The Human *KLK8* (*Neuropsin/Ovasin*) Gene: Identification of Two Novel Splice Variants and Its Prognostic Value in Ovarian Cancer

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ABSTRACT

***KLK8* (*neuropsin/ovasin*) is a new member of the human kallikrein gene family, which consists of enzymes with serine protease enzymatic activity. Recent reports have implicated *KLK8* in ovarian cancer. *KLK8* may have potential clinical value for disease diagnosis or prognosis and it may also be a useful therapeutic target.**

***Purpose:* We undertook this study to evaluate the prognostic value of *KLK8* in ovarian carcinoma by examining its expression in ovarian tumors.**

***Experimental Design:* The *KLK8* gene was analyzed by reverse transcription-PCR and direct sequencing in several human normal tissues. Subsequently, its expression was studied in a set of ovarian tumors, and statistical analysis was performed.**

***Results:* We have identified two novel mRNA splice variants of the *KLK8* gene, which are abundantly expressed in many tissues. These new variants were named *KLK8* type 3 and type 4. Study of the expression of the *KLK8* gene and its spliced variants in ovarian tumors indicated that the new variants were expressed very frequently and that full-length KLK8 expression is an independent and favorable prognostic marker for ovarian cancer. Patients with higher KLK8 expression in the tumor have lower grade disease, lower residual tumor left after surgery, live longer, and relapse less frequently. In multivariate analysis, higher KLK8 expression was significantly associated with longer disease-free survival.**

***Conclusions:* These results suggest that *KLK8* is a novel, favorable prognostic marker in ovarian cancer. Because KLK8 encodes for a predicted secreted protein, its detection in serum may aid in ovarian cancer diagnosis.**

INTRODUCTION

The human kallikrein gene family is a subfamily of serine proteases, located at the chromosomal locus 19q13.3–q13.4. Until recently, this family was known to include only three members: the pancreatic/renal kallikrein gene (*KLK1*), the human glandular kallikrein 2 gene (*KLK2*), and prostate-specific antigen (*KLK3*). In the past few years, another 11 kallikrein-like genes were discovered (reviewed in Ref. 1). Neuropsin is one of these genes that maps to this locus (1, 2). According to the approved human kallikrein gene nomenclature, neuropsin is also known as *KLK8* (3).

Neuropsin is a well-characterized, brain-related serine protease in mouse, where it has been shown to play an important role in neural plasticity (4), as well as in embryonic cell differentiation (5). Recently, the human homologue of mouse neuropsin was cloned and found to be highly expressed in brain, as well as in skin (6). Analysis of the genomic organization of the human kallikrein 8 gene (*KLK8*) revealed that it is composed of six exons and five introns, the first exon being non-coding (6). The cDNA has a single open reading frame of 780 bp encoding for a predicted 260-amino acid protein. Two alternatively spliced forms of KLK8 have been identified so far (7, 8). The first (type 2 neuropsin) has an insert of 45 amino acids between the leader (pre)peptide and the proenzyme (pro)peptide of the regular protein, because of the presence of a second in-frame splice site in exon 3, which gives rise to a different mRNA form (7). The second mRNA variant, known as *tumor-associated differentially expressed gene-14*, contains an additional new sequence of 491 bp of 5′ untranslated region; also, the nucleotides preceding the poly(A) tail in the 3′ untranslated region are not homologous with the regular form of neuropsin, but this mRNA form encodes for an identical protein as the human *neuropsin* gene (8). *Neuropsin/tumor-associated differentially expressed gene-14* was found to be overexpressed in ovarian carcinoma (8). In addition, a GenBank submission (accession number AF095742) refers to *KLK8* as *ovasin* and indicates that it is a potential marker for ovarian cancer.

In this report, we examine the expression of *KLK8* in ovarian tumors by reverse transcription-PCR. While examining the expression of the gene in normal tissues, however, we found, along with the expected PCR band, two additional bands present in all samples examined. Further analysis revealed that the two bands represent novel, alternatively spliced forms of the *KLK8* gene. We here describe the prognostic value of the expression level of *KLK8* and its two novel splice variants in a group of 143 patients with ovarian cancer.

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MATERIALS AND METHODS

Analysis of the *KLK8* Gene by RT-PCR[2] and Direct Sequencing. Total RNA isolated from 26 different human tissues was purchased from Clontech, Palo Alto, CA. Two μg of RNA were reverse-transcribed into first-strand cDNA using the Superscript preamplification system (Life Technologies, Inc., Gaithersburg, MD). The final volume was 20 μl. PCR was performed in a reaction mixture containing 1 μl of cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM deoxynucleotide triphosphates, and 2.5 units of HotStar Taq DNA polymerase (Qiagen, Mississauga, Ontario, Canada) in an Eppendorf thermal cycler. The primers used were described previously by Yoshida *et al.* (6), and their sequences were 5′-GTG-ACC-CCG-CCC-CTG-GAT-T-3′ (forward) and 5′-GGG-AGA-TCT-AGT-GCT-TAT-CCT-3′ (reverse). The cycling conditions were 95°C for 15 min to activate the HotStar Taq polymerase, followed by 40 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 1 min, and a final extension step at 72°C for 10 min. Equal amounts of PCR products were electrophoresed on 1% agarose gels and visualized by ethidium bromide staining. The three visible bands were cut out and purified using the Gel Purification kit (Qiagen), and the purified DNA was directly sequenced. To ensure that the first (non-coding) exon is included in the variant mRNAs, we constructed a new forward primer (5′-GTT-CCC-AGA-AGC-TCC-CCA-G-3′), binding at the beginning of exon 1, and performed new PCR analysis using the aforementioned reverse primer.

Patients with Ovarian Cancer. Included in this study were 143 consecutive patients with epithelial ovarian carcinoma, with ages ranging from 25 to 80 with a median of 58 years. Patients underwent surgery and treatment for primary ovarian carcinoma at the Department of Obstetrics and Gynecology, Gynecological Oncology Unit, University of Turin, between 1991 and 1999. Follow-up information (median follow-up period, 48 months) was available for 135 patients, among whom 81 (60%) had relapsed and 57 (42%) died. Patients were staged according to the criteria of the International Federation of Gynecology and Obstetrics (9); information was available for 138 of them (Table 1). Specifically, 27 (20%) patients had stage I disease, 10 (7%) had stage II, 90 (65%) had stage III, and 11 (8%) had stage IV disease. All patients were treated with chemotherapy regimens containing platinum compounds, alone or in association with other drugs; grade 1 and stage I patients received no further treatment. Investigations were performed in accordance with the Helsinki Declaration and were approved by the Institute of Obstetrics and Gynecology, University of Turin.

Ovarian Tumor Specimens and Extraction of Total RNA. Each tumor had been histologically typed and graded based on WHO criteria (10). Ten tumors (7%) were low potential malignancies, 17 (12%) were Grade 1, 23 (17%) were Grade 2, and 89 (64%) were Grade 3. With respect to histological type, 63 tumors were serous papillary, 25 were endometrioid, 22 were undifferentiated, 11 were mucinous, 11 were clear cell, 7 were Mullerian, and 2 were unclassified. In the data analysis, only serous, endometrioid, and undifferentiated cell carcinomas were considered. Because the numbers of the other histological types were too small to be analyzed reliably, they were combined together as one category called "others." The size of the residual tumors ranged from 0 to 9 cm, with a median of 1.5 (Table 1). Tumor specimens were stored in liquid nitrogen immediately after frozen section and shipped at −80°C. Tumor tissue (50–100 mg) was pulverized on dry ice, followed by total RNA extraction using the TRIzol method (Life Technologies, Inc.). RT-PCR was performed as described above. To ensure integrity of RNA and efficiency of the reverse transcription, the housekeeping gene *actin* was also analyzed by PCR.

Assessment of *KLK8* Expression. Agarose gel images were scored by two independent investigators for high (positive)

Table 1 Relationship between KLK8 (regular form) status and other variables in 143 patients with primary ovarian cancer

		No. of patients (%)		
	Patients	*KLK8* negative	*KLK8* positive	*P*
KLK8 type 3				
Negative	97	65 (67.0)	32 (33.0)	0.018[a]
Positive	46	21 (45.7)	25 (54.3)	
KLK8 type 4				
Negative	47	33 (70.2)	14 (29.8)	0.11[a]
Positive	96	53 (55.2)	43 (44.8)	
Stage				
I	27	12 (44.4)	15 (55.6)	
II	10	5 (50.0)	5 (50.0)	0.17[b]
III	90	59 (65.6)	31 (34.4)	
IV	11	8 (72.7)	3 (27.3)	
x[c]	5			
Grade				
GB[d]	10	2 (20.0)	8 (80.0)	
G1	17	8 (47.1)	9 (52.9)	0.007[b]
G2	23	12 (52.2)	11 (47.8)	
G3	89	62 (69.7)	27 (30.3)	
x	4			
Histotype				
Serous	63	37 (58.7)	26 (41.3)	
Endometrioid	25	13 (52.0)	12 (48.0)	0.32[b]
Undifferentiated	22	17 (77.3)	5 (22.7)	
Others	31	18 (58.1)	13 (41.9)	
x	2			
Residual tumor (cm)				
0	56	27 (48.2)	29 (51.8)	
1–2	27	16 (59.3)	11 (40.7)	0.044[b]
>2	53	38 (71.7)	15 (28.3)	
x	7			
Menopause				
Pre/peri	49	27 (55.1)	22 (44.9)	0.47[a]
Post	94	59 (62.8)	35 (37.2)	
Response to CTX[e]				
NC/PD	15	10 (66.7)	5 (33.3)	0.59[a]
CR/PR	118	69 (58.5)	49 (41.5)	
NE	10			

[a] χ^2 test.
[b] Fisher's exact test.
[c] x, unknown status.
[d] GB, borderline grade.
[e] CTX, chemotherapy; NC, no change; PD, progressive disease; CR, complete response; PR, partial response; NE, not evaluated.

[2] The abbreviations used are: RT-PCR, reverse transcription-PCR; PFS, progression-free survival; OS, overall survival; EST, expressed sequence tag; HR, hazard ratio; PSA, prostate-specific antigen.

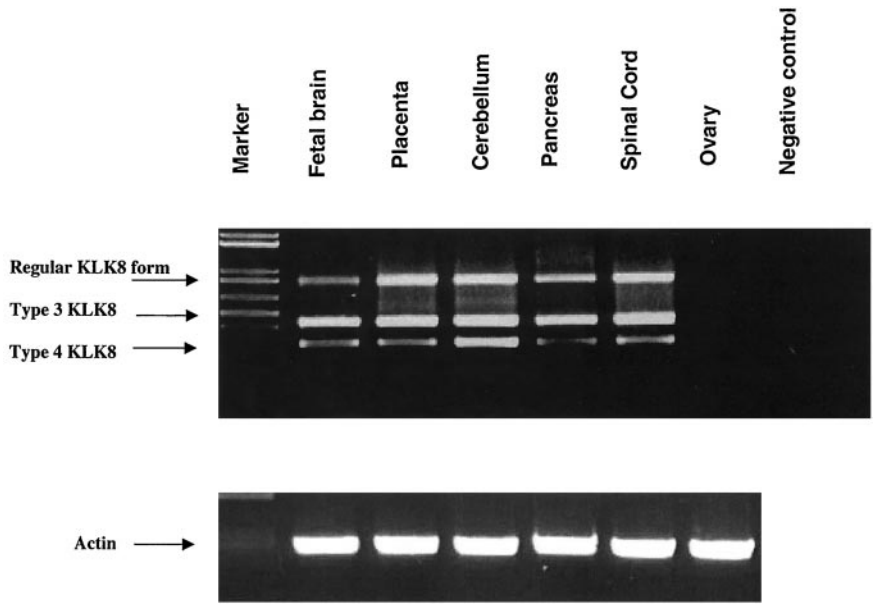

Fig. 1 Tissue expression of *KLK8* by RT-PCR. The expected PCR band (regular type) is 850 bp, type 3 is 450 bp, and type 4 is 300 bp long. There was no DNA template used in the PCR negative control. Actin was used as internal control for the integrity of the mRNA.

or low/undetectable levels of the three types of *KLK8* (regular form, type 3 and type 4; Fig. 1). Type 2 *KLK8* mRNA (described previously by Mitsui *et al.* (7)] was not detectable in any of the tumors. Concordance of scoring between the two investigators was 95%. In the remaining few discrepant cases, the final scoring was decided by re-review of the images.

Statistical Analysis. *KLK8* expression at the mRNA level was classified as positive or negative, and associations between *KLK8* status and other qualitative variables were analyzed using the χ^2 or the Fisher exact test, where appropriate. Cox proportional hazard regression model was developed to evaluate the association (*i.e.,* the hazard ratio and its confidence interval) between the potential prognostic marker and progression-free or overall survival. This analysis was conducted at both univariate and multivariate levels. The multivariate model was adjusted for *KLK8* expression in tumors, patient age, clinical stage, tumor grade, residual tumor size, and histological type. Survival analyses were performed by constructing Kaplan-Meier PFS and OS curves for *KLK8*-positive and *KLK8*-negative patients, and the log-rank test was used to examine the differences between them. Statistical analyses were performed using SAS software (SAS Institute, Cary, NC).

RESULTS

Identification of Two New *KLK8* mRNA Variants. After PCR amplification of the *KLK8* cDNA, we observed two additional bands, present in most of the normal human tissues examined as well as in ovarian tumors (Figs. 1 and 2). We determined the nucleotide sequence of these three PCR products. The sequence of the expected band was identical to that reported previously for the *KLK8* (human *neuropsin*) mRNA (6). The two new bands were also identical to the *KLK8* mRNA, except that whole exons were deleted. Specifically, the middle band (Fig. 1) was missing two exons (exons 3 and 4), whereas the lowest band was missing three exons (exons 3, 4, and 5).

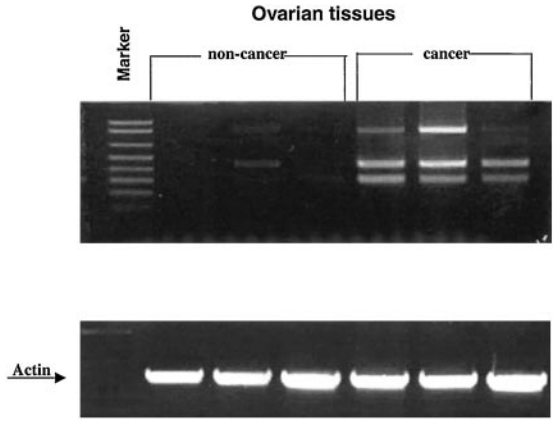

Fig. 2 PCR analysis of six ovarian tissues. *Lanes 1–3* are noncancerous tissues with undetectable or very low levels of *KLK8* (negative). The rest of them are cancerous tissues. The marker lane has seven visible bands with lengths in bp of 1050, 850, 600, 500, 400, 300, and 200 bp. Actin was used as internal control for the integrity of the mRNA.

These results revealed that these two bands corresponded to new, alternatively spliced variants; we named them *KLK8* type 3 and type 4, respectively (GenBank accession number AF251125). To further verify the presence of these variants in tissues, we conducted a homology search using the BLASTN algorithm (11) on the National Center for Biotechnology Information web server, against the human EST database (dbEST). We identified two partially sequenced EST clones. One clone (AI656124) included exons 2, 5, and 6; the other one (AA320217) included exons 2 and 6. These findings further support our proposal that the *KLK8* mRNA is alternatively spliced, yielding two new variants, as we had found by RT-PCR. All known *KLK8* variants are shown in Fig. 3.

Table 2 Univariate and multivariate analysis of *KLK8* with regard to PFS and OS

	PFS			OS		
	HR[a]	95% CI[b]	*P*	HR[a]	95% CI[b]	*P*
			Univariate analysis			
KLK8						
Negative	1.00			1.00		
Positive	0.44	0.27–0.71	<0.001	0.55	0.31–0.96	0.038
Stage of disease (ordinal)	2.91	2.04–4.16	<0.001	3.46	2.19–5.45	<0.001
Grading (ordinal)	2.21	1.56–3.10	<0.001	2.49	1.57–3.95	<0.001
Residual tumor (ordinal)	1.25	1.17–1.32	<0.001	1.30	1.21–1.40	<0.001
Histological type[c]	1.44	0.92–2.24	0.11	1.41	0.82–2.39	0.21
Age	1.01	0.99–1.03	0.26	1.01	0.99–1.04	0.27
			Multivariate analysis			
KLK8						
Negative	1.00			1.00		
Positive	0.57	0.34–0.94	0.027	0.81	0.45–1.47	0.49
Stage of disease (ordinal)	1.82	1.24–2.67	0.002	2.01	1.22–3.27	0.005
Grading (ordinal)	1.48	0.99–2.21	0.051	1.59	0.93–2.74	0.089
Residual tumor (ordinal)	1.12	1.04–1.21	0.002	1.19	1.09–1.31	<0.001
Histological type[c]	1.25	0.79–1.98	0.34	0.96	0.55–1.65	0.88
Age	1.01	0.98–1.03	0.48	1.01	0.98–1.04	0.29

[a] HR estimated from Cox proportional hazard regression model.
[b] Confidence interval of the estimated HR.
[c] Endometrial, undifferentiated, and others *versus* serous.

Tissue Expression. High levels of KLK8 mRNA were found in brain, cerebellum, pancreas, and placenta, as reported previously (Ref. 7; Fig. 1). We also detected high expression of the gene in the salivary gland, uterus, thymus, breast, testis, and kidney. In these tissues, we detected all three spliced variants (data not shown). Notably, there was no *KLK8* expression in the normal ovarian tissue (Fig. 1).

***KLK8* Expression as a Prognostic Marker for Ovarian Carcinoma.** Eighty-six patients (60%) showed overexpression of the regular form of KLK8, and 57 of them (40%) had very low or undetectable levels. Table 1 presents the relationships between KLK8 expression and other clinical or pathological variables, including clinical stage, histological grade, histotype, residual tumor after surgery, menopausal status, and response to chemotherapy. Of the 89 patients with poorly differentiated (G3) tumors, ~70% of them were negative for *KLK8* expression, and only 30% were positive. On the other hand, 80% of the patients with low potential malignancies were *KLK8* positive, and only 20% were negative. The association between *KLK8* expression and tumor grade was statistically significant by the χ^2 test ($P = 0.007$). The positivity rates for KLK8 expression/stage were 56% for Stage I disease, 50% for Stage II, 34% for Stage III, and 27% for Stage IV ($P = 0.17$). The positivity rates for *KLK8* expression were 52% in patients with no residual tumor after surgery, 41% in patients with a residual tumor of 1–2 cm, and 28% in patients with residual tumor >2 cm ($P = 0.044$). KLK8 expression was not related to cancer histotype, menopausal status, or response to chemotherapy (Table 1).

The strength of the associations between each clinicopathological variable and PFS or OS are demonstrated in the univariate analysis in Table 2. The stage of the disease was the strongest predictor for both PFS and OS, with a hazard ratio of approximately 3 and 3.5, respectively. The Cox regression model showed that the relative risk of relapse was significantly lower (by 56%) in *KLK8*-expressing ovarian tumors. Similarly, *KLK8* mRNA positivity indicated a 45% decrease in the relative risk of death for these patients. The Kaplan-Meier survival curves (Fig. 4) also demonstrate that patients with *KLK8*-positive tumors have substantially longer PFS ($P < 0.001$) and OS ($P = 0.034$) than patients with *KLK8*-negative tumors.

When all variables were included in the Cox model (multivariate analysis in Table 2), clinical staging and residual tumor status were found to be independent prognostic factors for PFS and OS. *KLK8* expression significantly added to the prognostic power in the same multivariate regression model for PFS (HR, 0.57; $P = 0.027$) but not for OS.

We have also performed association analysis between type 3 and type 4 variant expression and various clinicopathological variables and found no statistically significant associations (data not shown).

DISCUSSION

In this study, we have isolated and characterized two new variants of the mRNA encoding for *KLK8* (*neuropsin*). The existence of multiple splice variants of mRNA is frequent among kallikreins. Several distinct RNA species transcribed from the *KLK3/PSA* gene have been described in the literature (12–15). A number of alternate transcripts have also been reported for the *KLK2* gene (16, 17). *KLK12* (18) and *KLK13* (19), two newly identified kallikreins, are present in various spliced forms in most of the tissues examined.

The novel alternatively spliced forms of *KLK8* mRNA are

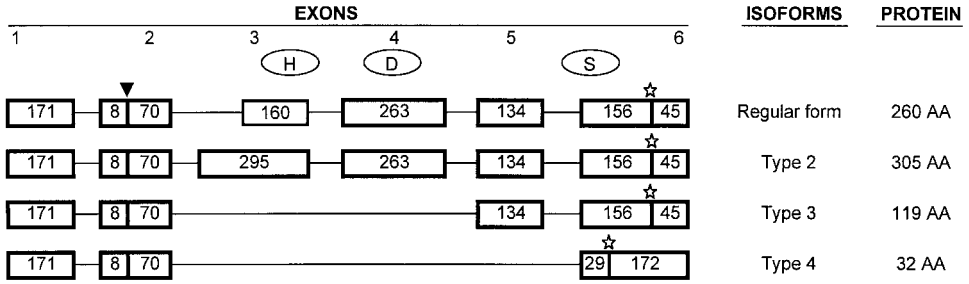

Fig. 3 Genomic organization of the different splice forms of the *KLK8* gene. *Arrow* and ∗, the position of the start and the termination codon, respectively. The first exon (*exon 1*) is untranslated. *H*, *D*, and *S* represent the approximate position of the catalytic amino acids within the exons. *AA*, amino acid. Type 2 *neuropsin* was described by Mitsui *et al.* (7). For more information, see text.

formed by deletion of whole exons. The first variant, called type 3 *KLK8*, is 584 bp long and is missing exons 3 and 4; the second variant, called type 4 *KLK8*, is 450 bp long and is missing exons 3, 4, and 5. The two variant mRNAs encode for putative proteins of 119 and 32 amino acids, respectively, which are missing two or three amino acids of the catalytic triad of serine proteases (Fig. 3). The signal peptide, which is necessary for the secretion of the regular form and is located within the first 32 amino acids, is also incompletely encoded, indicating that these variants encode for putative proteins that are likely not secreted. Expression analysis of *KLK8* showed that the variant mRNAs are abundant in several tissues (Fig. 1).

A previous report (8) suggested that *KLK8* might play a role as a prognostic marker in ovarian cancer. To investigate the potential clinical value of *KLK8* and its new variants, we studied their expression in a set of ovarian tumors. Statistical analysis showed that the two spliced variants had no prognostic value. However, the expression of the regular form of the gene was found to be inversely associated with tumor grade and residual tumor volume (Table 1). Therefore, high *KLK8* expression is associated with small, less aggressive tumors. These results suggest that *KLK8* is a favorable prognostic indicator for ovarian cancer. These observations are further corroborated by our finding that high *KLK8* expression is an independent prognostic factor of improved PFS (Fig. 4).

Nine of the 10 non-cancerous ovarian tissues examined showed no or very low levels of *KLK8* expression. It seems that *KLK8* is not expressed much in the normal ovarian tissue, but its expression increases in the first stages of tumorigenesis, and then it is progressively suppressed as the malignancy advances. Because there is no information available on the pathophysiological role of *KLK8* in ovarian tissue, it would be difficult to formulate a hypothesis that could explain the way of regulation of gene expression and the mechanism by which *KLK8* confers a favorable prognostic outcome in ovarian cancer.

There is now growing evidence that many kallikreins are related to malignancy. PSA is the most valuable clinical marker for diagnosis and management of prostate cancer (20). PSA is also recognized as a favorable prognostic factor for breast cancer (21, 22). Recent reports suggest that hK2 could be another useful diagnostic marker for prostate cancer (23, 24), whereas *KLK4* (*prostase*), another kallikrein gene, seems to be also implicated with this disease (25). *KLK13* is reported to be down-regulated in breast cancer (19), *KLK6* (*zyme*) was shown to be differentially expressed in primary breast and ovarian

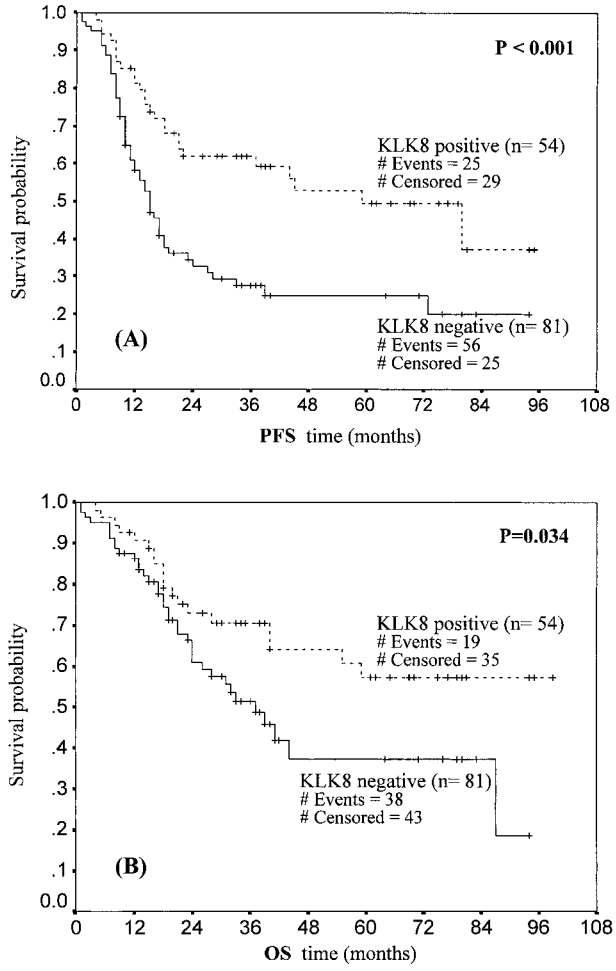

Fig. 4 Kaplan-Meier survival curves presenting the association between *KLK8* expression and PFS (*A*) or OS (*B*).

tumors (26), and *KLK7* (stratum corneum chymotryptic enzyme, *HSCCE*) has been shown to be expressed at abnormally high levels in ovarian cancer (27). All kallikreins are thought to have serine protease catalytic activity and may be able to activate each other or other molecules (growth factors, cytokines, and others) in a cascade of events associated with tumorigenesis. Our results suggest that KLK8 may be involved in such pro-

cesses. The finding that a protease like KLK8 is a favorable prognostic tumor marker is not new. Other proteases (*e.g.*, pepsinogen C and PSA) have also been associated with favorable cancer prognosis (21, 22, 28).

In summary, we here provide the first evidence that higher *KLK8* expression is associated with more favorable outcomes of patients with ovarian cancer. Because KLK8 encodes for a predicted secreted protein, analysis of this molecule in serum may aid in ovarian cancer diagnosis and prognosis. This possibility is under investigation.

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