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Alternative Splicing Variant of Kallikrein-Related Peptidase 8 as an Independent Predictor of Unfavorable Prognosis in Lung Cancer

Chris Planque,[1,2] Yun-Hee Choi,[3] Serge Guyetant,[1,2,4] Nathalie Heuzé-Vourc'h,[1,2] Laurent Briollais,[3] and Yves Courty[1,2*]

BACKGROUND: A relatively unexplored area for biomarker identification is alternative splice variants. We undertook this study to evaluate the usefulness of mRNA isoforms encoded by the *KLK8* (kallikrein-related peptidase 8) gene as prognostic markers for lung cancer.

METHODS: Real-time reverse-transcription PCR was used to analyze the mRNAs encoded by *KLK8* (particularly 2 mRNA splice variants, KLK8-T3 and KLK8-T4) in 60 non–small-cell lung cancer (NSCLC) tumors and in paired unaffected tissues. The ratios of these mRNAs to those encoded by the *KLK5*, *KLK6*, *KLK7*, *KLK10*, *KLK11*, *KLK13*, and *KLK14* genes were also determined and analyzed for correlations with various clinicopathologic variables.

RESULTS: KLK8-T3 and KLK8-T4 were the most abundant of the 6 mRNA isoforms identified in lung tissues. The overall expression of the *KLK8* gene and the amounts of the KLK8-T3 and KLK8-T4 mRNAs were significantly increased in lung tumor tissue ($P < 0.0001$). Univariate survival analysis revealed significant relationships of the relative concentrations of mRNA splice variants KLK8 ($P = 0.043$), KLK8-T3 ($P = 0.037$), and KLK8-T4 ($P = 0.009$) with overall survival (OS). Cox multivariate analysis indicated that the amount of KLK8-T4 mRNA was an independent prognostic factor for OS (relative risk = 3.90; $P = 0.016$) and that high KLK8-T4/KLK7, KLK8-T4/KLK10, and KLK8-T4/KLK11 mRNA ratios in NSCLC indicated increased risk of death. The increase was approximately 5-fold for the KLK8-T4/KLK7 and KLK8-T4/KLK10 ratios ($P = 0.006$, and $P = 0.011$, respectively) and 8-fold for the KLK8-T4/KLK11 ratio ($P = 0.001$).

CONCLUSIONS: The KLK8-T4 alternative splice variant, alone or in combination, may be a new independent marker of unfavorable prognosis in lung cancer.

Lung cancer is the leading cause of cancer-related deaths, and non–small-cell lung cancer (NSCLC)[5] accounts for almost 80% of these deaths. Despite improved understanding of the molecular biology of lung cancer, treatment decisions continue to be guided largely by the current tumor–node–metastasis (TNM) system. The clinical staging used to forecast the survival of individual patients remains far from accurate, however, because 50% of patients who undergo operation for early-stage disease develop recurrent disease *(1)*. Microarray gene expression profiling has been used to identify prognostic signatures for NSCLC *(2)*, but such array-based technology is not directly transferable to the clinical setting because it requires specialized laboratory facilities and complex statistical analyses. Prognostic models based on assaying the expression of a limited number of genes *(3, 4)* by quantitative real-time PCR may be more clinically practical. There is therefore a need to identify small signatures that can be easily analyzed in the clinical laboratory. A relatively unexplored area of biomarkers is alternative splice variants. Studies on specific genes and splice variations indicate that alternatively spliced products are particularly relevant in oncology *(5)*. They may contribute to the etiology of cancer, provide selective drug targets, or serve as markers for cancer diagnosis or prognosis. We have examined the prognostic

[1] Université François-Rabelais de Tours, Tours, France; [2] INSERM U618 "Protéases et Vectorisation Pulmonaires," Tours, France; [3] Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada; [4] Department of Pathology, CHRU de Tours, Tours, France.
* Address correspondence to this author at: INSERM U618, Faculté de Médecine, 10 Blvd. Tonnellé, F-37032 Tours Cedex 1, France. Fax +33-2-47-36-60-46; e-mail courty@univ-tours.fr.

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[5] Nonstandard abbreviations: NSCLC, non–small-cell lung cancer; TNM, tumor–node–metastasis; KLK, kallikrein-related peptidase; KLK8-T1, KLK8 mRNA splice variant type 1; RT-PCR, reverse-transcription PCR; OS, overall survival; RR, relative risk.

value of alternative mRNA variants of the *KLK8*[6] (kallikrein-related peptidase 8) gene. This gene belongs to the kallikrein-related peptidase (KLK) gene family, which is both an exciting source of potential cancer biomarkers *(6)* and a mine of splice variants *(7)*. The archetypical member of the KLK gene family is the *KLK3* gene, which encodes the most widely recognized marker in urologic oncology, prostate-specific antigen (also known as KLK3). This gene is also the source of at least 10 alternative mRNA transcripts. Although multiple mRNA variants encoded by KLK genes have been described, little is known about their function(s). They could act as regulators at the mRNA level only or encode proteins with similar or different functions *(8)*.

KLK8 was originally cloned from a human skin library *(9)* as a homolog of a gene encoding mouse neuropsin. The KLK8 peptidase is present in numerous human tissues *(10)* and is involved in several physiological and pathologic processes *(11, 12)*. Abnormal *KLK8* transcripts and/or the KLK8 protein have been found in several malignancies, including uterine endometrial carcinoma and ovarian, lung, and neck cancers *(11, 13–15)*. Five alternative mRNA variants encoded by *KLK8*, including the regular form, have been described *(7)*. Type 1 and type 2 KLK8 mRNA variants (KLK8-T1 and KLK8-T2) produce 2 zymogens that differ only in their propeptide sequences. In this case, alternative splicing produces the same final active protein, but the 2 zymogens are released in a cell type–dependent manner and are activated differently *(16)*. The KLK8-T3 mRNA variant encodes a truncated form of the KLK8 protein. The KLK8-T4 variant lacks exons 3–5. It encodes a putative protein of 32 amino acid residues that contains the KLK8 signal peptide and another peptide that is not related to KLK8 *(17)*. The KLK8-T3 and KLK8-T4 mRNAs are abundant in many tissues (brain, pancreas, skin) and are overproduced in ovarian cancers *(17)*.

We have examined the pattern of KLK8 mRNAs in NSCLC samples by reverse-transcription PCR (RT-PCR) and DNA sequencing. We identified 6 alternatively spliced transcripts, of which KLK8-T3 and KLK8-T4 were the most abundant. The concentrations of these 2 splice variants in a cohort of NSCLC patients were then measured by quantitative real-time PCR. Finally, we found that a splice variant mRNA, KLK8-T4, may be an independent indicator of a poor prognosis for lung cancer patients.

[6] Human genes: *KLK8*, kallikrein-related peptidase 8; *KLK3*, kallikrein-related peptidase 3; *KLK5*, kallikrein-related peptidase 5; *KLK6*, kallikrein-related peptidase 6; *KLK7*, kallikrein-related peptidase 7; *KLK10*, kallikrein-related peptidase 10; *KLK11*, kallikrein-related peptidase 11; *KLK13*, kallikrein-related peptidase 13; *KLK14*, kallikrein-related peptidase 14; *ERBB4*, v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian); *LOX*, lysyl oxidase.

Materials and Methods

CLINICAL SAMPLES

Matched samples of tumor and nontumor tissue were obtained from 60 patients who had undergone lung cancer resection as their primary therapy without preoperative radiation or chemotherapy. Tumor and nontumor tissue samples were selected by a pathologist from each fresh surgical sample, immediately frozen in liquid nitrogen, and stored at −80 °C. The nonmalignant tissue samples were taken from sites at least 3 cm away from the edge of the tumor. Histologic diagnosis was performed, and tumor grade was determined in accordance with the WHO classification of lung tumors. The tissue samples were banked with informed consent, in compliance with the Helsinki Accord and French bioethical regulations. The methods used for RNA extraction, cDNA synthesis, and conventional RT-PCR are described in the Supplemental Data section of the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol56/issue6.

QUANTITATIVE REAL-TIME RT-PCR ANALYSIS

To analyze *KLK8* gene expression, we based the design and synthesis of primer sets on the published mRNA sequence of KLK8-T1 (set KLK8, GenBank accession no. NM_007196), KLK8-T3 (NM_144506), and KLK8-T4 (NM_144507) (Table 1). Primers were designed to target 2 exons. The amounts of 18S rRNA and mRNAs encoded by *KLK5*, *KLK6*, *KLK7*, *KLK10*, *KLK11*, *KLK13*, and *KLK14* were measured as previously described *(18, 19)* (see Supplemental Data section in the online Data Supplement). Real-time PCR was carried out in iCycler 96-well PCR plates on an iCycler iQ Real-Time PCR System (Bio-Rad Laboratories) with the SYBR® Green I chemistry (see Supplemental Data section in the online Data Supplement). Each assay included 2 no-template controls, cDNA samples in duplicate, and serial dilutions of the appropriate plasmid DNA calibrator for constructing a calibration curve. The concentrations of all samples were calculated by plotting their quantification cycles against the calibration curve. The amount of the target molecule was then normalized by dividing by the amount of the endogenous reference (18S rRNA). Values are expressed in arbitrary units.

STATISTICAL ANALYSES

The KLK8 mRNAs were used to classify *KLK8* gene activity as negative or positive. The χ^2 test or the Fisher exact test was used as appropriate to analyze associa-

Table 1. Characteristics of the primers used for the quantification of the *KLK8* transcripts.

Primer set	Primer sequence	Position	Product length	Detected transcript
KLK8	Fwd:[a] 5′-CCAGAAGAAGTGTGAGGATG-3′	Exon 5	190 bp	KLK8-T1, -T2, -T3, -T5, and -T6
	Rev: 5′-GGTATAGACGCCAGGTTTG-3′	Exon 6		
KLK8-T3	Fwd: 5′-GGAGCCTGGGCAGAGAAT-3′	Exon 2–exon 5 junction	161 bp	KLK8-T3
	Rev: 5′-CCTCCAGAATCGCCCT-3′	Exon 6		
KLK8-T4	Fwd: 5′-TGGGCAGGGCGATTCT-3′	Exon 2–exon 6 junction	132 bp	KLK8-T4
	Rev: 5′-CAGTCCAGGTAGCGGCAG-3′	Exon 6		

[a] Fwd, forward; Rev, reverse.

tions between *KLK8* gene activity and other qualitative variables.

We used the Kaplan–Meier method for constructing overall survival (OS) curves to demonstrate differences in the survival of KLK8 mRNA–positive and KLK8 mRNA–negative patients. The OS time was defined as the time between the initial surgery and death. The survival times of patients who were still alive were noted along with the dates of the last follow-up appointment. The log-rank test was used to determine statistically significant differences between OS curves. The impact of *KLK8* gene activity on patient OS was assessed as the relative risk (RR) of death in the group with high *KLK8* activity, as calculated with the Cox univariate and multivariate proportional hazard regression models. In the multivariate analysis, we adjusted for the clinical and pathologic variables that could affect survival, including sex, age, stage of disease, histologic type, residual tumor size, and rate of smoking.

Results

IDENTIFICATION OF KLK8 mRNA VARIANTS IN LUNG TISSUE

To carry out the RT-PCR, we used RNA prepared from nonpathologic and tumoral lung tissues and the specific primer set to amplify the entire coding sequence of KLK8 mRNA. The RT-PCR produced several products (Fig. 1), and we determined the nucleotide sequences of 6 of them. The sequences of 5 products were identical to those of the regular KLK8 mRNA *(9)* and alternative *KLK8* gene transcript types 2–5 *(11, 17)*. We identified 1 new alternatively spliced mRNA, KLK8 type 6 (KLK8-T6), which lacked exon 4. For this variant, the alternative splicing creates a stop codon that prematurely terminates translation at amino acid residue 80. The predicted KLK8-T6 protein has the signal peptide, which is necessary for the secretion of the type 1 and type 2 forms *(16)*, suggesting that the type 6 variant is secreted. The truncated protein has only 1 amino acid residue of the catalytic triad and probably has no serine protease activity.

All 6 KLK8 mRNA forms were found mainly in lung cancer tissues; little or none was found in nonpathologic lung tissue (Fig. 1). The KLK8-T3 and KLK8-T4 mRNA variants appeared to be the most abundant forms in lung cancer.

REAL-TIME QUANTIFICATION OF mRNA KLK8 ISOFORMS IN NSCLC

We assayed the activity of the *KLK8* gene in lung tumors and nonpathologic lung tissues with 3 primer sets designed for quantitative real-time PCR analysis (Table 1). The first set (total KLK8 mRNA) was used to measure all *KLK8* gene transcripts except KLK8-T4; the other 2 sets were used to quantify KLK8-T3 or KLK8-T4 mRNA. We evaluated the specificity of PCR reactions by running quantitative real-time RT-PCR experiments with the primers specific for KLK8-T3 or KLK8-T4 in samples containing cloned cDNA that corresponded to the other transcript. Each transcript was amplified only by its specific primer pairs; the degree of cross-reaction was negligible. The dynamic ranges for KLK8-T3 and KLK8-T4 were $10–10^6$ copies. The Pearson correlation coefficient for the calibration curves was 0.99. The analytical limit of quantification was 15 copies per reaction (see Supplemental Data section in the online Data Supplement).

The total concentration of KLK8 mRNA in 60 NSCLC samples was significantly higher ($P < 0.0001$) than that in the paired, apparently unaffected control tissues (Fig. 2). Similarly, KLK8-T3 and KLK-T4 mRNAs were more abundant in NSCLC samples than in paired nonpathologic tissue samples ($P < 0.0001$).

We compared the mRNA amounts for total KLK8, KLK8-T3, and KLK8-T4 in patients who been classified according to conventional clinicopathologic parameters (Table 2). We used χ^2 analysis to identify an optimal cutoff value for each variable on the basis of the ability of the variable to predict the OS time of the study

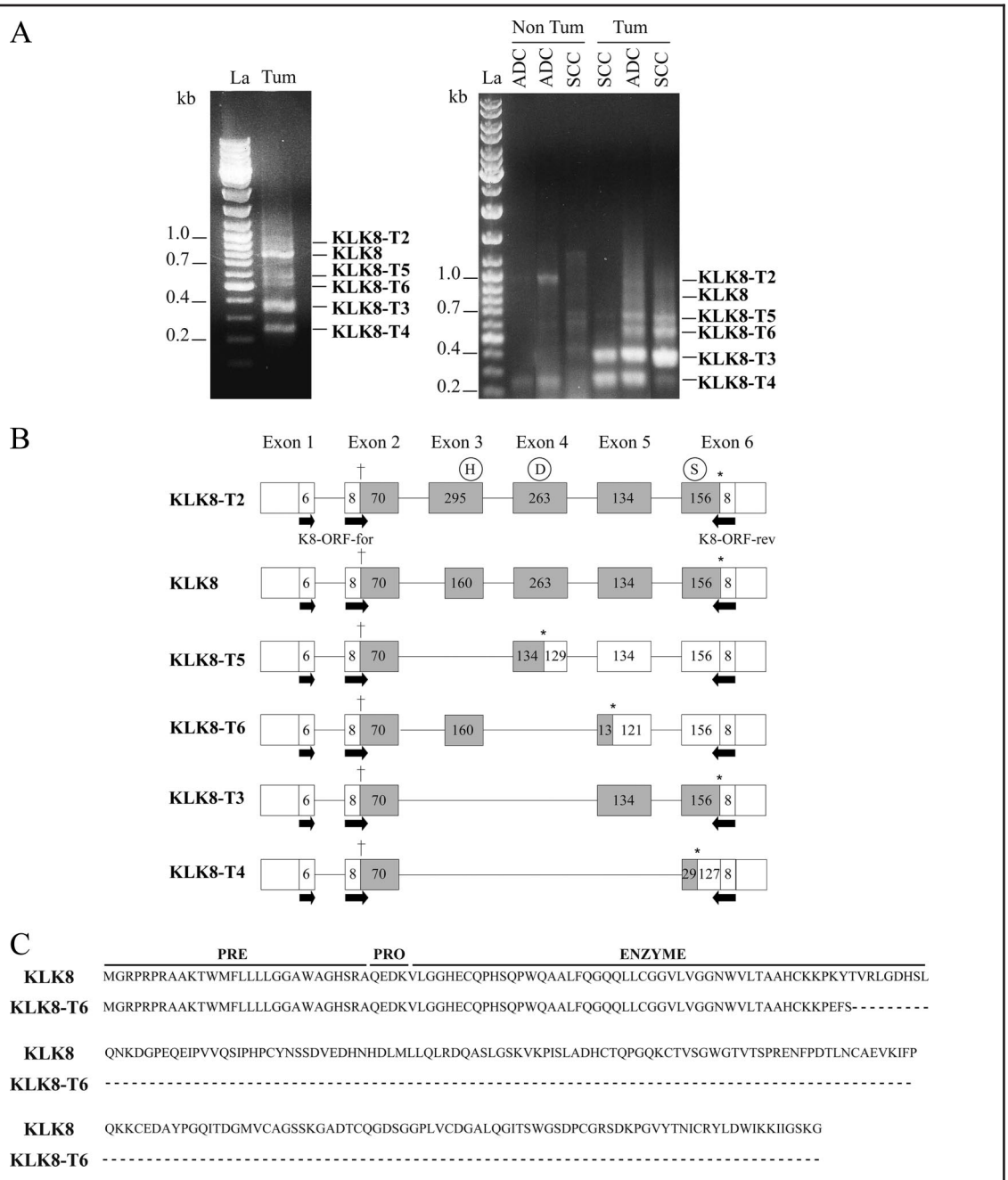

Fig. 1. *KLK8* gene expression pattern in lung tissues.

(A), *KLK8* gene expression profile in lung tissue samples. RT-PCR products from nontumoral (Non Tum) and tumoral (Tum) tissue samples were separated on agarose gels and stained with ethidium bromide. The PCR products were cloned into pcDNA5/FRT/V5-His-TOPO vector (Invitrogen) and sequenced. La, DNA ladder; ADC, adenocarcinoma samples; SCC, squamous cell carcinoma samples. (B), Structures of the *KLK8* gene transcripts. White and gray boxes represent noncoding and coding regions, respectively. The exons and the number of nucleotides in each exon are indicated for each splice variant. The *KLK8* gene is composed of 6 exons and 5 introns; the first exon is noncoding. Indicated are the locations of the start codon (†), the stop codon (*), and the approximate locations of the amino acid residues of the catalytic triad of serine proteases (H, D, S). The open reading frame (ORF) primers used for molecular cloning are represented by black arrows. (C), Alignment of the predicted protein sequence encoded by the KLK8–T6 splice variant with KLK8. Horizontal lines delimit the prepeptide, propeptide, and mature regions of KLK8.

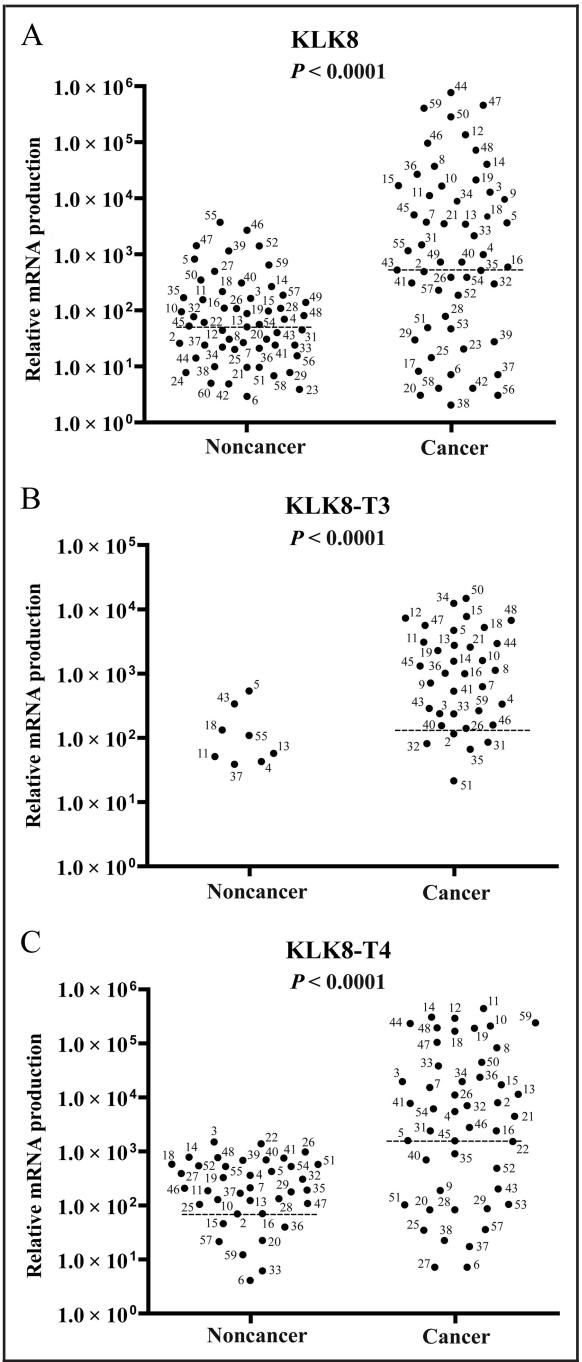

Fig. 2. KLK8, KLK8-T3, and KLK8-T4 mRNA production in cancerous and matched noncancerous tissues from 60 patients with NSCLC.

Gene expression is normalized to the amount of 18S rRNA and is reported in arbitrary units. Horizontal dashed lines indicate median values. Each individual is distinguished by a number. The difference between the amounts of transcripts in nontumoral and tumoral tissues was evaluated by a Wilcoxon matched-pairs test.

population. With this cutoff, we separated the lung tumors into positive and negative groups (i.e., above and below the cutoff) and were able to correlate patients with KLK8 mRNA–positive and KLK8-T3–positive tumors with the squamous cell carcinoma histotype ($P < 0.05$). The KLK8-T4–positive tumors were mainly of T3-T4 status, although the result was marginally nonsignificant ($P = 0.066$). We found no relationship between KLK8, KLK8-T3, or KLK8-T4 mRNA status and tumor grade, size, nodal status, or stage of cancer.

KLK8 mRNA ISOFORMS AND OS

We examined several variables, including total KLK8 mRNA and the KLK8-T3 and KLK8-T4 mRNAs. The KLK8 mRNAs were considered alone or as a ratio with the KLK5, KLK6, KLK7, KLK10, KLK11, KLK13, or KLK14 mRNA in the same samples. An optimal cutoff value was identified for each variable by χ^2 analysis, as described above, and the lung tumors were stratified into positive and negative groups (above and below the cutoff). Table 3 shows the strength of the association between positive tumors and OS. Univariate analysis indicated that clinical stage was significantly associated with a poor prognosis (RR = 4.14; $P = 0.001$), as was the presence of KLK8, KLK8-T3, or KLK8-T4 mRNA [RR = 2.56 ($P = 0.043$), 2.64 ($P = 0.037$), and 3.37 ($P = 0.009$), respectively]. Several ratios also were significantly associated with OS (Table 3). The KLK8, KLK8-T3, and KLK8-T4 mRNA ratios with the clearest associations with OS were KLK8/KLK13 (RR = 6.32; $P = 0.003$), KLK8-T3/KLK13 (RR = 4.25; $P = 0.009$), and KLK8-T4/KLK11 (RR = 8.16; $P = 0.001$). Multivariate Cox regression analysis did not confirm the relationship between variables that included KLK8-T3 mRNA and OS. Conversely, the KLK8/KLK10, KLK8/KLK11, and KLK8/KLK13 mRNA ratios remained independent prognostic factors for survival. Almost all of the variables that included KLK8-T4 mRNA were significantly associated with OS in the Cox multivariate regression analysis (Table 3). The RR of cancer-related death was >8-fold higher (RR = 8.37; $P = 0.001$) for patients with a KLK8-T4/KLK11 mRNA ratio above the cutoff than for those with a KLK8-T4/KLK11 ratio below the cutoff. Kaplan–Meier survival curves further demonstrated that patients with KLK8-T4/KLK11–positive tumors had substantially shorter OS times ($P < 0.001$) than those with KLK8-T4/KLK11–negative tumors (Fig. 3).

Discussion

The *KLK8* gene encodes at least 6 mRNA variants in lung tissue, including the classic KLK8-T1 form. The alternative splicing events imply exon skipping (mRNAs KLK8-T3, -T4, -T5, and -T6) and exon extension

Table 2. Relationship of KLK8, KLK8-T3, and KLK8-T4 mRNA status with clinicopathologic variables.[a]

Variable	KLK8			KLK8-T3			KLK8-T4		
	Negative, n (%)	Positive, n (%)	*P*	Negative, n (%)	Positive, n (%)	*P*	Negative, n (%)	Positive, n (%)	*P*
Histotype									
ADC[b] (n = 33)	16 (48.5)	17 (51.5)	**0.029**[c]	17 (51.5)	16 (48.5)	**0.035**[c]	16 (48.5)	17 (51.5)	0.881[c]
SCC (n = 17)	2 (11.8)	15 (88.2)		3 (17.6)	14 (82.4)		7 (41.2)	10 (58.8)	
Other subtypes (n = 10)	5 (50.0)	5 (50.0)		6 (60.0)	4 (40.0)		5 (50.0)	5 (50.0)	
Tumor grade									
Poorly diff (n = 19)	7 (36.8)	12 (63.2)	1.000[d]	8 (42.1)	11 (57.9)	1.000[d]	7 (36.8)	12 (63.2)	0.417[d]
Diff (n = 39)	14 (35.9)	25 (64.1)		16 (41.0)	23 (59.0)		19 (48.7)	20 (51.3)	
Unknown (n = 2)									
Tumor size									
≤3 cm (n = 20)	8 (40.0)	12 (60.0)	1.000[d]	9 (45.0)	11 (55.0)	1.000[d]	9 (45.0)	11 (55.0)	1.000[d]
>3 cm (n = 40)	15 (37.5)	25 (62.5)		17 (42.5)	23 (57.5)		19 (47.5)	21 (52.5)	
Nodal status									
N0 (n = 40)	15 (37.5)	25 (62.5)	1.000[d]	18 (45.0)	22 (55.0)	0.787[d]	19 (47.5)	21 (52.5)	1.000[d]
N1/N2 (n = 20)	8 (40.0)	12 (60.0)		8 (40.0)	12 (60.0)		9 (45.0)	11 (55.0)	
Tumor status									
T1/T2 (n = 47)	20 (42.6)	27 (57.4)	0.334[d]	23 (48.9)	24 (51.1)	0.122[d]	25 (53.2)	22 (46.8)	0.066[d]
T3/T4 (n = 13)	3 (23.1)	10 (76.9)		3 (23.1)	10 (76.9)		3 (23.1)	10 (76.9)	
Stage									
I/II (n = 37)	16 (43.2)	21 (56.8)	0.416[d]	19 (51.4)	18 (48.6)	0.180[d]	19 (51.4)	18 (48.6)	0.430[d]
III/IV (n = 23)	7 (30.4)	16 (69.6)		7 (30.4)	16 (69.6)		9 (39.1)	14 (60.9)	

[a] The cutoffs used were equal to the 38th, 43rd, and 47th percentiles for KLK8, KLK8-T3, and KLK8-T4 mRNAs, respectively. Statistically significant differences ($P < 0.05$) are in boldface.
[b] ADC, adenocarcinoma; SCC, squamous cell carcinoma; diff, differentiated.
[c] χ^2 test.
[d] Fisher exact test.

(KLK8-T2), which are the most common mechanisms generating mRNA variants in the KLK gene family *(7, 8)*. The *KLK8* gene is much more active in NSCLC ($P < 0.0001$) than in healthy lung tissue, as it is for several other malignancies, including uterine endometrial carcinoma and ovarian and neck cancers *(13–15)*. Several KLK8 mRNAs are abnormally abundant in NSCLC, suggesting that a common mechanism, such as an increase in the transcription rate of the *KLK8* gene, affects the steady-state concentration of these mRNAs; however, the concentrations of each splice variant are not similarly increased. Therefore, specific mechanism(s) may also regulate the steady-state concentration of individual KLK8 splice variants. Traditional models of how alternative splicing is regulated involve auxiliary splicing factors that bind to the pre-mRNA and enhance or repress the ability of the spliceosome to recognize particular splice sites *(20)*. According to these models, changes in the relative distributions of auxiliary factors affect the pattern of alternative splicing. Various auxiliary splicing factors are up-regulated in lung cancer *(21)*, and differential production of alternative splice variants seems to be common in this disease *(22)*. We therefore postulate that the differential production of alternative KLK8 mRNAs in NSCLC is due to overall alterations in the splicing machinery.

Our findings concerning the KLK8 mRNA variable are in general agreement with a previous study on the expression of the *KLK8* gene in lung cancer *(11)*. In both of these studies, the primer set used to evaluate *KLK8* gene activity hybridizes with all of the alternative *KLK8* gene transcripts, except KLK8-T4 (Table 1). We found no statistically significant associations between *KLK8* gene activity and several clinicopathologic variables (grade, stage, size or status of the tumor, and nodal status) except for the squamous cell carcinoma histotype ($P = 0.018$). Neither Sher et al. *(11)* nor we

Table 3. Univariate and multivariate analysis of various prognostic factors in patients with NSCLC.[a]

Prognostic factor	Cutoff (percentile)	Univariate analysis		Multivariate analysis	
		RR (95% CI)	*P*	RR (95% CI)	*P*
Sex		0.53 (0.16–1.79)	0.309	0.41 (0.08–2.08)	0.282
Age (>median vs <median)		1.01 (0.97–1.05)	0.596	1.01 (0.97–1.06)	0.605
Histotype (ADC[b] vs SCC)		1.76 (0.74–4.19)	0.202	1.94 (0.64–5.87)	0.242
Histotype (ADC vs other histotypes)		1.62 (0.57–4.65)	0.367	2.13 (0.51–8.91)	0.301
Tumor size (>3 cm vs ≤3 cm)		1.08 (0.45–2.57)	0.867	1.10 (0.40–3.02)	0.850
Differentiation (diff vs poorly diff)		1.56 (0.71–3.42)	0.271	1.80 (0.58–5.91)	0.307
Stage (III/IV vs I/II)		**4.14 (1.84–9.33)**	**0.001**	**3.63 (1.53–8.60)**	**0.003**
Smoking status		1.01 (0.99–1.02)	0.475	1.00 (0.97–1.02)	0.762
mRNA production status (positive vs negative)					
KLK8	38	2.56 (1.03–6.39)	0.043	1.45 (0.51–4.09)	0.486
KLK8/KLK5	43	2.22 (0.93–5.30)	0.073	1.67 (0.66–4.24)	0.283
KLK8/KLK6	22	0.79 (0.32–1.96)	0.606	0.45 (0.16–1.31)	0.143
KLK8/KLK7	21	2.99 (0.89–10.02)	0.076	1.94 (0.44–8.63)	0.386
KLK8/KLK10	42	**3.84 (1.50–9.81)**	**0.005**	**4.20 (1.38–12.83)**	**0.012**
KLK8/KLK11	40	**4.57 (1.68–12.46)**	**0.003**	**4.22 (1.32–13.52)**	**0.015**
KLK8/KLK13	36	**6.32 (1.87–21.31)**	**0.003**	**5.35 (1.24–23.02)**	**0.024**
KLK8/KLK14	40	4.14 (1.41–12.17)	0.010	2.34 (0.67–8.10)	0.180
KLK8-T3	43	2.64 (1.06–6.59)	0.037	1.44 (0.51–4.05)	0.488
KLK8-T3/KLK5	46	2.65 (1.11–6.32)	0.028	1.53 (0.61–3.85)	0.370
KLK8-T3/KLK6	42	2.53 (1.02–6.31)	0.046	1.38 (0.49–3.89)	0.540
KLK8-T3/KLK7	38	2.36 (0.95–5.88)	0.066	1.25 (0.45–3.49)	0.672
KLK8-T3/KLK10	36	3.58 (1.22–10.51)	0.020	1.83 (0.53–6.39)	0.341
KLK8-T3/KLK11	40	3.47 (1.29–9.34)	0.014	2.63 (0.82–8.40)	0.103
KLK8-T3/KLK13	40	4.25 (1.45–12.50)	0.009	2.71 (0.73–10.15)	0.138
KLK8-T3/KLK14	53	2.61 (1.12–6.10)	0.027	1.74 (0.66–4.65)	0.264
KLK8-T4	47	**3.37 (1.35–8.41)**	**0.009**	**3.90 (1.29–11.75)**	**0.016**
KLK8-T4/KLK5	54	**3.06 (1.33–7.06)**	**0.009**	**3.38 (1.26–9.07)**	**0.015**
KLK8-T4/KLK6	43	2.22 (0.93–5.30)	0.071	1.77 (0.70–4.46)	0.227
KLK8-T4/KLK7	40	**2.74 (1.10–6.85)**	**0.031**	**5.51 (1.62–18.74)**	**0.006**
KLK8-T4/KLK10	42	**4.97 (1.69–14.58)**	**0.004**	**5.13 (1.45–18.09)**	**0.011**
KLK8-T4/KLK11	44	**8.16 (2.40–27.75)**	**0.001**	**8.37 (2.26–30.94)**	**0.001**
KLK8-T4/KLK13	50	**3.97 (1.56–10.09)**	**0.004**	**3.45 (1.17–10.15)**	**0.024**
KLK8-T4/KLK14	52	**3.94 (1.55–10.03)**	**0.004**	**3.33 (1.22–9.11)**	**0.019**

[a] Statistically significant differences ($P < 0.05$) are highlighted in boldface.
[b] ADC, adenocarcinoma; SCC, squamous cell carcinoma; diff, differentiated.

found any association between *KLK8* activity and OS by multivariate analysis [RR = 1.45 ($P = 0.486$) in our Cox multivariate analysis]. Sher et al., however, reported that early-stage (I–II) NSCLC patients with high *KLK8* gene expression in their tumors had significantly longer remission times and lower rates of recurrence. Similar observations were reported in patients with ovarian cancer *(17)*, with a multivariate analysis showing high KLK8 mRNA production to be associated with disease-free survival but not with OS. The duration of progression-free survival in cancer patients depends on the probability for and percentages of tumor cells to pass from one step of the metastatic process to the next. These steps include local invasion, intravasation of cells from the primary tumor into the circulatory system, survival of these cells within the

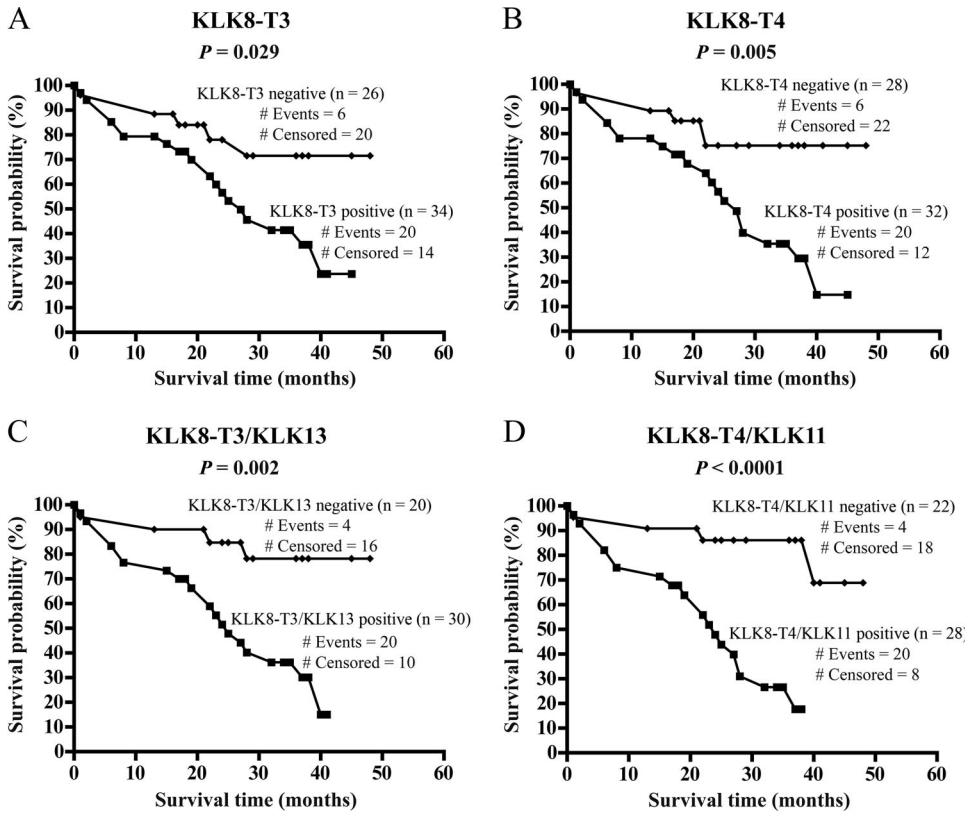

Fig. 3. Kaplan–Meier OS curves.

Patients were separated into 2 groups according to the amount of KLK8-T3 mRNA (A), the amount of KLK8-T4 mRNA (B), the KLK8-T3/KLK13 ratio (C), and the KLK8-T4/KLK11 ratio (D). Survival rates were calculated with the Kaplan–Meier method, and the rates for the 2 groups were compared. The differences between the survival curves were tested for statistical significance with the log-rank test. An optimal cutoff value was identified by χ^2 analysis, based on the ability of KLK8 mRNA variants to predict the OS of the study population. The cutoffs used were the 43rd percentile for KLK8-T3 mRNA, the 47th percentile for KLK8-T4 mRNA, the 40th percentile for the KLK8-T3/KLK13 ratio, and the 44th percentile for the KLK8-T4/KLK11 ratio. The influence of the KLK8 mRNA variants was categorized as negative or positive on the basis of these cutoffs.

blood or lymphatic system, evasion of the immune system, arrest at a secondary site distant from the site of origin, extravasation, initiation of either intra- or extravascular growth within this secondary site, and, finally, maintenance of growth leading to the formation of overt, vascularized, clinically detectable metastases *(23)*. Overproduction of type 1 and type 2 KLK8 mRNAs has been shown to decrease the in vitro invasiveness of lung cancer cells *(11)*. These protective effects of KLK8-T1 and KLK8-T2 mRNAs have been ascribed to the proteolysis of extracellular fibronectin by the encoded KLK8 peptidase, KLK8-mediated degradation of fibronectin-suppressed integrin signaling, and decreased lung cancer cell motility through inhibition of actin polymerization. In nude mice, production of these splice variants decreases tumor growth and reduces intravasation *(11)*. The protective role of KLK8 in invasiveness may explain the longer disease-free survival times of ovarian cancer patients with high concentrations of KLK8 mRNA in their cancer tissue *(17, 24)*, or higher concentrations of the KLK8 peptidase in their tissues *(13, 25)* or ascites fluids *(26)*. Collectively, these findings suggest that the KLK8 peptidase may influence the initial course of ovarian and lung cancers by delaying some early steps of the metastatic process without having any implication on their final outcomes.

In contrast, our multivariate Cox proportional hazards regression analysis and the Kaplan–Meier survival curves indicate that KLK8-T4 mRNA is an independent predictor of an unfavorable prognosis in lung cancer. Although several studies have shown that splice

variants of various *KLK* genes are differentially regulated in cancer tissues *(8)*, our results are the first evidence of the clinical value of one alternative transcript of a KLK gene. Cancer deaths are generally due to the physiological effects of local or distant metastases, rather than to the primary tumor. KLK8-T4 mRNA production may therefore have a negative impact on the final NSCLC outcome by facilitating the occurrence of life-threatening metastases. Our observations, together with those of Sher et al. on *KLK8 (11)*, suggest that the *KLK8* gene generates 2 products that have opposite actions on NSCLC progression and metastasis. Divergent biological functions of splice variants have previously been reported for 2 isoforms encoded by *ERBB4* [v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)] that exhibit markedly opposing effects on mammary epithelium growth and differentiation *(27)*. Moreover, metastasis requires functionally distinct classes of genes and activities that regulate metastasis initiation, progression, and virulence functions *(23)*. In this context, it is conceivable that the KLK8-T1 and KLK8-T4 mRNAs participate in distinct metastatic processes. Given that KLK8 limits the early steps of metastasis, the prometastatic activity of the KLK8-T4 mRNA could be achieved through actions on later steps, such as the initial seeding or persistent growth of metastatic tumor cells in the target site. For example, expression of the hypoxia-regulated gene *LOX* (lysyl oxidase) predicts relapse in human breast tumors *(28)*, but recent studies suggest that systemic secretion of lysyl oxidase into the lung and liver might facilitate the homing of disseminated cancer cells to these organs through the formation of a prometastatic microenvironment *(29)*.

KLK8 has recently been reported to be downregulated in nodal metastasis, compared with primary head and neck squamous cell carcinoma *(15)*. The existence of a similar situation in lung metastases would imply actions of the KLK8-T4 mRNA at the level of the primary tumor site that have long-term consequences. Long-term effects have been described in antitumoral immunity. For example, distinct antitumoral immune profiles involving effector memory T cells are generated at the primary tumor site in colorectal cancer. Because of their memory properties, effector memory T cells may provide long-term protection against outgrowth of disseminated occult tumor cells after surgical resection of primary tumors *(30)*. This long-term action might explain why the amplitude of the adaptive immune reaction within the primary tumor was found to be a better predictor of survival in colon cancer than traditional clinical parameters *(31)*. Patients with cancers at nonmetastatic stages had prognoses as poor as patients with metastatic tumors if they presented a low intratumoral adaptive immune reaction. Conversely, patients with metastatic tumors eliciting a high intratumoral immune reaction had a better prognosis. Little is known about the influence of the products of the KLK family on the adaptive immune reaction. Immunoregulatory functions of KLK3 have been described. Kennedy-Smith et al. *(32)* showed that KLK3 inhibited mitogen and recall antigen–induced T-cell proliferation. Aalamian et al. *(33)* demonstrated KLK3 inhibition of dendritic cell maturation. Clearly, additional experiments are necessary to elucidate the exact mechanism by which the KLK8-T4 mRNA influences the OS of patients with NSCLC and to determine whether a balance between the KLK8 peptidase and KLK8-T4 mRNA or protein is involved in NSCLC progression.

The KLK8-T4/KLK11 mRNA ratio in lung cancer tissues appears to be a much better predictor of OS (RR = 8.37; $P = 0.001$) than KLK8-T4 mRNA alone (RR = 3.90; $P = 0.016$) or pathologic TNM staging (RR = 3.63; $P = 0.003$). Our previous study found no correlation between *KLK11* gene expression and OS in NSCLC *(19)*. Two other variables (*KLK7*, *KLK10*) that are not linked to OS in lung cancer *(18, 19)* have a similar effect on the prognostic value of KLK8-T4 mRNA. One possible explanation is that the basal transcription rate of several KLK genes (*KLK7*, *KLK8*, *KLK10*, *KLK11*) is concomitantly altered in some individuals because of polymorphisms in common regulatory factors. In these patients, the KLK8-T4 mRNA concentration would not necessarily be related to cancer aggressiveness but would reflect the individual variation in the *KLK8* basal transcription rate. Thus, the other variables (i.e., *KLK7*, *KLK10*, *KLK11*) in the 2-gene index would operate as internal controls to normalize the KLK8-T4 mRNA values for changes in the basal transcription rate unrelated to the pathologic status, thereby improving the prognostic significance of the KLK8-T4 variable. As an example, the expression of multiple KLK genes is coordinated in breast cancer cell lines *(34)*. Further studies are required to determine whether the basal expression of KLK genes in lung tissue is governed by a locus control region, as in other clustered gene families, or whether individual genes are coregulated by the same regulatory factors. Alternatively, these findings could indicate that several KLKs cooperate with KLK8-T4 mRNA production for the occurrence of life-threatening metastases in NSCLC. Several studies found KLKs to be involved in cancer progression through actions on tumor cell growth, invasion, and angiogenesis *(35)*. For example, KLK11 may play a role in breast cancer progression by increasing the bioavailability of insulinlike growth factors via degradation of insulinlike growth factor–binding protein 3 *(36)*. KLK5, KLK13, and KLK14 may also contribute to tumor cell invasion via degradation of extracellular matrix components *(37–39)*. Finally, several

KLKs regulate proteinase-activated receptor–mediated signaling through receptor activation or disarming. These G protein–coupled receptors may play roles in cancer-associated inflammation and can promote tumor growth and invasion *(40)*.

In summary, we have obtained evidence that an alternative transcript of the *KLK8* gene is an independent predictor of an unfavorable prognosis in NSCLC. The KLK8-T4 /KLK11 mRNA index is a better predictor of OS than clinical stage or the concentration of KLK8-T4 mRNA alone. This 2-gene index may provide a new prognostic marker for NSCLC.

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