rates, largely because the disease is often diagnosed at an advanced, less curable stage. Several lines of evidence suggest that members of the kallikrein family are involved in various malignancies such as prostate (PSA, KLK2, KLK15), ovarian (KLK4, KLK5, KLK6, KLK8, KLK10), and breast cancer (KLK10, KLK13, KLK14). Recent evidence has indicated that expression of KLK7 appears to be increased in ovarian cancer. We hypothesized that overexpression of the KLK7 gene in ovarian cancer may serve as a prognostic marker of the disease.

Methods: Using the LightCycler™ technology we quantified the level of KLK7 mRNA expression in 125 ovarian tumors. Different disease stages and tumor grades were analyzed. Univariate and multivariate analyses were performed to establish the associations between clinicopathological parameters and KLK7 expression.

Results: We here report that patients with KLK7-negative tumors have a significantly higher disease-free survival than patients with KLK7-positive tumors. KLK7 expression levels were significantly higher in patients with grade 3 than in patients with grade 1 to 2 tumors ($p = 0.030$). KLK7 status also correlated with size of residual tumor postsurgery. KLK7 expression is an independent predictor of both disease-free and overall survival for patients with low grade [1–2] tumors. In this subgroup of patients the hazard ratios for disease-free and overall survival were 3.28 and 3.09, respectively. Similarly, patients who had undergone optimal debulking but harbored KLK7-positive tumors had a high hazard ratio (HR) for relapse (HR = 8.2) and death (HR = 4.6).

Conclusions: We conclude that higher KLK7 expression in ovarian cancer tissue is associated with poorer prognosis of ovarian cancer patients, especially those with lower grade disease and those who have been optimally debulked.

Non-Standard abbreviations: HSCCE, human stratum corneum chymotryptic enzyme.

Keywords: Kallikreins; Ovarian cancer; Serine proteases; Cancer genes; Prognostic markers; Cancer predictive markers; KLK7; Tumor markers; Human stratum corneum chymotryptic enzyme

1. Introduction

Ovarian cancer represents a major clinical problem in gynecological oncology. Since most patients are asymptomatic until the disease has metastasized, two thirds are diagnosed with advanced disease [1]. In the United States, around 23,000 new cases of ovarian cancer and about 14,000 deaths from the disease were expected for the year 2000 [2]. Ovarian cancer has the highest mortality rate among all gynecological malignancies.

Currently, the only tumor marker that has a clinically validated role in the management of ovarian cancer is the carbohydrate antigen CA125. Serum CA125 has been evaluated for ovarian cancer screening, differential diagnosis of benign and malignant ovarian masses and for prognosis [3–6]. This tumor marker does not have a clear place in

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0009-9120/03/$ – see front matter
doi:10.1016/S0009-9120(02)00446-0

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 for ovarian cancer have been evaluated, either alone or in combination with CA125, including CA15 to 3, CA19 to 9, OVX1, lysophosphatidic acid (LPA) and carcinoembryonic antigen (CEA) [7,10,11]. These new markers are not used routinely at present, and only the combination of CA125 with ultrasonography yields the highest available sensitivity and specificity [8].

Kallikreins are serine proteases with diverse physiologic functions. We, and others, have recently identified 12 new members of the human kallikrein gene family on chromosome 19q13.3-q13.4 [12–21]. Several groups have shown that many human kallikrein genes are differentially expressed in various malignancies (reviewed in Ref [22–24]. PSA is the premier tumor marker for prostate cancer [25]. hK2 (encoded by the KLK2 gene) is an emerging tumor marker for prostate cancer [26–29]. KLK10 (NES1) appears to be a tumor suppressor gene [30], and KLK5 is a poor prognostic marker for ovarian cancer [31]. Two new kallikrein proteins, hK6 and hK10 are novel serologic markers of ovarian carcinoma [32,33].

The human kallikrein gene 7 (KLK7, also known as the human stratum corneum chymotryptic enzyme, HSCCE) is a new member of the human kallikrein gene family [34,35]. Recent reports indicate that it is expressed at abnormally high levels in ovarian cancer [36]

Recently, accumulating literature suggests that a number of serine proteases of the kallikrein family, including KLK4 [37], KLK5 [31], KLK6 [38,39], KLK7 [36], KLK9 [40], KLK10 [33,41] and KLK11 [42] are differentially expressed in ovarian cancer. However, no study has reported the prognostic value of KLK7 expression in this disease. In this paper we have quantitatively analyzed KLK7 expression in a large series of ovarian tumors and report that higher expression of this gene is associated with unfavorable outcome, especially in subgroups of patients who have lower grade disease.

2. Materials and Methods

2.1. Study population

Included in this study were tumor specimens from 125 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 and the availability of sufficient tumor for RNA extraction.

Patient ages ranged from 27 to 82 with a median of 57 yr. Residual tumor after surgery sizes ranged from 0 to 9 cm, with a median of 2 cm. Follow-up information (median follow-up period 64 months) was available from 121 patients, among whom 94 (78%) had relapsed and 50 (41%) died. With respect to histologic type, 56 tumors were serous papillary, 22 were endometrioid, 18 were undifferentiated, 11 were mucinous, 9 were clear cell, 5 were Mullerian and 2 were sarcomas.

Classification of histologic types followed the World Health Organization criteria [43]. All patients were staged according to the International Federation of Gynecology and Obstetrics staging system [44]. Grading information was available for 114 patients; 42 (37%) had grade 1 or 2 and 72 (63%) had grade 3 ovarian carcinoma. Grading was established for each ovarian tumor according to the criteria of Day *et al.* [45]. All patients were treated with postoperative platinum-based regimen chemotherapy. The first-line chemotherapy regimens included *cis*-platin in 56% patients, carboplatin in 30%, cyclophosphamide in 41%, doxorubicin in 7%, epirubicin in 12%, paclitaxel in 16%, and methotrexate in 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 less than 25% in the product of the diameters of all measurable lesions; an increase of at least 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. Histologic examination, performed during intra-surgery frozen section analysis, allowed representative portions of each tumor containing $>$ 80% tumor cells to be selected for storage until analysis.

2.2. 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 homogenized and total RNA was extracted using Trizol™ reagent (Gibco BRL, Gaithersburg, MD) following the manufacturer's instructions. Sterile, RNase-free solutions and equipments were used for RNA extraction. The concentration and purity of RNA were determined spectrophotometrically. 2 μg of total RNA was reverse-transcribed into first strand cDNA using the

Superscript™ preamplification system (Gibco BRL). The final volume was 20 μL.

2.3. Quantitative real-time RT-PCR analysis

Based on the published genomic sequence of KLK7 (GenBank accession # AF166330), two gene-specific primers were designed (HS1: GTG AAG AAA GTC AGG CTG CC, and HS2: TGC CAG CGC ACA GCA TGG AA). These primers spanned more than 2 exons to avoid contamination by genomic DNA.

Real-time monitoring of PCR reactions was performed using the LightCycler™ system (Roche Molecular Systems, Indianapolis, IN, USA) and the SYBR green I dye, which binds preferentially to double stranded DNA. Fluorescence signals, which are proportional to the concentration of the PCR product, are measured at the end of each cycle and immediately displayed on a computer screen, permitting real time monitoring of the PCR reaction [46]. The reaction is characterized by the point during cycling, when amplification of PCR products is 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 is observed [47]. The threshold cycle is defined as the fractional cycle number at which fluorescence passes a fixed threshold above baseline [48].

2.4. 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 below). The amount of the target molecule was then divided by the amount of the endogenous reference, to obtain a normalized target value [47].

2.5. Calibration curves

Separate calibration (standard) curves for actin and KLK7 were constructed using serial dilutions of total cDNA from thymus tissue, purchased from Clontech, Palo Alto, CA, as described previously [47,49]. The four standard curve calibrators were included in each run. The LightCycler™ software automatically calculates the standard curve by plotting the starting dilution of each standard sample vs. the threshold cycle, and the sample concentrations were then calculated accordingly (Figure 1). Standards for both KLK7 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.

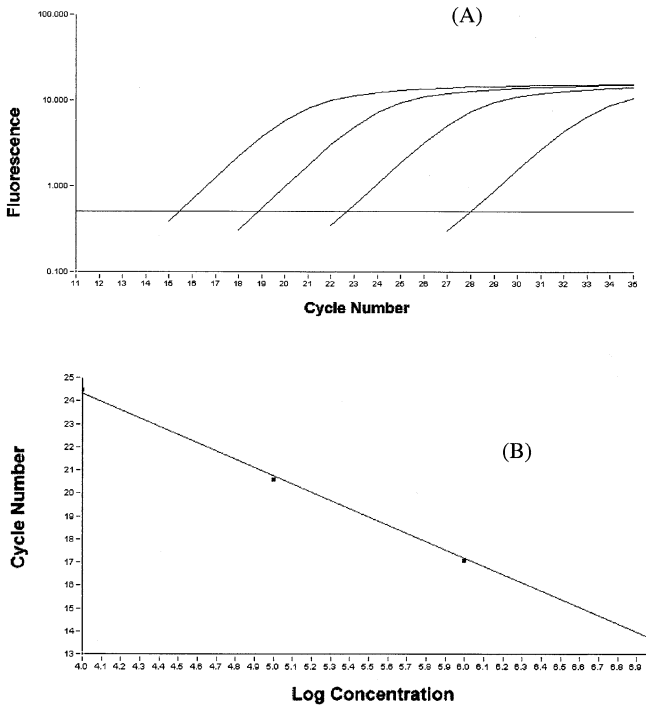

Fig. 1. Real-time PCR. (Top): Standard curve based on serial dilutions of total RNA from thymus tissue. RNA copy number was arbitrarily assigned to each sample according to the dilution factor. Each sample was tested in duplicate. The *x*-axis represents the cycle number and the *y*-axis the logarithm of the fluorescent signal. (Bottom): Representative graph of the standard curve. The log of the concentration of each dilution was plotted against cycle number.

2.6. 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 60°C for 5 s, and extension at 72°C for 16 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.

2.7. 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 [50]. To verify the melting curve results, representative samples of the PCR products were run on 1.5%

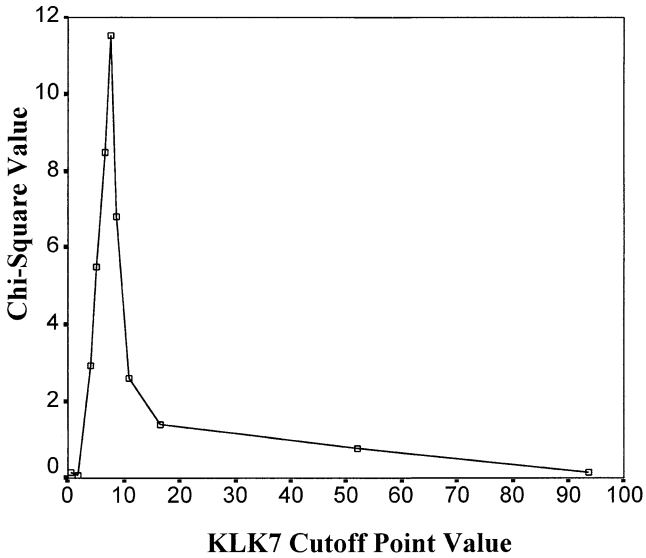

Fig. 2. Graph showing the optimal cut-off value of KLK7 as determined by chi-square analysis. For more details see text.

agarose gels, purified, and cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The inserts were sequenced using vector-specific primers, with an automated DNA sequencer.

2.8. Statistical analysis

Associations between clinicopathological parameters such as stage, grade, histotype, and residual tumor, and KLK7 expression were analyzed by the chi-square 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 progression free and overall survival, respectively. Progression free survival was defined as the time interval between the date of surgery and the date of identification of recurrent or metastatic disease. Overall survival was defined as the time interval between the date of surgery and the date of death.

The Cox univariate and multivariate proportional hazard regression model [51] was used to evaluate the hazard ratio (relative risk of relapse or death in the KLK7-positive group). In the multivariate analysis, the models were adjusted for KLK7 expression, clinical stage, histologic grade, residual tumor and age.

Kaplan–Meier survival curves were constructed for KLK7-positive and KLK7-negative patients. For further analysis, patients were divided into two groups either by the tumor grade (grade 1–2 vs. grade 3), tumor stage (stage I-II vs. stage III-IV), or by the success of debulking (optimal vs. suboptimal debulking group). In each category, survival rates (disease-free survival and overall survival) were compared between KLK7-positive and KLK7-negative groups. The differences between the survival curves between groups were tested for statistical significance by the log rank test [52]

Table 1
Relationship between KLK7 status and other variables in 125 ovarian cancer patients

Variable	Patients	No. of patients (%)		P value
		KLK7 negative	KLK7 positive	
Stage	23	16 (69.6)	7 (30.4)	
I	9	6 (66.7)	3 (33.3)	0.25[a]
II	80	40 (50.0)	40 (50.0)	
III	10	7 (70.0)	3 (30.0)	
x	3			
Grade				
G1	17	12 (70.6)	5 (29.4)	
G2	25	19 (76.0)	6(24.0)	0.030[a]
G3	72	35 (48.6)	37 (51.4)	
x	11			
Histotype				
Serous	56	33 (58.9)	23 (41.1)	0.41[b]
Others	67	37 (55.2)	30 (44.8)	
x	2			
Residual tumor (cm)				
0	45	32 (71.1)	13 (28.9)	
1–2	25	9 (36.0)	16 (64.0)	0.014[a]
>2	50	26 (52.0)	24 (48.0)	
x	5			
Debulking success[c]				
OD	56	35 (62.5)	21 (37.5)	0.19[b]
SO	64	32 (50.0)	32 (50.0)	
Menopause				
Pre/Peri	42	25 (59.5)	17 (40.5)	0.84[b]
Post	83	47 (56.6)	36 (43.4)	
Response to CTX[d]				
NC/PD	15	8 (53.3)	7 (46.7)	0.99[b]
CR/PR	103	58 (56.3)	45 (43.7)	
NE	7			

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

3. Results

3.1. KLK7 expression and relation to other variables

First, an optimal cutoff value was defined by chi-square analysis, based on the ability of KLK7 values to predict the progression-free survival of the study population. As shown in Fig. 2a value of 7.5 (this is a unitless ratio) was shown to be the optimal cutoff ($\chi^2 = 11.5$, $p = 0.001$). This cutoff (58^{th} percentile) identifies 42% of patients as being KLK7 positive.

The relationship between KLK7 expression and other clinical or pathologic variables, including stage, grade, histotype, menopausal status or response to chemother-

Table 2
Univariate and Multivariate Analysis of KLK7 with Regard to DFS and OS

Variable	Disease-free survival (DFS)			Overall survival (OS)		
	HR[a]	95% CI[b]	P value	HR[a]	95% CI[b]	P value
	Univariate analysis					
KLK7						
negative	1.00		1.00			
positive	1.94	1.22–3.09	0.005	1.50	0.86–2.62	0.15
as a continuous variable	1.01	0.99–1.02	0.25	1.01	0.99–1.03	0.42
Stage of disease (ordinal)	4.60	2.86–7.49	< 0.001	5.11	2.83–9.23	< 0.001
Grading (ordinal)	2.49	1.69–3.66	< 0.001	2.84	1.68–4.79	< 0.001
Residual tumor (ordinal)	1.28	1.19–1.37	< 0.001	1.29	1.19–1.41	< 0.001
Histologic type[c]	0.76	0.48–1.21	0.25	0.91	0.52–1.61	0.76
Age	1.01	0.99–1.03	0.15	1.03	0.99–1.05	0.061
	Multivariate analysis					
KLK7						
negative	1.00			1.00		
positive	1.57	0.96–2.57	0.067	1.18	0.63–2.20	0.60
as a continuous variable	1.001	0.99–1.003	0.081	1.001	0.99–1.03	0.60
Stage of disease (ordinal)	3.31	1.88–5.79	< 0.001	3.69	1.79–7.58	< 0.001
Grading (ordinal)	1.53	0.96–2.43	0.068	1.71	0.89–3.25	0.11
Residual tumor (ordinal)	1.13	1.04–1.23	0.0026	1.17	1.05–1.29	0.002
Histologic type[c]	0.88	0.54–1.43	0.61	1.01	0.58–1.80	0.98
Age	1.01	0.98–1.04	0.27	1.03	0.99–1.06	0.053

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

apy is shown in Table 1. KLK7 expression was significantly higher in patients with high grade (G3) tumors ($p = 0.03$). Also, higher KLK7 expression is seen in patients who have residual tumor postsurgery ($p = 0.01$). KLK7 expression was not significantly associated with stage, histotype, menopausal status or response to chemotherapy.

3.2. Survival analysis

The strength of the associations between each individual predictor and disease-free or overall survival are shown in the univariate analysis in Table 2. Stage of disease, histologic grade, and residual tumor showed a strong association with cancer relapse and death. The stage of disease showed the strongest association, with hazard ratios of 4.60 and 5.11, respectively. The hazard ratio for patients with KLK7-positive tumors was 1.94 and 1.50 for disease-free and overall survival.

When all the predictors were included in the Cox model (multivariate analysis, Table 2), the stage of disease and residual tumor size were the only predictors significantly associated with disease-free and overall survival. Using this analysis the KLK7-positive tumors were shown to have no independent value as predictors of survival in ovarian cancer patients.

When the Cox proportional hazard regression analysis was applied to subgroups of patients (Table 3). KLK7 positive tumors were shown to be independent predictors of both disease-free and overall survival for patients with low grade tumors *i.e.*, grade 1 and 2. The hazard ratio for disease-free and overall survival was 3.28 and 3.09, respectively. Similarly, high hazard ratios (8.18 and 4.58), with respect to disease-free survival and overall survival were seen in patients who were optimally debulked but were KLK7-positive. In conclusion, patients with well or moderately differentiated tumors had higher risk of developing recurrent disease or dying if were KLK7-positive, when compared to patients with tumors of the same grade but who were KLK7-negative. Patients who had undergone optimal debulking also had increased risk for relapse when the tumors were KLK7-positive. The risk for cancer relapse or death was not significantly different between KLK7-positive and KLK7-negative tumors in patients with tumor grade 3 or with suboptimal debulking.

3.3. Kaplan-Meier survival analysis

Overall, patients with KLK7-negative tumors have improved progression-free survival than patients with KLK7-positive tumors ($p = 0.0035$; Fig. 3). However, this trend was not seen in the overall survival of these patients. Shown in Fig. 4 are the disease-free and overall survival curves for cancer patients with histologic grades 1 and 2. Patients with KLK7-negative tumors had substantially longer disease free ($p < 0.001$) and overall survival ($p = 0.041$) than did

Table 3
Cox proportional hazard regression analysis for subgroups of patients

Variable	Disease-free survival (DFS)			Overall survival (OS)		
	HR[a]	95% CI[b]	P value	HR[a]	95% CI[b]	P value
Tumor grade 1-2						
KLK7 unadjusted	3.28	1.28–8.36	0.012	3.09	1.03–9.41	0.046
KLK7 adjusted[c]	6.14	1.81–20.84	0.004	3.74	1.15–12.13	0.027
Tumor grade 3						
KLK7 unadjusted	1.48	0.85–2.56	0.16	1.17	0.61–2.24	0.62
KLK7 adjusted[c]	1.21	0.68–2.14	0.51	0.91	0.46–1.81	0.79
Optimal debulking success						
KLK7 unadjusted	8.18	2.31–29.14	0.0012	4.58	0.88–23.8	0.071
KLK7 adjusted[e]	7.77	2.10–28.72	0.002	4.02	0.69–25.9	0.12
Suboptimal debuling success						
KLK7 unadjusted	0.94	0.56–1.58	0.83	0.91	0.49–1.69	0.78
KLK7 adjusted[e]	1.13	0.64–1.99	0.65	0.94	0.47–1.88	0.87

[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.
[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.

patients with KLK7-positive tumors. These differences were not seen in patients with grade 3 tumors. Similarly, patients with KLK7-negative tumors who had undergone optimal debulking had a higher probability of disease-free and overall survival than did patients who had KLK7-positive tumors (Fig. 5). No differences in the disease-free or overall survival of patients were observed when surgical debulking was suboptimal.

4. Discussion

In this study, we analyzed the expression of KLK7 (also known as the human stratum corneum chymotryptic enzyme, HSCCE), a member of the human kallikrein gene family, in a series of primary ovarian cancers. Our aim was to establish the relationship between expression of KLK7 with pathologic and clinical parameters, as well as with

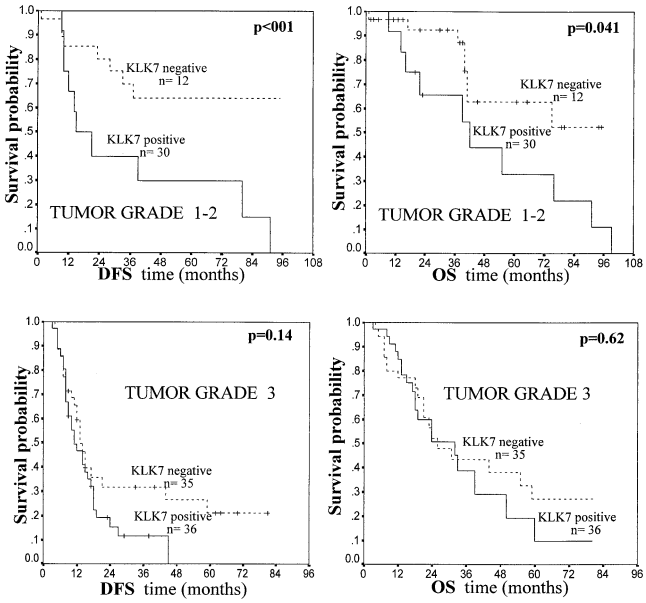

Fig. 4. Kaplan–Meier survival curves for patients with KLK7-positive and KLK7-negative tumors of Grade 1, 2 and 3. DFS: disease-free survival, OS: overall survival.

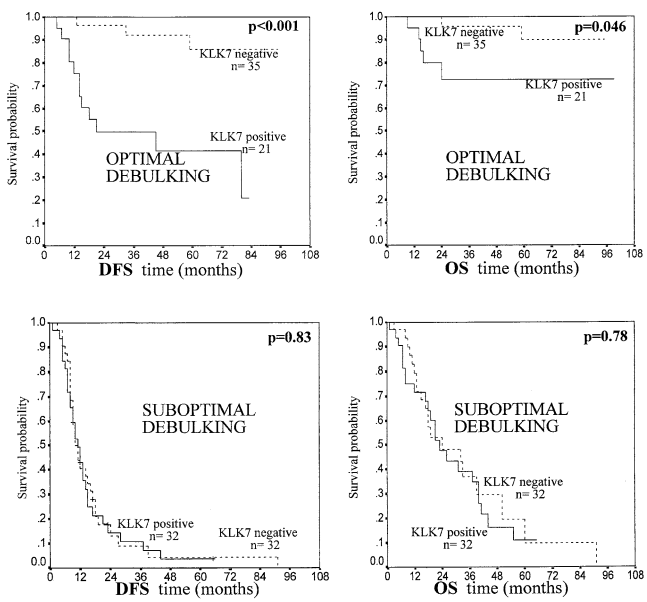

Fig. 5. Kaplan-Meir survival curve for patients with KLK7-positive and KLK7-negative tumors of patients who have undergone optimal or suboptimal debulking. DFS: disease-free survival, OS: overall survival.

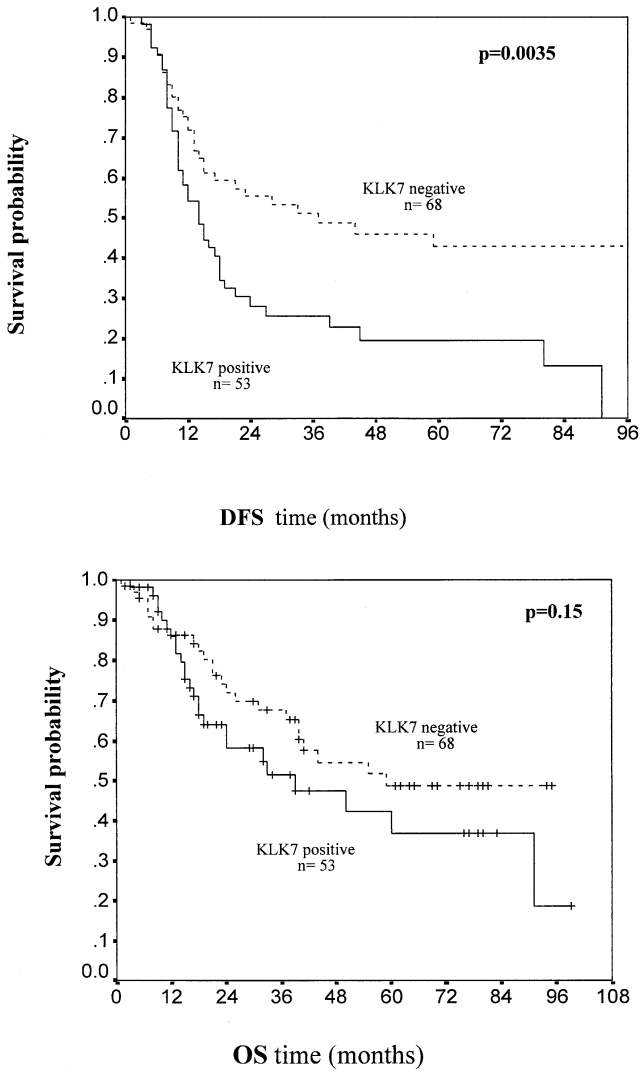

Fig. 3. Kaplan–Meier survival curves for patients with KLK7-positive and KLK7-negative ovarian tumors. DFS: disease-free survival, OS: overall survival.

clinical outcome. Expression levels of KLK7 were measured using a semiquantitative real-time RT-PCR assay on the LightCycler™ system.

To our knowledge, only one previous study has examined KLK7 gene expression in ovarian cancer [36]. However, a relatively small number of patients was included and the patients had primarily advanced disease. Therefore, no conclusive evidence could be presented on the prognostic significance of KLK7 expression in epithelial ovarian carcinoma. Our results suggest that increased expression of this gene is associated with high grade tumors and significantly reduced relapse-free survival (Figure 3). In addition, overexpression of KLK7 is significantly associated with reduced relapse-free and overall survival of patients with grade 1 to 2 tumors (Figure 4). Similarly, overexpression of KLK7 correlates with reduced DFS and OS of patients who had undergone optimal debulking (Figure. 5). This is the first report describing that KLK7 gene overexpression is associated with poor prognosis in a subset of ovarian cancer patients.

It has been shown that KLK7 expression is primarily restricted to the skin and specifically the stratum corneum [35,53,54]. The mechanism by which KLK7 is overexpressed in ovarian cancer is not known.

In the skin, KLK7 has been shown to participate in the degradation of intercellular structures of the matrix, thus promoting epithelial cell desquamation and shedding [53,54]. It has been suggested that overexpression of KLK7 in ovarian cancer may be associated with the shedding or desquamation of ovarian tumor cells [36]. This association is likely, since progression is characterized by early foci of peritoneal metastases and cancer cell spread into the peritoneum, along with ascites production.

We have previously shown that KLK7 expression is regulated by steroid hormones [35]. Recent studies have shown that progesterone receptor positivity is a favorable prognostic marker in ovarian cancer [55–58]. Progesterone promotes cell differentiation and apoptosis and thus, diminished response to progesterone could lead to disregulation of differentiation/apoptosis pathways. Indeed, grade 3 ovarian tumors are predominately PR-negative. Taken together this evidence may pinpoint to a link between KLK7 overexpression and progesterone receptor negativity.

Data from our laboratory and others, indicate that a number of kallikreins (KLK4, KLK5, KLK6, KLK7, KLK8, KLK9, KLK10) are up-regulated in ovarian cancer [31,33,36–41,59]. While most kallikrein overexpression is associated with unfavourable outcome, KLK8 and KLK9 overexpression is associated with a favorable outcome. These data suggest that the kallikrein gene family may encode for proteases that act in concert in ovarian cancer tissues to either promote or inhibit more aggressive phenotypes. In addition, we speculate that some kallikrein proteins (as is the case with hK3 and hK2) may be valuable diagnostic and prognostic markers and possibly therapeutic targets.

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