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Report

The prognostic value of the human kallikrein gene 9 (*KLK9*) in breast cancer

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Summary

Background. Many members of the human kallikrein gene family were found to be differentially expressed in various malignancies and some of them are useful diagnostic/prognostic markers. *KLK9* is a newly discovered human kallikrein gene that is expressed in several tissues including thymus, spinal cord, testis, prostate, breast, and ovary. Like other kallikreins, the *KLK9* gene was found to be regulated by steroid hormones, mainly estrogens and progestins, in cancer cell lines.

Experimental design. We studied the expression of *KLK9* by quantitative RT-PCR in 169 breast cancer patients of different stages, grades and histological types. We also compared the relation between *KLK9* expression and other clinicopathological variables and patient survival.

Results. KLK9 expression is significantly higher in patients with early stage cancers ($p = 0.039$) and in patients with small tumor size (<2 cm) ($p = 0.028$). Kaplan-Meier survival curves demonstrated that *KLK9*-positive patients have longer disease-free and overall survival ($p = 0.015$ and 0.036, respectively). Univariate and multivariate analysis also indicates that *KLK9* expression is associated with increased disease-free and overall survival. When the Cox proportional hazard regression analysis was applied to subgroups of patients, *KLK9* expression was found to be a significant predictor of disease-free survival in the estrogen receptor (ER) and progesterone receptor (PR) negative subgroups of patients (Hazard Ratio 'HR' = 0.28, and 0.38, respectively, and $p = 0.011$ and 0.028, respectively). After adjusting for other known prognostic variables, *KLK9* retained its independent prognostic value in these subgroups of patients. Similar results were obtained for overall survival.

Conclusions. KLK9 is a new potential independent marker of favorable prognosis in breast cancer.

Abbreviations: KLK: human kallikrein (gene); hK: human kallikrein (protein); PCR: polymerase chain reaction; PSA: prostate specific antigen; RT: reverse transcription; ER: estrogen receptor; PR: progesterone receptor; DFS: disease-free survival; OS: overall survival

Introduction

Breast cancer is the most common malignancy among females in North America. In the United States alone, about 200,000 new cases are diagnosed every year, and about 50,000 women die annually from the disease [1]. The selection of therapies for breast cancer is based on grouping of patients according to the

presence or absence of certain clinical characteristics. Since these carcinomas display high variability in their biological and clinical behavior, efforts have been directed at finding specific markers that could reflect the characteristics of each particular tumor. The identification of new prognostic/predictive markers will contribute to more optimal patient sub-grouping and individualization of treatment [2]. The classical prognostic markers for breast cancer, including lymph node status, tumor size and stage, have proven prognostic importance [3]. Many other potential prognostic/predictive markers have been identified, including steroid receptors, indicators of cell proliferation (Ki 67, TLI), apoptosis (Bax, BCL2) and angiogenesis (VEGF, PD-ECGF), tumor suppressor genes (p53, nm23), oncogenes (c-erbB2, ras) and proteolytic factors (uPA, PAI-1) [2–6]. However, only hormone receptor status is recommended for routine use by the American Society of Clinical Oncology [7] and the College of American Pathologists Consensus Statement [3]. None of the remaining biomarkers has sufficient prognostic/predictive value by itself.

The human kallikrein gene family comprises 15 genes that co-localize on a 300-kb region of chromosome 19q13.4 [8–17]. Several groups have shown that many human kallikrein genes are differentially expressed in various malignancies (reviewed in Refs. [18–20]). This family contributed the best tumor marker available so far for prostate cancer, prostate specific antigen (PSA) [21] and hK2 (encoded by the *KLK*2 gene) is a useful adjuvant marker for certain subtypes of prostate cancer [22, 23]. In addition to its clinical utility in prostate cancer, recent reports suggest that hK3(PSA) can also be used as a breast cancer prognostic marker [24]. Another kallikrein, *KLK*10 (NES1) is a breast cancer tumor suppressor gene [25].

We have recently cloned the human kallikrein gene 9 (*KLK9*, formerly known as *KLK*-L3), a new member of the human kallikrein gene family [10], which is expressed in many tissues including cerebellum, spinal cord, and hormonally-regulated organs such as the testis, breast, prostate and ovary. *KLK9* is under steroid hormonal regulation, mainly by estrogens and progestins, in cancer cell lines [26]. More recently, we have shown that *KLK9* is differentially expressed in ovarian cancer and is an independent marker of favorable prognosis [26]. In this investigation, we analyzed *KLK9* gene expression in breast cancer tissues by quantitative RT-PCR and examined its prognostic significance.

Materials and methods

Study population

Included in this study were tumor specimens from 169 consecutive patients undergoing surgical treatment for primary breast carcinoma at the Department of Gynecologic Oncology at the University of Turin, Turin, Italy. The diagnosis was confirmed by histopathology for all samples. Tumor tissues had been frozen in liquid nitrogen immediately after surgery. The patient ages ranged from 29 to 83 with a median of 57 years. Tumor sizes ranged from 0.1 to 15 cm with a median of 2.1 cm. Follow-up information (median follow-up period 78 months) was available for 161 patients, among whom 50 (31%) had relapsed and 43 (27%) died. The histological type and steroid hormone receptor status of each tumor as well as the number of positive axillary nodes were established at the time of surgery. Out of the 169 patients, 111 (66%) had ductal carcinoma, 28 (17%) lobular carcinoma and 29 (17%) had other histological types. The histological type was not identified for one patient. Patients from clinical stages I–III were included in the study; staging was determined according to the TNM classification. Grading of tumors was done according to the Bloom–Richardson grading system [27]. Thirty-eight patients (22%) received no adjuvant treatment, 78 (46%) received tamoxifen, and 53 (31%) received chemotherapy with or without tamoxifen. Estrogen and progesterone receptor status was established as described by the European Organization for Research and Treatment of Cancer [28]. This study has been approved by the Institutional Review Board of the University of Turin.

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, homogenized and total RNA was extracted using Trizol^{TM} reagent (Gibco BRL, Gaithersburg, MD) following the manufacturer's instructions. The concentration and purity of RNA were determined spectrophotometrically. Total RNA of 2 μg was reverse-transcribed into first strand cDNA using the Superscript^{TM} preamplification system (Gibco BRL).

Quantitative real-time RT-PCR analysis

Based on the published genomic sequence of *KLK9* (GenBank accession # AF135026), two gene-specific

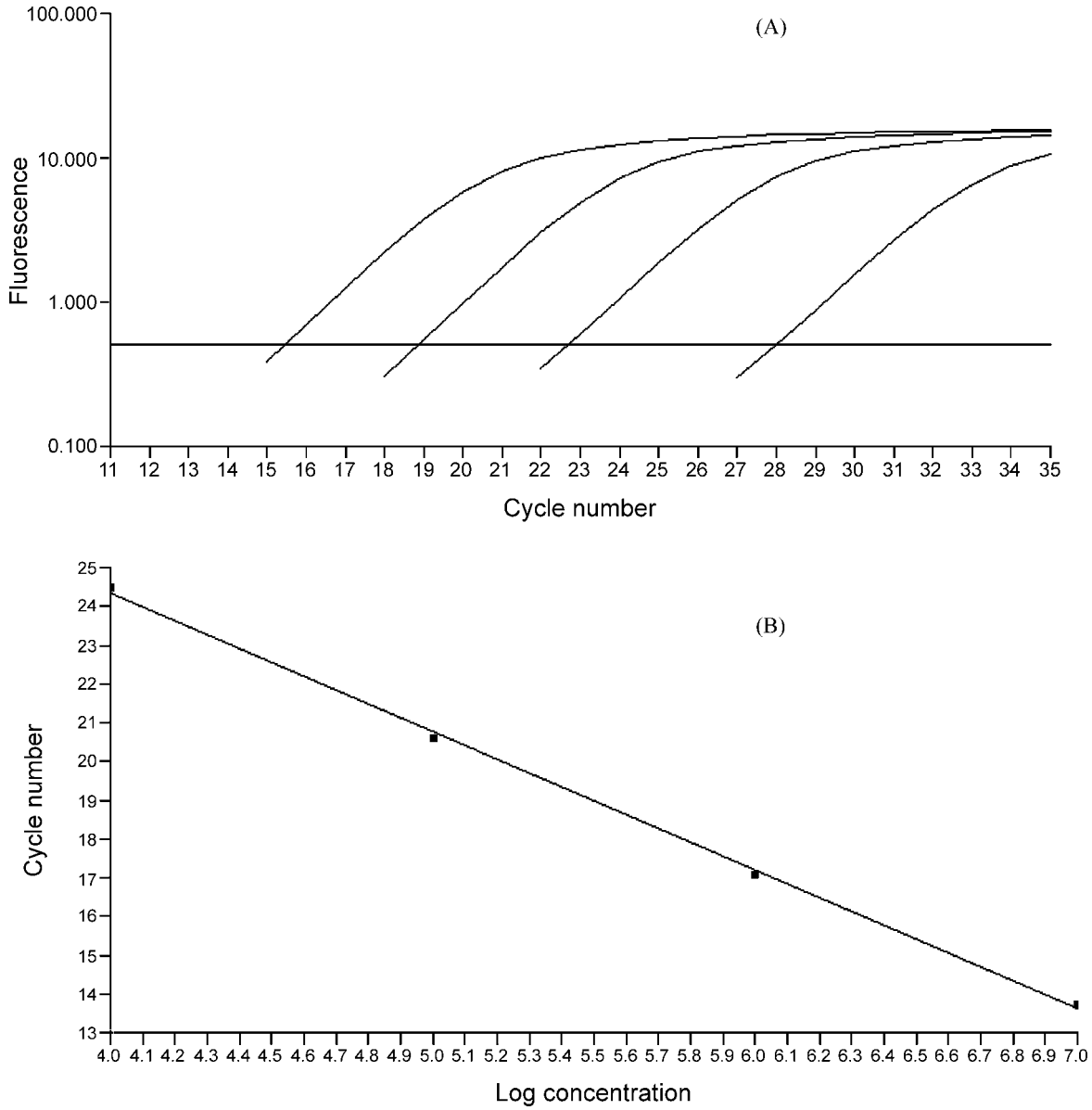

Figure 1. Quantification of *KLK9* gene expression by real-time PCR. (A): A logarithmic plot of fluorescence signal (Y-axis) of the standard curve dilutions above the noise level during amplification (the X-axis represent the cycle number). Serial dilutions of a *KLK9* cDNA plasmid were used and an arbitrary copy number was assigned to each sample according to the dilution factor. Each sample was done in duplicate. (B): A representative graph of the standard curve.

primers were designed (3F5: 5′ GAG AGC ACC ACC TCT GGA AA 3′, and 3R5: 5′ GTG GCA TAC GCT GGT GTA GA 3′). These primers spanned more than two exons to avoid contamination by genomic DNA.

Real-time monitoring of PCR reactions was performed using the LightCycler^{TM} 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, permitting real time monitoring of the PCR reaction. 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

Table 1. Distribution of numerical variables in the study

Variable	No. of patients	Mean ± SE[a]	Median	Range
KLK9 (arbitrary units)	169	98.3 ± 17.8	6.67	0.002–939
Age (years)	169	57.1 ± 0.93	57.0	29–83
Tumor size (cm)	169	2.54 ± 0.12	2.10	0.1–15.00
Lymph nodes[b]	159	3.1 ± 0.4	1	0–29

[a] SE, standard error.
[b] Number of lymph nodes positive for malignancy.

quantity of the template, the earlier a significant increase in fluorescence is observed. The threshold cycle is defined as the fractional cycle number at which fluorescence passes a fixed threshold above baseline (Figure 1). *KLK9* calibration curves were constructed using serial dilutions of a *KLK9* mRNA plasmid. Standard curve calibrators were included in each run. Standards for both *KLK9* and actin RNAs were defined to contain an arbitrary starting concentration, and serial dilutions (with concentrations defined according to the dilution factor) were used to construct the standard curve.

PCR amplification

The PCR reaction was carried out on the LightCycler™ system. For each run, a master mixture was prepared on ice, containing 1 μl of cDNA, 2 μl of LC DNA Master SYBR green I mix, 50 ng of primers and 1.2 μl of 25 mM $MgCl_2$. The final volume was adjusted with H_2O to 20 μl. After the reaction mixture was loaded into a glass capillary tube, the cycling conditions were carried out as follows: initial denaturation at 94°C for 10 min, followed by 45 cycles of denaturation at 94°C for 0 s, annealing at 55°C for 10 s, and extension at 72°C for 30 s. The temperature transition rate was set at 20°C per second. Fluorescent product was measured by a single acquisition mode at 86°C after each cycle. 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.

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. The amount of the target molecule was then divided by the amount of the endogenous reference, to obtain a normalized target value, as described elsewhere [26].

Melting curve

For distinguishing specific from non-specific 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 per s, with the signal acquisition mode set at step. To verify the melting curve results, representative samples of the PCR products were run on 1.5% agarose gels, purified, and cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The inserts were sequenced using vector-specific primers, with an automated DNA sequencer.

Statistical analysis

Associations between clinicopathological parameters such as stage, grade and histotype and *KLK9* 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 disease-free and overall survival, respectively. Disease-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 [29] was used to evaluate the hazard ratio (relative risk of relapse or death in the *KLK9*-positive group). In the multivariate analysis, the models were adjusted for *KLK9* expression, clinical stage, histologic grade, residual tumor and age. Kaplan-Meier survival curves [30] were constructed for *KLK9*-positive and *KLK9*-negative patients.

Table 2. Associations between *KLK9* status[a] and other variables

Variable	Total	No. of patients (%)		*p* value
		KLK9-negative	*KLK9*-positive	
Age (years)				
<45	32	18 (56.3)	14 (43.8)	
45–55	47	27 (57.4)	20 (42.6)	0.78[d]
>55	90	56 (62.2)	34 (37.8)	
Menopausal status				
Pre/peri	58	32 (55.2)	26 (44.8)	0.41[e]
Post	111	69 (62.2)	42 (37.8)	
Tumor size (cm)				
<2	79	40 (50.6)	39 (49.4)	0.028[e]
≥2	90	61 (67.8)	29 (32.2)	
Nodal status				
Negative	71	44 (62.0)	27 (38.0)	0.63[e]
Positive	88	51 (58.0)	37 (42.0)	
x	10			
Stage[c]				
I	77	38 (49.3)	39 (50.6)	
II	64	44 (68.7)	20 (31.2)	0.039[d]
III	20	14 (70.0)	6 (30.0)	
x	8			
Grade[b]				
I	63	35 (55.6)	28 (44.4)	
II	58	34 (58.6)	24 (41.4)	0.65[e]
III	45	29 (64.4)	16 (35.6)	
x	2			
Histology				
Ductal	111	68 (61.3)	43 (38.7)	
Lobular	28	15 (53.6)	13 (46.4)	0.75[d]
Other	29	17 (58.6)	12 (41.4)	
x	1			
ER status				
Negative	65	37 (56.9)	28 (43.1)	0.75[e]
Positive	99	59 (59.6)	40 (40.4)	
x	5			
PR status				
Negative	75	45 (60.0)	30 (40.0)	0.87[e]
Positive	90	52 (57.8)	38 (42.2)	
x	4			
Adjuvant treatment				
None	38	20 (52.6)	18 (47.4)	
Tamoxifen	78	51 (65.4)	27 (34.6)	0.36[d]
Chemotherapy ± tamoxifen	53	30 (56.6)	23 (43.4)	

[a] Cutoff point: 16.4, equal to the 60th percentile.
[b] Bloom–Scarff–Richardson grading system.
[c] TNM system.
[d] χ^2 test.
[e] Fisher's exact test.
x Status unknown.

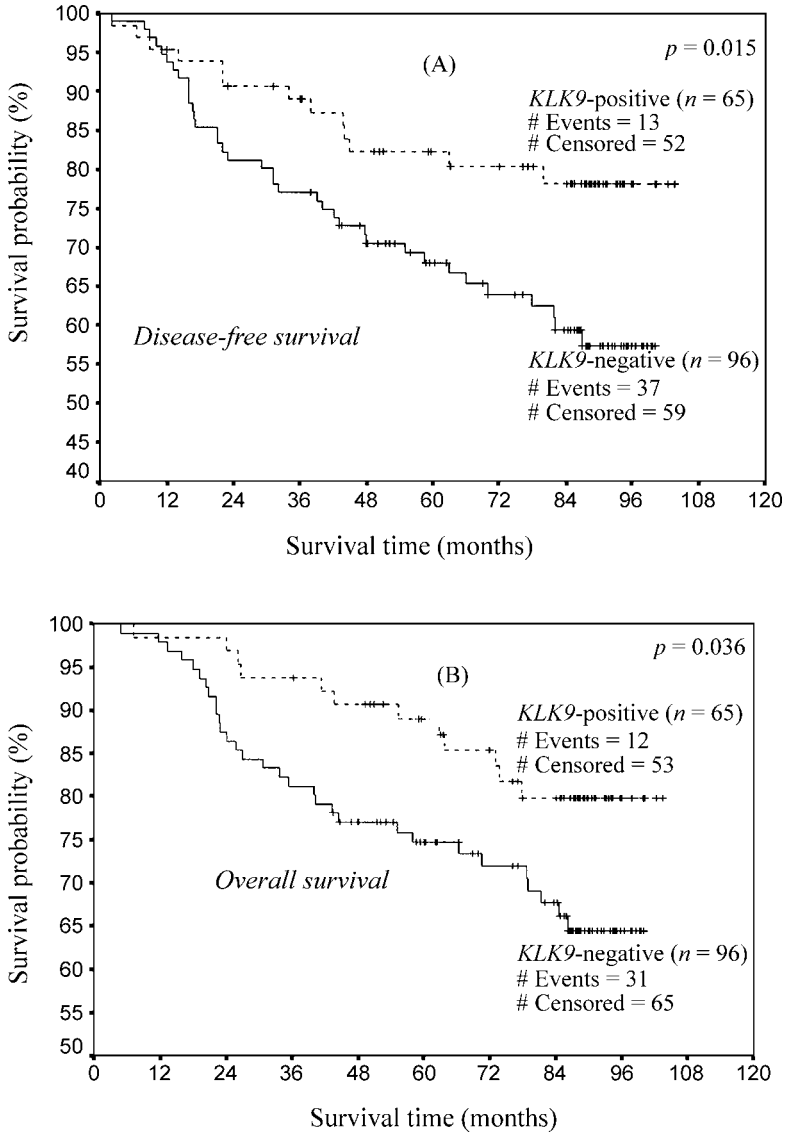

Figure 2. Kaplan-Meier survival curves for patients with *KLK9* positive and negative breast cancer in relation to disease-free survival (A) and overall survival (B). n = number of samples.

Results

The distribution of *KLK9* expression levels in the tumor specimens was not Gaussian and was scewed to the right. Numerical values of some study parameters are shown in Table 1. An optimal cutoff value was defined by χ^2 analysis, based on the ability of *KLK9* to predict the overall survival (OS) of the study population. A value of 16.4 (arbitrary units) was shown to be the statistically optimal cutoff (60th percentile).

KLK9 *expression in relation to other variables*

Table 2 shows the distribution of qualitative *KLK9* expression (positive or negative according to the cut-off) in breast cancer tissues, in relation to age, menopausal status, tumor size, nodal status, clinical stage, grade, histological type and steroid receptor status. *KLK9* expression is significantly higher in patients with early stages compared with advanced stages ($p = 0.039$) and in patients with tumor size <2 cm compared with larger tumors ($p = 0.028$). No other significant

Table 3. Associations between *KLK9* expression and disease-free and overall survival

KLK9 status	Disease-free survival			Overall survival		
	HR[a]	95% CI[b]	*p* value	HR[a]	95% CI[b]	*p* value
Univariate analysis ($n = 161$)						
Categorical variable[c]						
Negative	1.00			1.00		
Positive	0.46	0.24–0.88	0.018	0.49	0.25–0.97	0.040
Continuous variable	0.99	0.99–1.00	0.31	0.99	2.99–1.00	0.41
Multivariate analysis[d] ($n = 148$)						
Categorical variable[c]						
Negative	1.00			1.00		
Positive	0.42	0.20–0.87	0.021	0.50	0.25–0.97	0.041

[a] Hazard ratio (HR) estimated from Cox proportional hazard regression model.
[b] Confidence interval of the estimated HR.
[c] *KLK9* status based on a cutoff point equal to the 60th percentile of the distribution of *KLK9* values.
[d] Multivariate models were adjusted for lymph node status; tumor size; patient age; grade; histologic type; ER and PR expression.

Table 4. Associations between *KLK9* and DFS and OS in subgroups of patients defined by tumor size, nodal status, estrogen and progesterone receptor status

Variable	Disease-free survival (DFS)			Overall survival (OS)		
	HR[a]	95% CI[b]	*p* value	HR[a]	95% CI[b]	*p* value
Tumor size $\leq 2\,cm$ ($n = 77$)						
KLK9 unadjusted	0.31	0.10–0.97	0.044	0.36	0.09–1.41	0.14
KLK9 adjusted[d]	0.30	0.08–1.04	0.059	0.31	0.06–1.58	0.16
Tumor size $> 2\,cm$ ($n = 84$)						
KLK9 unadjusted	0.72	0.34–1.55	0.41	0.72	0.33–1.56	0.41
KLK9 adjusted[d]	0.81	0.31–2.09	0.67	0.86	0.31–2.36	0.77
Node negative ($n = 69$)						
KLK9 unadjusted	0.22	0.02–1.77	0.15	0.38	0.04–3.45	0.39
KLK9 adjusted[c]	0.15	0.01–1.55	0.11	0.24	0.51–3.03	0.29
Node positive ($n = 83$)						
KLK9 unadjusted	0.51	0.24–1.02	0.058	0.46	0.22–0.97	0.043
KLK9 adjusted[c]	0.48	0.22–1.07	0.073	0.39	0.17–0.91	0.031
ER negative ($n = 63$)						
KLK9 unadjusted	0.28	0.10–0.74	0.011	0.29	0.11–0.79	0.015
KLK9 adjusted[e]	0.29	0.09–0.87	0.029	0.28	0.08–0.91	0.035
ER positive ($n = 93$)						
KLK9 unadjusted	0.65	0.28–1.53	0.33	0.79	0.31–2.06	0.64
KLK9 adjusted[e]	0.68	0.24–1.89	0.46	0.63	0.21–1.97	0.43
PR negative ($n = 71$)						
KLK9 unadjusted	0.38	0.16–0.91	0.028	0.43	0.18–0.99	0.046
KLK9 adjusted[f]	0.31	0.11–0.91	0.033	0.31	0.10–0.94	0.038
PR positive ($n = 86$)						
KLK9 unadjusted	0.51	0.19–1.31	0.16	0.55	0.19–1.59	0.27
KLK9 adjusted[f]	0.82	0.28–2.34	0.71	0.86	0.24–3.02	0.82

[a] Hazard ratio (HR) estimated from Cox proportional hazard regression model.
[b] Confidence interval of the estimated HR.
[c] Multivariate models were adjusted for tumor size, grade, ER, PR, histologic type and age.
[d] Multivariate models were adjusted for tumor grade, nodal status, ER, PR, histologic type and age.
[e] Multivariate models were adjusted for tumor size, grade, nodal status, PR, histologic type and age.
[f] Multivariate models were adjusted for tumor size, grade, nodal status, ER, histologic type and age.

associations were found between *KLK9* expression and clinicopathological variables.

Survival analysis

Kaplan-Meier survival curves demonstrate that patients with *KLK9*-positive tumors have significantly longer disease-free survival (DFS) ($p = 0.015$) and overall survival (OS) ($p = 0.036$) compared to those who are *KLK9*-negative (Figure 2).

Cox proportional hazard regression analysis (univariate analysis, Table 3) showed that when *KLK9* expression was analyzed as a categorical variable, it associates higher DFS and OS (hazard ratio of 0.46 and 0.49 and p value of 0.018 and 0.040, respectively). When all other variables were controlled in the multivariate analysis, *KLK9* retained its prognostic significance (hazard ratio of 0.42 and 0.50 and p value of 0.021 and 0.041, respectively, for DFS and OS). No statistically significant associations were obtained when *KLK9* was analyzed as a continuous variable (Table 3).

When the Cox proportional hazard regression analysis was applied to subgroups of patients (Table 4), *KLK9* expression was found to be a significant predictor of overall survival in the estrogen receptor-negative and progesterone receptor-negative subgroups of patients (HR = 0.29 and 0.43, respectively, and $p = 0.015$ and 0.046, respectively). Similar results were obtained in the node-positive subgroup of patients (HR = 0.46 and $p = 0.043$). After adjusting for other known prognostic variables, *KLK9* retained its independent prognostic value in all these subgroups of patients. With respect to the disease-free survival, *KLK9* expression was a favorable prognostic marker for the subgroup of patients with tumor size <2 cm ($p = 0.04$), and those who are estrogen receptor-negative ($p = 0.011$), or progesterone receptor-negative ($p = 0.028$).

Discussion

Accumulating reports suggest a relationship between kallikreins and breast cancer. Many kallikreins were found to be potential prognostic markers for this malignancy, including hK3 [24], *KLK5* [31], *KLK13* [32] and *KLK15* (our unpublished data). The mechanism by which kallikreins are involved in the initiation and/or progression of breast cancer is not known. However, reports indicate that most kallikreins are down-regulated in breast cancer tissues and cell lines [11, 12, 14, 16] and in all cases, except for *KLK5*, overexpression associates with longer survival. A recent report provided evidence that one of the kallikreins, *KLK10*, acts as a tumor suppressor gene in breast cancer [25]. This gene appears to be down-regulated due to hypermethylation of regions of exon 3 [33]. Further, the dramatic down-regulation of the gene in most invasive breast carcinomas suggests that it may be a potential prognostic marker [34].

Compelling evidence indicates a relationship between estrogen, androgen and progesterone, and their receptors, in the development and progression of breast cancer [35, 36]. However, the precise role of steroid hormones in breast cancer remains poorly defined. We speculate that the enzymatic activity of these serine proteases might terminate or initiate certain biological events, for example, onset of angiogenesis, activation or inactivation of growth factors, receptors, cytokines, etc. A recent report provided evidence that another closely related kallikrein, hK3 (PSA), has antiangiogenic activity, and that this activity may be related to its action as a serine protease [37]. This study suggested also that other members of the kallikrein multigene family should be evaluated for potential antiangiogenic action. Other studies suggested that hK3 inhibits growth of MCF-7 breast cancer cell lines and prolongs the doubling time of PC-3 prostate cancer cell lines [38, 39]. In addition, identification of hormonally regulated genes in breast cancer, such as *KLK9*, may help to better understand the pathogenesis of this disease and may also lead to new possibilities for hormonal treatment.

Although *KLK9* might not be a powerful marker by itself, there is now growing interest in neural networks which show promise of combining weak, but independent, information from various biomarkers to produce a prognostic/predictive index that is more informative than each individual biomarker alone [40, 41]. As is the case with HER-2 evaluation, which is useful for selection of patients for Herceptin therapy [2], *KLK9* can be also used to modify treatment decisions in certain subgroups of patients, for example, those who are ER and PR negative (Table 4).

Considering that the chromosomal region 19q13.4 harbors many steroid hormone-regulated, closely located, kallikreins [18, 42] and that most of them have prognostic value, it is reasonable to hypothesize that they may participate in an enzymatic cascade pathway. This possibility is currently under investigation.

In conclusion, we studied the quantitative *KLK9* expression in breast tumors and found that higher expression is associated with increased DFS and OS at both univariate and multivariate analysis. Larger studies will be necessary to confirm this data and further establish the clinical value of this biomarker in breast cancer.

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