Evaluation of Kallikrein-related Peptidase 5 Expression and its Significance for Breast Cancer Patients: Association with Kallikrein-related Peptidase 7 Expression

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Abstract. *Background: Kallikrein-related peptidases (KLKs) have been proposed as potential cancer biomarkers. Contradictions in literature led us to clarify the role of KLK5 as a breast cancer predictor, as well as its association with KLK7 expression. Patients and Methods: Semi-quantitative RT-PCR detected KLKs 5 and 7 in 80 breast tissues, 74 neoplastic and 6 normal. Steroid hormone receptors were quantified in all samples. Associations between KLK5 status and clinicopathological variables, as well as disease-free survival (DFS) and overall survival (OS) of patients were analyzed. Results: Forty tumor tissues showed high KLK5 expression, which was significantly associated with estrogen receptor status. Significant co-expression of KLKs 5 and 7 was observed in the same cancer samples (p=0.02). Increased KLK5 expression was a statistically significant independent prognostic factor for DFS (p=0.009 univariate analysis and p=0.028 multivariate analysis) and OS of patients (p=0.014, univariate analysis). Conclusion: Increased KLK5 expression can contribute to the prognosis of DFS and OS of breast cancer patients. KLKs 5 and 7 are co-expressed in breast cancer.*

Breast cancer is the most common cancer after lung and the fifth most common cause of death worldwide. Breast cancer accounts for 17% of all European female cancer deaths and 26% of all new cancer cases among women in the USA (1). In Greece, approximately 3500 and worldwide 1.15 million new cases are diagnosed annually. Consequently, biological markers for early diagnosis, prognosis and therapeutic treatment, improving the course of the disease are urgently needed.

Cell migration is a fundamental process in early morphogenesis and cancer metastasis that involves a multi-step cascade of events coupled to proteolytic remodelling of the extracellular matrix (ECM). Multiple sets of proteolytic enzymes, are dysregulated and activated during cancer progression (2). Clinical reports have shown that dysregulation of certain proteases correlates with poor prognosis in different malignancies (3).

Human kallikrein-related peptidases (KLKs) are serine proteases constituting the largest cluster of protease genes in the human genome and consisting of a group of 15 members. KLKs are expressed as inactive zymogens that require activation by other protease(s) of the same or different families. Crosstalk between KLKs and other proteolytic systems have been proposed to enhance ECM degradation (4-6).

KLKs play important roles in different physiological processes such as the regulation of cell growth and differentiation, tissue remodeling, angiogenesis, skin desquamation, human semen liquefaction, dental enamel formation, neuronal plasticity, inflammation, cervico-vaginal physiology, vascularization and antimicrobial defense *via* activation of cathelicidines and the production of antimicrobial peptides in skin and human genital track. Furthermore, KLKs have digestive roles in exocrine pancreatic function. KLKs are implicated in different pathologies, such as skin diseases including Netherton syndrome, psoriasis, atopic dermatitis and rosascea, and neurodegenarative diseases such as Alzheimer and multiple sclerosis (7). Several members of the family have been reported as potential cancer biomarkers.

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Human *KLK5* gene was originally cloned by Brattsand and Egelrud (8) and was characterized as trypsin-like serine protease with possible function in desquamation. Its molecular characterization, mapping, tissue expression and hormonal regulation was concurrently reported by Yousef and Diamandis (9). Consequently, the gene was named tissue kallikrein 5 (10) and finally the term kallikrein-related peptidase 5 was adopted for it (11). *KLK5* codes for the secreted protease KLK5, which consists of 293 amino acids and is synthesized as a pre-pro-enzyme. The activation of the enzyme requires the cleavage of an arginine residue (Arg^{66}-Ile^{67}). KLK5 has trypsin-like activity (12), and its gene is regulated by steroid hormones (8, 9, 13).

Six splice variants have been reported for *KLK5*, namely the classical form, splice variant 1, splice variant 2 and three more that produce truncated isoforms (14), all having tissue-specific expression (13-15).

The highest concentration of KLK5 is in adult and fetal skin, moderate concentration in breast and testis and low in lung (16). *KLK5* has been shown to be differentially expressed in steroid hormone-regulated carcinomas, such as ovarian, testicular and prostate cancer at the mRNA and/or protein level, (15-17), as well as in steroid hormone-independent carcinomas, such as, lung, colon, urinary bladder and oral cavity (17-21). In breast cancer, KLK5 has been shown to be a potential novel serum biomarker (22) and in breast cancer tissues, *KLK5* overexpression was shown to be an independent indicator of poor prognosis (23). In contrast, Yousef *et al*. (24) showed down-regulation of *KLK5* splice variant 2 in breast cancer, but up-regulation of the same splice variant in ovarian cancer, whereas Kurlender *et al*. (13) reported no significant differential expression of *KLK5* splice variant 1 between normal and malignant mammary tissues. In order to investigate these controversies, the expression of *KLK5* in breast cancer tissues was examined and association with clinical and pathological data, as well as with patient outcome, was investigated.

Moreover, contradictory results were also found for *KLK7* gene expression in breast cancer between our group (25) and Holzscheiter *et al*. (26), although different populations were studied and different methods used. Additionally, the expression of *KLK7* in the same breast samples and its putative association with *KLK5* were investigated.

Patients and Methods

Study population. The study group consisted of 74 patients with breast malignancies who underwent surgery for primary breast cancer at Saint Savvas Oncologic Hospital of Athens and 6 normal breast tissues. No chemotherapy or radiotherapy had been administered before surgery. Histological diagnoses and grading of the tumors were made based on the revised World Health Organization (WHO) classification for breast tumors. Cancer and normal tissues were evaluated by eosin-hematoxylin staining of paraffin sections. Patients' mean age was 64.2±1.6, with a range of 35-88 years. Sixteen patients (22%) received no adjuvant treatment, 22 (30%) received tamoxifen and 18 (24%) received chemotherapy with or without tamoxifen. Estrogen (ER) and progesterone receptor (PR) status was established as previously described (27). Clinical and pathological information documented at the time of surgery included stage and grade of the disease, histological type, size and nodal status of the tumors and the existence of ER and PR. The staging of the tumors followed the TNM system. The agreement of the Institute's Ethics Committee for the scientific analysis of tumor tissues was available, as well as patients' written informed consent. Investigations were carried out in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 1983.

Cell lines and culture conditions. Human breast cancer cell lines MCF-7, MDA-MB-435, BT-20, the ovarian cancer cell line CaOV-3 and the prostate cancer cell line PC-3 were obtained from the American Tissue Culture Collection and the breast cancer cell line MCF-10A was provided by the Department of Pharmacology, Wayne State University, Detroit, USA. All the culture media were obtained from Invitrogen (Carlsbad, CA, USA) and supplemented with 10% fetal bovine serum, as well as 40 mg/l gentamicin sulfate. The media for MCF-7 were additionally supplemented with 10 ng/ml insulin whereas the media for MCF-10A were additionally supplemented with 10 ng/ml insulin, 20 ng/ml EGF, and 1.3 nM hydrocortisone. All the cells were grown at 37˚C in an atmosphere of 5% CO_2.

RNA isolation – semi-quantitative RT-PCR. Upon collection, the breast tissues were snap frozen in liquid nitrogen and subsequently kept at –80˚C until required. The tissues were pulverized and total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The purity and concentration of RNA were determined by spectrophotometric methods. Two micrograms of total RNA were reverse-transcribed into first-strand cDNA using Superscript™ pre-amplification system (Invitrogen), following the manufacturer's instructions. The integrity of cDNA was examined by PCR amplification of *GAPDH* housekeeping gene, as previously described (27). In order to optimize PCR conditions, different quantities of cDNA (0.005-2 μl) from the CaOV-3 ovarian cancer cell line were amplified under exponential, non-saturating conditions, for 27, 31, 35, 37 and 40 cycles to confirm that amplification was in the linear range and to determine the appropriate cycle number for semi-quantitative PCR as described previously (28). For the amplification of *KLK5*, the forward primer anneals to exon 3 and the reverse primer anneals to exon 5 (forward 5'-CCA CTA CTC CCT GTC ACC AG-3'; reverse 5'-GTA ATC TCC CCA GGA CAC GA-3'), detecting the classical *KLK5* form as well as splice variants 1 and 2 and giving an amplicon of 435 bp. For the amplification of *GAPDH* (amplicon 233 bp) the primers used were: forward 5'-ATG GGG AAG GTG AAG GTC G-3'; reverse 5'-GGG TCA TTG ATG GCA ACA ATA TC-3'. After optimization, PCR was carried out in a 20 μl reaction mixture containing 0.8 μl of cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM dNTPs (deoxynucleoside 5'-triphosphate), 5 μM of each primer and 2.5 units of Taq DNA polymerase (New England Biolabs, Frankfurt am Main, Germany) on a thermal cycler (MJ Research, Waltham, Massachusetts, USA). The cycling conditions for *KLK5* were: a denaturation step at 94˚C for 5 min, followed by 36 cycles of 94˚C for 30 sec, an annealing step at 68˚C for 50 s and 72˚C for 45 s and a final extension step at 72˚C for 10

min. The cycling conditions for *GAPDH* were: a denaturation step at 95˚C for 10 min, followed by 30 cycles of 94˚C for 30 s, an annealing step at 60˚C for 1 min and 72˚C for 1 min and a final extension step at 72˚C for 8 min. Equal quantities of PCR products for the *KLK5* and *GAPDH* genes were electrophoresed on 1.5% agarose gels and visualized by ethidium bromide staining. The primers spanned more than two exons to avoid contamination by genomic DNA. For the study of *KLK7* expression the same primers and conditions as those we reported previously were used (25).

Following densitometric measurements of the band intensities using a Gel Logic 100 Imaging System and 1D Image Analysis Software, version 3.6 (Eastman Kodak Company, Rochester, NY, USA), the ratio of *KLK5* and *KLK7* to *GAPDH* band intensity was calculated. Based on this ratio, *KLK5* and *KLK7* expression was characterized as low or high compared to the ratio for non-cancer samples. A cut-off value of the mean ratio for non-cancer samples +2SD was calculated. *KLK5* and *KLK7* expression in samples with ratios higher than this value was considered high, whereas *KLK5* and *KLK7* expression in samples with ratios less than or equal to this value was characterized as low, as previously described (28). Expression analysis was performed twice for each sample. The identity of PCR products was verified by sequencing with an automated DNA sequencer.

Steroid hormone receptor analysis. Steroid hormone receptors were quantified as described elsewhere (29). The results of the dual ligand binding assay, in which dextran-coated charcoal was used to separate bound ligand from free ligand, were interpreted by Scatchard analysis. Tumors with ER and PR concentrations of ≤10 fmol/mg protein were characterized as negative, whereas concentrations of 10-300 fmol/mg protein were characterized as positive.

Statistical analysis. Associations between *KLK5* status and qualitative variables were analyzed using the Chi-square test or the Fisher's exact test, where appropriate. A Cox proportional hazard regression model was developed to evaluate the association (*i.e.* hazard ratio and its confidence interval) between the potential prognostic marker and disease-free survival (DFS) or overall survival (OS). This analysis was conducted at both univariate and multivariate levels. Survival analysis was performed by constructing Kaplan-Meier DFS and OS curves for patients with *KLK5* status low or high and the Wilcoxon signed ranks test was used to compare survival between subgroups of patients. DFS was defined as the time between the date of surgical removal of the primary tumor and the date of the first documented evidence of relapse. OS was defined as the time interval between the date of surgery and the date of death, or the date of last follow-up for those who were alive at the end of the study. Associations between *KLK5* expression status and hormone receptor status were evaluated by the use of Mann-Whitney test. Finally, association between *KLK5* and *KLK7* expression was studied by the use of Fisher's exact test.

Results

KLK5 gene expression and relation to other variables. KLK5 expression in a representative group of samples is shown in Figure 1a. *GAPDH*, which was used as an internal control, showed a consistent pattern of expression in all samples, indicating the integrity of RNA, as well as equal loading. The *KLK5* PCR product from the ovarian cancer cell line CaOV-3 and one highly expressing breast cancer tissue sample were sequenced and *KLK5* sequences were shown to be identical to those reported previously (8, 9). As shown in Figure 1b, only MCF-10A showed weak *KLK5* expression, whereas all the other breast cell lines showed no expression. The prostate cancer cell line PC-3 showed strong positive *KLK5* expression, as did the CaOV-3 cell line.

Table I. *Distribution of numerical variables (patient and tumor characteristics).*

Variable	Mean±SE	Median	Range
Age (years)	64.2±1.6	65.0	35-88.0
Tumor size (cm)	2.8±0.2	2.5	0.3-8.0
Lymph nodes[a]	2.8±0.6	1	0-19
ER (fmol/mg protein)	55.6±12.7	17.0	0.0-438.0
PR (fmol/mg protein)	77.7±16.9	25.0	0.0-634.0
DFS time (months)	50.3±4.0	47.0	1-154
OS time (months)	56.8±4.3	54.0	1-163

[a]Number of lymph nodes positive for malignancy. DFS: Disease-free survival; OS: overall survival.

Forty out of the 74 tumor samples (54%) and none of the 6 normal breast samples (0%) were characterized as having a high *KLK5* status. Table I shows the distribution of numerical variables. Statistical analysis of the results obtained indicated that high *KLK5* expression was associated in a statistically significant manner with ER status (Table II). However, increased *KLK5* expression seemed to be independent of the other clinical and histomorphological variables studied, namely PR, menopausal and nodal status of patients, size and grade of the tumors and stage of the disease. Low *KLK5* gene expression status was more frequent in patients with strongly positive ER (p=0.004) and PR status (p=0.011) (Figures 2 and 3).

Follow-up information (median follow-up period 54 months, range 1-163 months) was available for 54 patients. During follow-up, 4 patients (7.4%) experienced disease relapse and 10 (18.5%) died. Both univariate and multivariate analyses revealed that increased *KLK5* expression was a statistically significant independent prognostic factor for DFS (p=0.009 and p=0.028, respectively) (Table III). Furthermore, univariate analysis revealed that increased *KLK5* expression was a statistically significant independent prognostic factor for OS (p=0.014), whereas multivariate analysis for the same parameters did not show statistical significance (Table III). Kaplan-Meier survival curves demonstrated that longer DFS and OS were associated with low *KLK5* gene expression status (p=0.028 and p=0.014, respectively) (Figures 4 and 5).

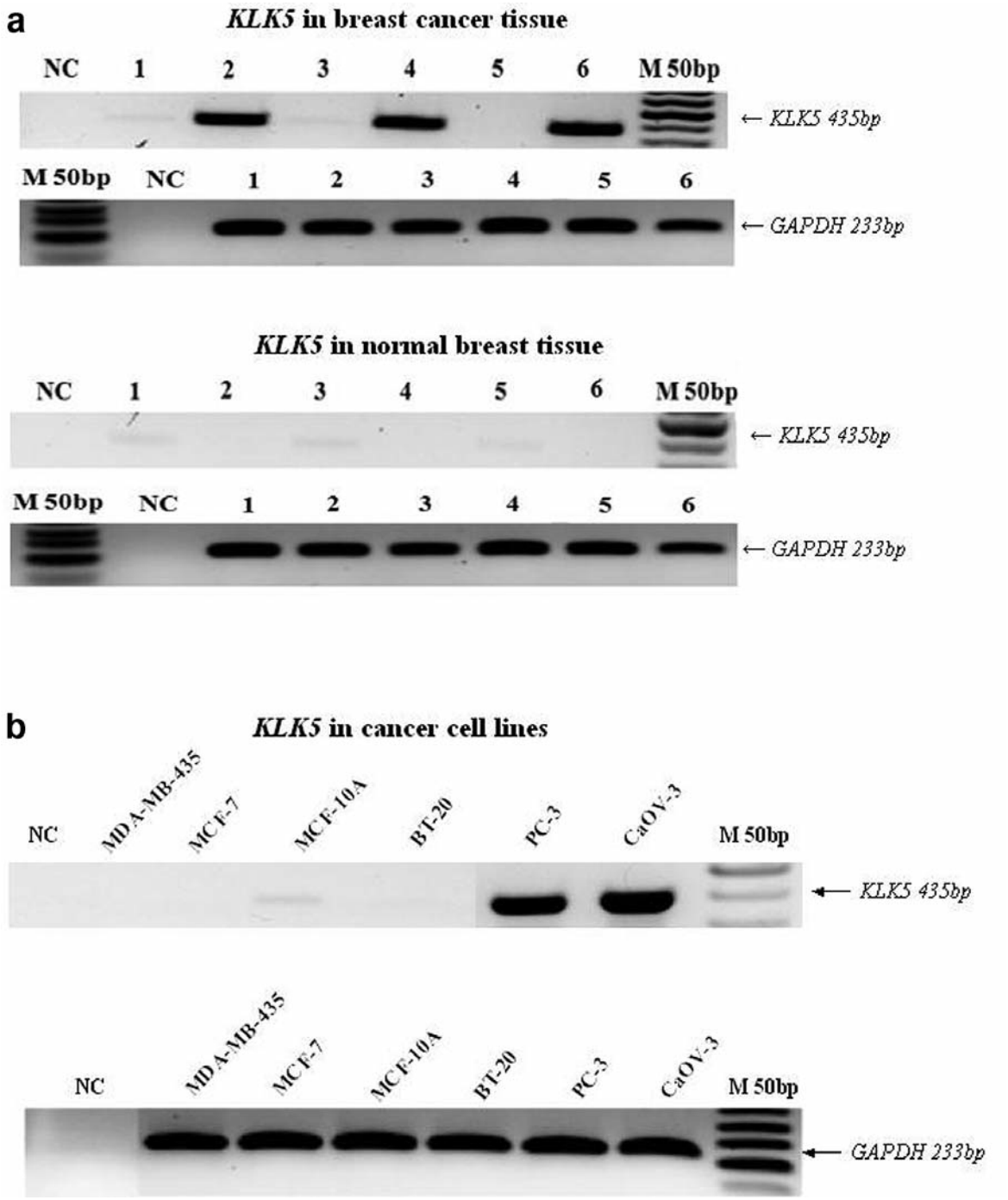

Figure 1. *KLK5 expression. a: Representative breast tissue samples. b: Cancer cell lines. GAPDH: Loading control, NC: negative control.*

Association of KLK5 with KLK7 gene expression. Table IV shows the association of *KLK5* with *KLK7* expression in the same samples. Statistically significant co-expression of *KLKs 5* and *7* (p=0.020) was observed in the breast cancer tissue.

Discussion

Many kallikreins have been shown to be differentially expressed in hormone-related malignancies such as breast cancer. In this study, the expression of *KLK5* in 74 breast

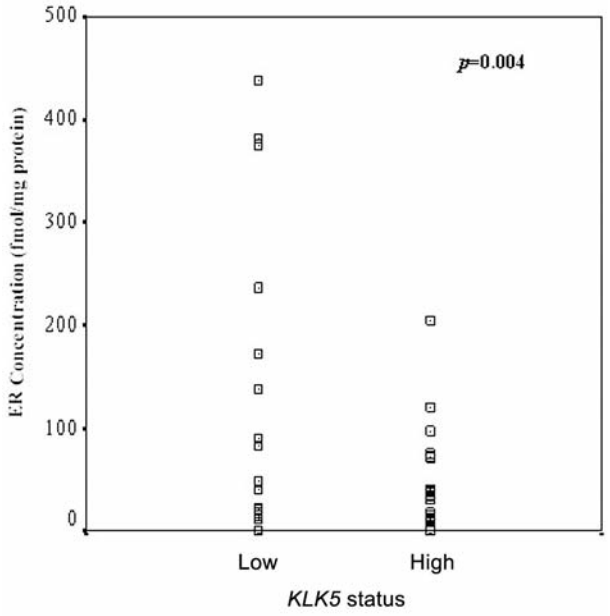

Figure 2. *Association of KLK5 status with estrogen receptor (ER) concentration. p-Value was calculated by the Mann-Whitney test.*

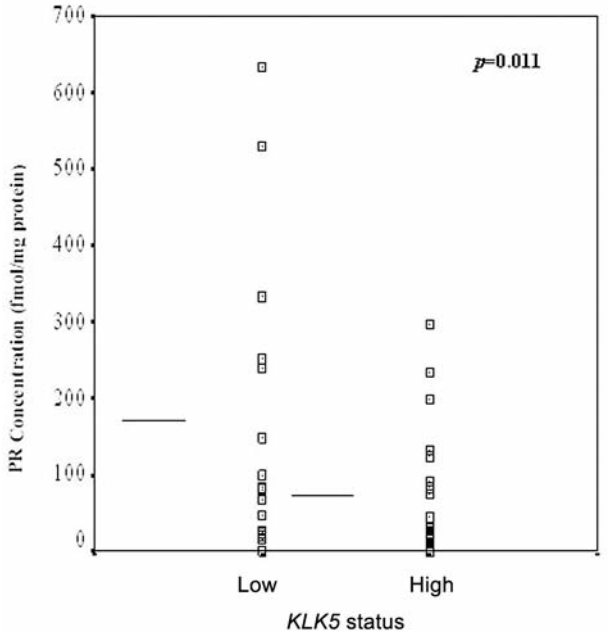

Figure 3. *Association of KLK5 status with progesterone receptor (PR) concentration. p-Value was calculated by the Mann-Whitney test.*

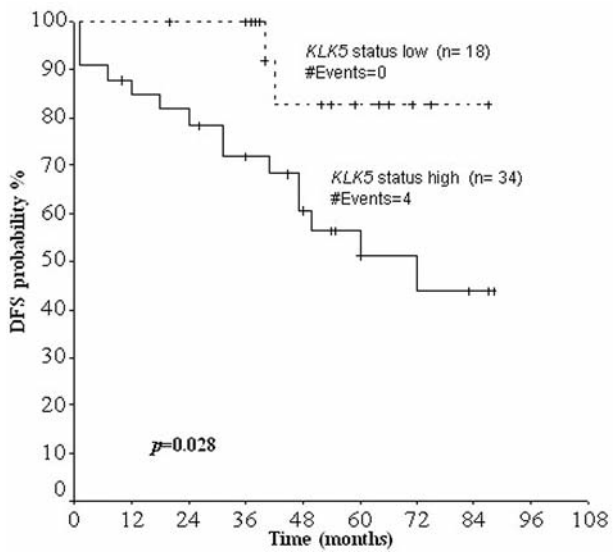

Figure 4. *Disease-free survival (DFS) in relation to KLK5 status.*

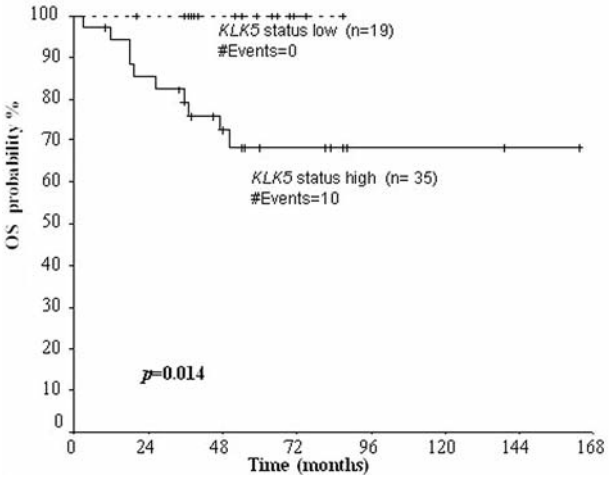

Figure 5. *Overall survival (OS) in relation to KLK5 status.*

cancer tissues and 6 normal breast tissues, 4 breast cancer cell lines, 1 ovarian and 1 prostate cancer cell line were examined by semi-quantitative RT-PCR method as previously described (28).

In the breast cancer cell lines, *KLK5* expression was negative or weak, while in line with earlier reports (17, 22), the ER-positive ovarian cancer cell line CaOV-3 and the prostate cancer cell line PC-3 showed strong positive *KLK5* expression. High *KLK5* expression was found in some breast cancer tissue samples and was associated with the ER status of patients. *KLK5* expression was also an indicator of poor prognosis in breast cancer, in agreement with Yousef *et al*. (23). This was in contrast to recent work by Li *et al*. (30) showing the down-regulation of *KLK5* in breast cancer by examining common sequences of all known splice variants. In another study, in which only

Table II. *Association of KLK5 expression status with other dichotomous variables.*

Variable	No. of patients (%)			*p*-Value
	Total	*KLK5* status		
		Low	High	
ER status[e]				
Negative	17	2 (11.8)	15 (88.2)	0.018[a]
Positive	40	18 (45.0)	22 (55.0)	
x	17			
PR status[e]				
Negative	14	2 (14.3)	12 (85.7)	0.063[a]
Positive	42	18 (42.9)	24 (57.1)	
x	18			
Menopausal status				
Pre/peri	13	5 (38.5)	8 (61.5)	0.99[a]
Post	51	19 (37.3)	32 (62.7)	
x	10			
Tumor size (cm)				
<2	21	7 (33.3)	14 (66.7)	0.78[a]
≥2	42	16 (38.1)	26 (61.9)	
x	11			
Nodal status				
Negative	22	7 (31.8)	15 (68.2)	0.57[a]
Positive	34	14 (41.2)	20 (58.8)	
x	18			
Stage[c]				
I	10	3 (30.0)	7 (70.0)	0.84[b]
II	33	13 (39.4)	20 (60.6)	
III	12	4 (33.3)	8 (66.7)	
x	19			
Grade[d]				
I	3	1 (33.3)	2 (66.7)	0.47[b]
II	38	17 (44.7)	21 (55.3)	
III	21	6 (28.6)	15 (71.4)	
x	12			

[a]Fisher's exact test, [b]Chi-square test, [c]TNM system, [d]Bloom-Scarff-Richardson grading system, [e]cut-off ≤10 fmol/mg, x: status unknown.

KLK5 splice variant 2 was examined, down-regulation of *KLK5* was also reported (24). In the present study, the classical *KLK5* form, as well as splice variants 1 and 2, were detected as the primers were designed based on the common sequences of all known splice variants as in the study of Li *et al*. (30) and the different findings may be attributed to differences between Asian and Western populations. The difference with Yousef *et al*.'s study (24) may have been due to the different variants examined.

The discrepancy between *in vitro* and *in vivo* systems noticed in the present work confirms the fact that tumor establishment is a multifactorial process and *in vitro* systems can only provide some evidence of the actual mechanisms that orchestrate within organisms.

In the present study, the co-expression of *KLK5* and *KLK7* was observed in the same samples, which supports the hypothesis that these two genes may take part in an activation cascade in breast cancer, possibly similar to the one that exists in skin, helping in remodeling of breast matrix or simply acting as activators of other proteases (31). Li *et al*. (30) observed parallel *KLK5* and *KLK7* under-expression in Asian population, whereas the present work found parallel overexpression of the two genes in Greek population. Similar co-expression of *KLK5* and *KLK7* has also been shown in ovarian cancer (15).

The potential of KLK5 as a serum biomarker for breast cancer was shown by ELISA measurements in that it was almost undetectable in normal individuals, whereas in the serum of breast cancer patients it was found in high concentrations (22). This finding is supported the present results, showing the presence of KLK5 protein, which is the product of *KLK5* gene expression, in breast cancer patients. Additionally, in the same report, MCF-7 cell line weakly expressed *KLK5* while CAOV-3 cell line expressed it intensely, which was very similar to the present findings.

The relationship of KLK5 to cancer progression may be attributed to the fact that KLK5 digests ECM components such as collagens I, II, III, IV, fibronectin and laminin, promoting cancer invasion and metastasis (12). In order to clarify the role of *KLK5* expression in breast cancer, a multiplex study is needed to analyze all splice variants in the same samples, and with a larger number of samples.

Conflict of Interest Statement

The Authors report no conflicts of interest. The Authors alone were responsible for the content and writing of the paper.

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Table III. *Association of KLK5 expression status with disease-free and overall survival.*

Variable	Disease-free survival			Overall survival		
	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
			Univariate analysis			
KLK5 status						
Low	1.00			1.00		
High	8.50	1.71-42.27	0.009	2.50	1.20-5.20	0.014
Nodal status	3.52	0.82-15.05	0.09	4.76	1.83-12.57	0.001
Grading (ordinal)	1.48	0.51-4.34	0.46	1.88	1.37-2.57	<0.001
Tumor size	1.84	1.17-2.89	0.008	1.47	1.16-1.85	0.001
			Multivariate analysis			
KLK5 status						
Low	1.00			1.00		
High	9.55	1.26-71.94	0.028	0.79	0.06-1.73	0.57
Nodal status	6.06	0.77-47.7	0.087	1.32	0.19-8.96	0.77
Grading (ordinal)	0.33	0.056-1.92	0.22	7.17	1.68-30.57	0.008
Tumor size	1.95	1.06-3.62	0.033	0.68	0.38-1.22	0.21

HR, Hazard ratio (HR) estimated from logistic proportional hazard regression model; CI: confidence interval of the estimated HR.

Table IV. *Association of KLK5 with KLK7 expression status.*

Variable	No. of patients (%)			*p*-value*
	Total	*KLK5* status		
KLK7 status		Low	High	
Low	62	23 (46.0)	27 (54.0)	0.020
High	12	4 (16.7)	20 (83.3)	

*Fisher's exact test.

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