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Human kallikrein 11: an indicator of favorable prognosis in ovarian cancer patients

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Abstract

Objectives: Human kallikrein 11 (hK11) is a secreted serine protease, highly expressed in hormonally regulated tissues, including the prostate and the ovary. Our preliminary studies indicate that hK11 may represent a diagnostic and prognostic biomarker for ovarian cancer. The aim of the present study was to examine the prognostic value of hK11 expression in ovarian tumors.

Methods: Using our established immunofluorometric assay, hK11 levels were quantified (ng per mg of total protein) in 134 ovarian tumor extracts and correlated with various clinicopathological variables and outcome [progression-free survival (PFS), overall survival (OS)], over a median follow-up period of 42 months.

Results: hK11 concentration in ovarian tumor cytosols ranged from 0 to 155 ng/mg of total protein, with a median of 1.45 ng/mg. An optimal cutoff value of 6.3 ng/mg was selected to categorize tumors as hK11-positive or negative. hK11-positive tumors were most often of early stage (Stage I/II) and grade (G1/G2) ($P < 0.05$). Univariate analysis revealed that patients with hK11-positive tumors had a significantly longer PFS (HR of 0.39, $P = 0.005$) and OS (HR of 0.44, $P = 0.033$). Cox multivariate analysis indicated that hK11 was an independent prognostic indicator of PFS (HR of 0.47, $P = 0.042$). Kaplan–Meier survival curves further confirmed that women with hK11-positive tumors have longer PFS and OS ($P = 0.003$ and $P = 0.028$, respectively). Also, a weak positive correlation was found between the expression levels of tissue hK11 and tissue CA125 ($r_s = 0.508$; $P < 0.001$).

Conclusions: These results further validate our initial findings that hK11 is an independent marker of favorable prognosis in ovarian cancer patients.

Keywords: Serine proteases; Kallikreins; Cancer biomarkers; Prognostic markers; Ovarian cancer; Human kallikrein 11; hK11

Introduction

Human tissue kallikreins are serine proteases encoded by 15 structurally similar hormonally regulated genes that colocalize to chromosome 19q13.4 [1]. Accumulating evidence indicates that many kallikreins are differentially expressed in ovarian cancer at both the mRNA and protein levels and several possess prognostic value [2]. Furthermore, several kallikrein proteins, including hK6 and hK10, represent putative serum-based screening and/or diagnostic ovarian cancer biomarkers [3–6].

We have recently developed a highly specific and sensitive immunofluorometric assay for human kallikrein 11 (hK11, TLSP, PRSS20; encoded by *KLK11*) [7]. Using this method, we observed elevated serum hK11 levels in 70% of women with ovarian cancer, thereby suggesting that hK11, similar to hK6 and hK10, is a candidate screening and/or diagnostic biomarker. In a subsequent study, we quantified hK11 in ovarian tumor cytosolic extracts and found that hK11-positive tumors were most often of early stage (Stage I/II) disease and from women with pre/peri-

Abbreviations: KLK, human kallikrein gene; hK, human kallikrein protein; PFS, progression-free survival; OS, overall survival; HR, hazard ratio.

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Table 1
Distribution of hK11 values in cancer and low malignant potential (LMP) ovarian tissues

hk11 concentration (ng/mg protein)	Mean ± SE[a]	Range	Percentiles (median)					*P* value[b]
			10	25	50	75	90	
LMP tissues (*N* = 22)	11.6 ± 6.8	0.00–152	0.19	0.88	3.28	7.0	23.5	0.14
Cancer tissues (*N* = 134)	8.6 ± 1.9	0.00–155	0.00	0.16	1.45	6.3	18.5	

[a] Standard error.
[b] Calculated by the Mann–Whitney test.

menopausal status, who exhibited complete or partial response to chemotherapy [8]. Furthermore, hK11 was found to be an independent indicator of favorable prognosis [8]. Thus, hK11 may possess prognostic value, in addition to its screening/diagnostic potential. By immunohistochemical analysis, we have also demonstrated that hK11 is present in the cytoplasm of epithelial cells derived from invasive papillary serous carcinoma of the ovary [7]. Also, similar to other kallikreins, the *KLK11* gene was found to be up-regulated by estradiol in two breast cancer cell lines [7], further suggesting a role for this protease in ovarian cancer and other endocrine-related malignancies. In the present study, we further examine the prognostic value of hK11 expression in a different patient population with ovarian cancer, to extend and confirm our previously published data [8].

Materials and methods

Ovarian cancer patients and specimens

One hundred and thirty-four German patients with primary epithelial ovarian cancer and 22 with low malignant potential (LMP) tumors were examined in this study, ranging in age from 20 to 85 and years, with a median age of 58 (Table 1). Histological examination, performed during intrasurgery frozen section analysis, allowed representative portions of each tumor containing more than 80% tumor cells to be selected for storage until analysis. Patients were monitored for survival and disease progression (no apparent progression or progression) for a median duration of 42 months (range of 1–125 months). Follow-up information was available for 134 patients, among which 77 (57%) had relapsed and 57 (42%) had died.

Clinical and pathological information documented at the time of surgery included tumor stage, grade, histotype, residual tumor size, debulking success, and volume of ascites fluid (Table 2). The staging of tumors was in accordance with the FIGO criteria [9]; grading was established according to Day et al. [10]; and the classification of histotypes was based on both the WHO and FIGO recommendations [11].

Patients with disease at clinical stages I–III and grades [1–3] were represented in this study. Of the 134 ovarian tumors, the majority (95; 71%) were of the serous papillary histotype, followed by mucinous (12; 9%), undifferentiated (12; 9%), endometrioid (6; 4%), clear cell (4; 3%), or were unclassified (5; 4%). The residual size of tumors ranged from 0 to 6 cm.

Table 2
Relationship between hK11 status[a] and other variables in 134 ovarian cancer patients

Variable	Patients	No. of patients (%)		*P* value
		hK11-negative	hK11-positive	
Stage				
I/II	32	19 (59.4)	13 (40.6)	0.02[b]
III	102	82 (80.4)	20 (19.6)	
Grade				
G1/G2	53	35 (66.0)	18 (34.0)	0.038[b]
G3	80	66 (82.5)	14 (17.5)	
x	1			
Histotype				
Serous	95	75 (78.9)	20 (21.1)	0.078[c]
Mucinous	12	5 (41.7)	7 (58.3)	
Endometrioid	6	5 (83.3)	1 (36.7)	
Clear cell	4	3 (75.0)	1 (25.0)	
Undifferentiated	12	8 (66.7)	4 (33.3)	
x	5			
Residual tumor (cm)				
0	69	49 (71.0)	20 (29.0)	0.45[c]
≤2	38	31 (81.6)	7 (18.4)	
>2	23	18 (78.3)	5 (21.7)	
x	4			
Debulking success[d]				
SD	61	49 (80.3)	12 (19.7)	0.23[b]
OD	69	49 (71.0)	20 (29.0)	
x	4			
Ascites fluid (ml)				
0	41	28 (68.3)	13 (31.7)	0.22[c]
≤500	45	33 (73.3)	12 (26.7)	
>500	44	37 (84.1)	7 (15.9)	
x	4			

x = status unknown.
[a] Equal to 75th percentile (6.3 ng/mg protein).
[b] Fisher's Exact Test.
[c] χ^2 test.
[d] OD, optimal debulking (0–1 cm), SO; suboptimal debulking (>1 cm).

Investigations were carried out in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 1983, and were approved by the IRB of the Technical University of Munich.

Preparation of cytosolic extracts

Tumor specimens were snap-frozen in liquid nitrogen immediately after surgery and stored at −80°C until extraction. Frozen tissues (20–100 mg) were pulverized on dry ice to a fine powder and added to 10 volumes of extraction buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 10 g/l of NP-40 surfactant, 1 mM phenylmethyl sulfonyl fluoride, 1 g/l of aprotinin, 1 g/l of leupeptin). The resulting suspensions were incubated on ice for 30 min, with repeated shaking and vortexing every 10 min. The mixtures were then centrifuged at 14,000 rpm at 4°C for 30 min and the supernatant (cytosolic extract) was collected and stored at −80°C until further analysis. Protein concentration of the extracts was determined using the bicinchoninic acid method, with albumin as standard, as per the manufacturer's recommendations (Pierce Chemical Co., Rockford, IL).

Measurement of hK11 in ovarian cytosolic extracts

The concentration of hK11 in the cytosolic extracts was quantified using a highly sensitive and specific noncompetitive ''sandwich-type'' immunoassay for hK11, previously described and evaluated [7]. Briefly, a mouse anti-hK11 monoclonal antibody was captured with sheep antimouse IgG, Fc fragment-specific antibodies (Jackson Immunoresearch, West Grove, PA) on 96-well polystyrene microtiter plates. hK11 calibrators (recombinant hK11 in 60 g/L BSA) or cytosolic extracts (100 μl) were then applied to each well in duplicate, incubated for 2 h with gentle shaking and washed. Rabbit anti-hK11 polyclonal antiserum was subsequently applied, incubated, and washed. Finally, alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson Immunoresearch) was added, incubated, and washed as before. Signal detection and data reduction were performed automatically by the CyberFluor 615 Immunoanalyzer, which uses time-resolved fluorometry, as described elsewhere [12]. The detection range of this assay is 0.1–50 μg/l. hK11 concentrations in μg/l were converted to ng of hK11/mg of total protein to adjust for the amount of tumor tissue extracted.

Statistical analysis

Statistical analyses were performed with SPSS software (SPSS Inc., Richmond, CA). An optimal cutoff was identified by χ^2 analysis, based on the ability of hK11 values to predict the PFS of the study population. Based on this cutoff, the hK11 status of ovarian tumor extracts was categorized as either hK11-positive or hK11-negative. The relationship between hK11 status and various clinicopathological variables was analyzed with the χ^2 test and the Fisher's Exact Test, as appropriate.

For survival analysis, two different end points—cancer relapse (either local recurrence or distant metastasis) and death—were used to calculate progression-free (PFS) and overall survival (OS), respectively. PFS was defined as the time interval between the date of surgery and the date of identification of recurrent metastatic disease. OS was defined as the time interval between the date of surgery and the date of death. The impact of hK11 on patient survival (PFS and OS) was assessed with the hazard ratio (relative risk of relapse or death in the hK11-positive group) calculated with the Cox univariate and multivariate proportional hazard regression model [13]. In the multivariate analysis, the clinical and pathological variables that may affect survival, including age, stage of disease, tumor grade, histotype, and residual tumor size were adjusted.

Kaplan–Meier PFS and OS curves [14] were constructed to demonstrate survival differences between the hK11-positive and hK11-negative patients. The differences between the survival curves were tested for statistical significance using the log rank test [15].

Results

Distribution of hK11 concentration in ovarian tumor and LMP tissue extracts

As shown in Table 1, hK11 concentration in ovarian tumor cytosols from 134 patients ranged from 0 to 152 ng/mg of total protein, with a mean of 8.6 ng/mg total protein and a median of 1.5 ng/mg total protein. The hK11 levels in tumors of low malignant potential ranged from 1 to 152 ng/mg total protein, with a mean of 11.6 ng/mg total protein and a median of 3.3 ng/mg total protein. Although mean hK11 levels were higher in LMP tumors vs. cancerous tissues (Table 1), this difference was not statistically significant ($P = 0.14$). An optimal cutoff value of 6.3 ng/mg total protein was identified by χ^2 analysis (Fig. 1). Based on this cutoff (75th percentile), 25% of the ovarian tumors were categorized as being hK11-positive.

Relationships between hK11 status and other clinicopathological variables

The distributions of various clinicopathological variables between hK11-postitive and hK11-negative patients are summarized in Table 2. The relationships between hK11 and these variables were examined with either the χ^2 or Fisher's Exact Test. No relationship was observed between hK11 status and residual tumor size, debulking

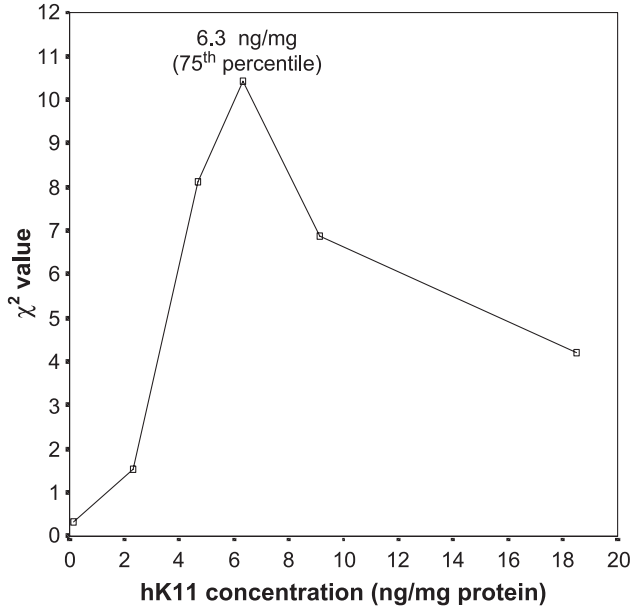

Fig. 1. Determination of the optimal cutoff point for hK11 expression by χ^2 analysis. For details, see text.

success, or volume of ascites fluid. However, patients with hK11-positive ovarian tumors were more likely to have early stage (Stage I/II) and grade (G1/G2) disease ($P < 0.05$). Although marginally significant, hK11-positive tumors were mainly of the mucinous histotype ($P = 0.078$).

Univariate and multivariate survival analysis

The strength of association between hK11-positive tumors and survival outcome is presented in Table 3. In univariate analysis, hK11-positive patients had a significantly longer PFS (HR of 0.39, $P = 0.005$) and OS (HR of 0.44, $P = 0.033$). However, only the favorable effects of hK11 positivity on PFS remained when hK11 was treated as a continuous variable (HR of 0.96, $P = 0.025$). As expected, disease staging, grading, and debulking success were found to be strongly associated with decreased PFS and OS ($P < 0.05$).

Furthermore, when survival outcomes were adjusted for all other variables in the multivariate analysis (Cox proportional hazard regression model), the association with PFS remained (HR of 0.47, $P = 0.042$). Also, disease staging, grading, and debulking success were found to be the strongest independent indicators of poor prognosis.

Kaplan–Meier survival curves (Fig. 2) further demonstrate that women with hK11-positive tumors have longer PFS and OS ($P = 0.003$ and $P = 0.028$, respectively) compared with those who are hK11-negative.

Table 3
Univariate and multivariate analysis of hK11 status with regard to PFS and OS

Variable	Progression-free survival			Overall survival		
	HR[a]	95% CI[b]	*P* value	HR[a]	95% CI[b]	*P* value
Univariate analysis						
hK11 ($N = 130$)						
Negative	1.00			1.00		
Positive	0.39	0.21–0.75	0.005	0.44	0.21–0.93	0.033
As continuous variable	0.96	0.94–0.99	0.025	0.97	0.95–1.01	0.12
Stage of disease (ordinal)	2.29	1.61–3.27	<0.001	3.38	2.18–5.23	<0.001
Grading (ordinal)	1.48	1.08–2.02	0.013	1.63	1.14–2.32	0.007
Debulking success	4.04	2.46–6.63	<0.001	8.00	4.12–15.52	<0.001
CA125	1.00	0.99–1.00	0.29	1.00	0.99–1.00	0.33
Age	1.02	0.99–1.03	0.061	1.024	1.00–1.05	0.026
Multivariate analysis[c]						
hK11 ($N = 120$)						
Negative	1.00			1.00		
Positive	0.47	0.22–0.98	0.042	0.49	0.19–1.22	0.13
As continuous variable	0.96	0.92–1.00	0.077	0.96	0.92–1.02	0.22
Stage of disease (ordinal)	1.64	1.07–2.51	0.023	2.22	1.27–3.85	0.004
Grading (ordinal)	1.24	0.87–1.77	0.22	1.41	0.091–2.21	0.13
Debulking success	2.75	1.58–4.81	<0.001	4.97	2.31–10.71	<0.001
CA125	1.00	0.99–1.00	0.71	1.00	0.99–1.00	0.54
Age	1.00	0.98–1.03	0.47	1.01	0.98–1.04	0.33

[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, debulking success, tumor grade, CA125, and age.

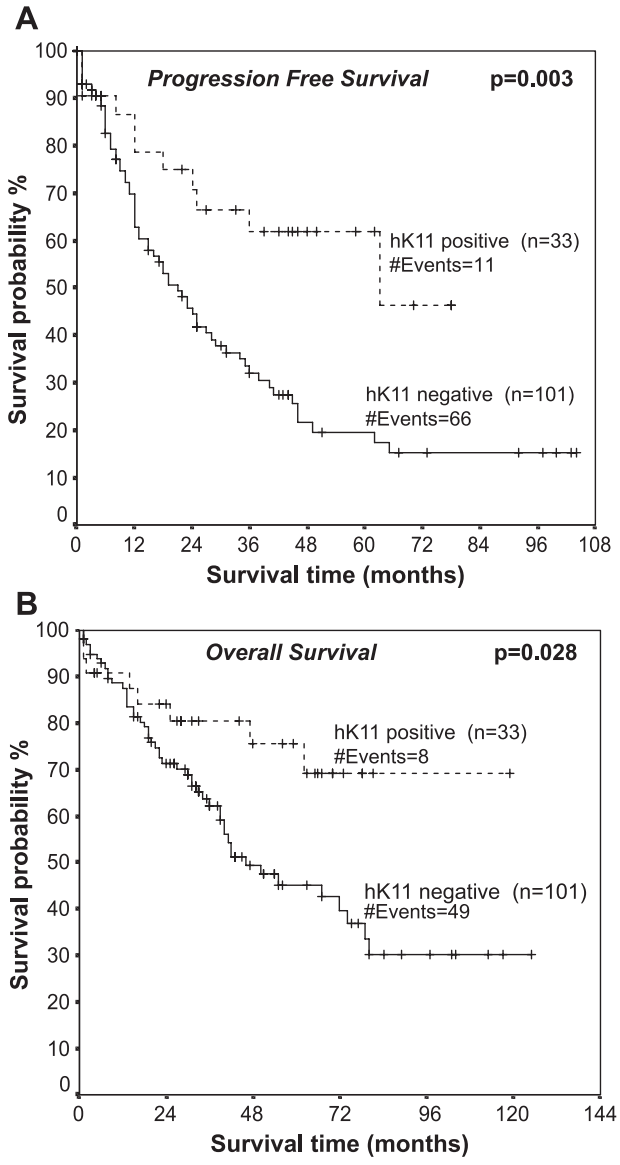

Fig. 2. Kaplan–Meier survival curves for (A) progression-free survival and (B) overall survival in patients with hK11-positive and -negative ovarian tumors. *n* = number of samples.

Lastly, a weak positive correlation was found between the expression levels of hK11 and CA125 in the ovarian tumor extracts (r_s = 0.51; $P < 0.001$; Fig. 3).

Discussion

Epithelial ovarian cancer is the sixth most common malignancy among women worldwide and the most lethal of all gynecological cancers [16,17]. With a lack of early warning symptoms or reliable screening methods, the majority of patients are diagnosed at advanced International Federation of Gynecologists and Obstetricians (FIGO) Stages III/IV, when 5-year survival rates are only 18% [18]. This figure increases to 87%, when tumors are detected at FIGO Stages I/II.

In addition to pelvic examination and transvaginal ultrasonography, serum CA125, the most extensively evaluated ovarian cancer biomarker, has been implemented in screening, as well as in diagnosis and monitoring [19]. However, only a multimodal screening approach based on the sequential use of ultrasound and CA125 levels, and the parallel use of CA125 with other tumor markers, has resulted in higher sensitivity, specificity, and positive predictive value for early stage disease, compared to CA125 measurements alone [20–22]. The identification of additional reliable screening and/or diagnostic biomarkers would be expected to improve the outcome of ovarian cancer patients by enabling early detection. The discovery of prognostic and predictive biomarkers would aid in the optimal management of these patients, predict disease outcome, and determine effective, individualized therapeutic strategies.

In the present study, we have evaluated hK11 expression in epithelial ovarian tumors in relation to other established prognostic indicators and patient survival. hK11 was most frequently expressed in early stage (Stage I/II) and grade (G1/G2) tumors and this overexpression was significantly associated with an increased PFS and OS in univariate analysis. This relationship was further illustrated in the Kaplan–Meier survival curves. Multivariate analysis also indicated that hK11 was an independent indicator of PFS.

In comparison to our earlier study of Italian ovarian cancer patients (N = 104) [8], the present report included a slightly higher number of German patients (N = 134). While both studies established significant associations ($P < 0.05$) between hK11-positive ovarian tumors and early stage (Stage I/II) disease as well as with prolonged

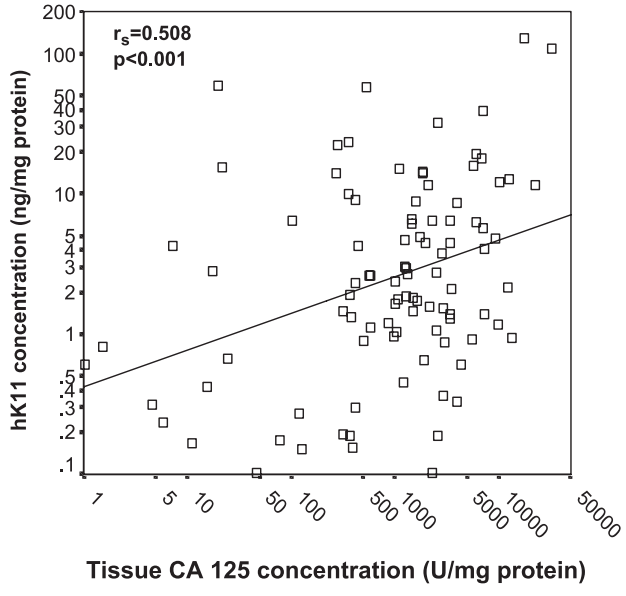

Fig. 3. Correlation between tissue CA125 and hK11 in ovarian tumor extracts. r_s, Spearman correlation coefficient.

PFS and OS, the current study demonstrates additional associations between hK11 and other prognostic variables. For example, a connection between high hK11 levels and a lower tumor grade (G1/G2) was not apparent in the original study. Furthermore, mucinous tumors were more frequently hK11-positive than endometrioid and serous tumors ($P = 0.078$), while in the previous study all three histotypes had approximately equal hK11-positivity ($P = 0.32$). We speculate that some of these discrepancies may be due to the different ethnic origin of the ovarian cancer patients studied.

Analogous to hK11, other members of the human tissue kallikrein family, including *KLK8*, *KLK9*, and *KLK14* are also most frequently expressed in early stage ovarian tumors [23–25]. Because high mRNA levels of these genes were also associated with a favorable prognosis, it would be interesting to determine their combined prognostic potential together with hK11. As well, it is also possible that these kallikreins function in concert or in a cascade, inhibiting ovarian carcinogenesis [26]. Furthermore, we have recently reported that presurgical serum hK6 [4] and hK10 [6] levels are indicators of poor prognosis for ovarian cancer patients. It would be interesting to evaluate the prognostic value of serum hK11 levels in the future.

We have also discovered a weak, positive correlation between hK11 and CA125 levels in ovarian tumor tissues. In general, high tissue CA125 levels are associated with serous and endometriod ovarian tumors [27]; however, CA125 is usually not informative in patients with nonserous tumors [28,29]. Out of the 33 hK11-positive tumors examined, significantly higher levels were found in tumors of the nonserous histotype ($P = 0.078$; Table 2), suggesting that hK11 may be useful in evaluating prognosis in the subgroup of patients with tumors of this histotype.

In conclusion, we provide further evidence to support the potential clinical utility of hK11 as an independent indicator of favorable prognosis in ovarian cancer patients. Additional studies are warranted to determine the usefulness of hK11 in ovarian cancer prognosis, as well as in cancer screening and diagnosis.

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