Human Kallikrein 8 Protease Confers a Favorable Clinical Outcome in Non–Small Cell Lung Cancer by Suppressing Tumor Cell Invasiveness

Yuh-Pyng Sher,[1,3] Cheng-Chung Chou,[4] Ruey-Hwang Chou,[6] Han-Ming Wu,[2] Wun-Shaing Wayne Chang,[6] Chun-Houh Chen,[2] Pan-Chyr Yang,[1,4,5] Cheng-Wen Wu,[1,6] Chia-Li Yu,[3,5] and Konan Peck[1]

Institutes of [1]Biomedical Sciences and [2]Statistical Science, Academia Sinica; [3]Institute of Molecular Medicine and [4]Center for Genomic Medicine, National Taiwan University; [5]Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan, Republic of China; and [6]National Institute of Cancer Research, National Health Research Institutes, Miaoli, Taiwan, Republic of China

Abstract

The human *kallikrein 8* (*KLK8*) gene, a member of the human tissue kallikrein gene family, encodes a serine protease. The *KLK8* protein (hK8) is known to be a favorable prognostic marker in ovarian cancer, but the biological basis of this is not understood. We found that overexpressing the *KLK8* gene in highly invasive lung cancer cell lines suppresses their invasiveness. This role in invasiveness was further confirmed by the fact that inhibition of endogenous *KLK8* expression with a specific short hairpin RNA reduced cancer cell invasiveness. *In situ* degradation and cell adhesion assays showed that proteins produced from *KLK8* splice variants modify the extracellular microenvironment by cleaving fibronectin. DNA microarray experiments and staining of cells for actin filaments revealed that the degradation of fibronectin by hK8 suppresses integrin signaling and retards cancer cell motility by inhibiting actin polymerization. In addition, studies in a mouse model coupled with the detection of circulating tumor cells by quantitative PCR for the human *Alu* sequence showed that *KLK8* suppresses tumor growth and invasion *in vivo*. Finally, studies of clinical specimens from patients with non–small cell lung cancer showed that the time to postoperative recurrence was longer for early-stage patients (stages I and II) with high *KLK8* expression (mean, 49.9 months) than for patients with low *KLK8* expression (mean, 22.9 months). Collectively, these findings show that *KLK8* expression confers a favorable clinical outcome in non–small cell lung cancer by suppressing tumor cell invasiveness. (Cancer Res 2006; 66(24): 11763-70)

Introduction

Human tissue kallikreins (hK) are encoded by a family of 15 structurally homologous genes (*KLK*) clustered together on chromosome 19q13.4. Aberrant amounts of *KLK* transcripts and/or hK proteins have been found in several hormonal malignancies, such as breast, prostate, testicular, and ovarian cancers, making the hKs useful diagnostic and/or prognostic biomarkers (1).

Cancer metastasis consists of a series of linked sequential steps. It is not surprising that proteases, such as the hKs, are reported to promote tumor invasion (2–5). Human *kallikrein 8* (*KLK8*; neuropsin/ovasin) is a member of the *KLK* family and the hK8 protein is homologous to mouse neuropsin (6). Most studies have focused on the clinical value of hK8 as a serologic or histologic biomarker of tumors. For instance, a high level of hK8 expression is detected in cervical cancer (7), and patients with a higher level of hK8 in ovarian tumor tissue have a lower grade of disease, a longer progression-free survival, and relapse less frequently (8). Despite this information, little is known about the biological function of hK8, why it is associated with favorable outcome in cancer, and what role it plays in metastasis.

To investigate the role of hK8 in cancer metastasis, we first analyzed *KLK8* expression in a panel of cell lines with different degrees of invasiveness. Surprisingly, although proteolytic enzymes are believed to participate in tumor progression by degrading the extracellular matrix (ECM), we found that *KLK8* transcripts were highly expressed in cancer cell lines of low invasiveness. We therefore investigated the likely reasons and mechanisms by which hK8 suppresses invasion by performing a series of molecular, cellular, and animal studies using a group of model lung adenocarcinoma cell lines with different levels of invasiveness. Finally, based on a study of clinical specimens from non–small cell lung cancer (NSCLC) patients, we conclude that hK8 suppresses tumor cell invasiveness and results in a favorable clinical outcome in patients with early-stage NSCLC.

Materials and Methods

Cell lines. The following cell lines were obtained from the American Type Culture Collection (Manassas, VA): breast cancer cell lines SK-BR3, T47D, ZR-75-1, and Hs578T; colon cancer cell lines Colo320DM, Colo320HSR, WiDr, and LoVo; ovarian cancer cell lines OVCAR-3, A59, ES2, PA1, and A59-4; bladder cancer cell lines HT1197, HT1197-4, EJ, and NTUB1; and lung cancer cell lines A549 and H928. The cell lines were grown in the recommended culture media. The invasiveness of the cell lines was examined in a Matrigel invasion assay system as previously described (9). Two human lung adenocarcinoma cell lines of different invasiveness (CL1-0, weakly invasive; CL1-5, highly invasive) were derived as previously described (10). The materials and methods for the reverse transcription-PCR (RT-PCR) analysis of *KLK8* expression in these cell lines are described in the Supplementary Data.

***KLK8* gene transcript construction and retroviral infection.** The *KLK8* splice variants, K8-2 and K8-R, were amplified by RT-PCR from

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

P-C. Yang, C-L. Yu, and K. Peck codirected the project and made equal contributions.

Current address for C-C. Chou: Department of Life Science and Institute of Molecular Biology, National Chung Cheng University, Chia-Yi, Taiwan 621, Republic of China.

Requests for reprints: Konan Peck, Institute of Biomedical Sciences, Academia Sinica, 128, Sec. 2, Academia Road, Taipei, Taiwan 115, Republic of China. Phone: 886-2-2652-3072; Fax: 886-2-2785-8594; E-mail: konan@ibms.sinica.edu.tw.

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CL1-0 cells using a forward primer (5′-GGGGGCCCAGCCGGCCGCGT-GTGGAAGCCTGGACCTC-3′ for K8-2 or 5′-GGGGGCCCAGCCGGCC-GGACACTCCAGGGCACAGGAGG-3′ for K8-R) and reverse primer (5′-CTTATCGATGAATCAGCCCTTGCTGCCTATGA-3′, for both K8-2 and K8-R). The amplified products were inserted into the pLNCX retroviral vector (BD Clontech, Palo Alto, CA) with a human influenza hemagglutinin tag after the leader sequence for secreted proteins to produce the pLNCX/*KLK8* splice variants. Retroviruses were generated as previously described (11). The virus-infected cells were selected in 1 mg/mL G418 (Invitrogen, Carlsbad, CA) to generate CL1-5/Vector, CL1-5/K8-2, and CL1-5/K8-R cells.

Lentiviral short hairpin RNA–mediated knockdown of *KLK8* in CL1-0 cells. The short hairpin RNA (shRNA) vector for the knockdown of *KLK8* (TRCN0000050182; shK8; target sequence of 5′-GCCTTGTTCCAGGG-CCAGCAA-3′) was obtained from the RNA interference consortium shRNA library (Open Biosystems, Huntsville, AL). Lentivirus was generated by cotransfecting TE671 cells with lentiviral vector and packaging DNA mix using GeneJammer (Stratagene, La Jolla, CA). The lentiviruses were then used to infect CL1-0 cells for 24 hours in the presence of 8 μg/mL polybrene. The infected cells were grown for 48 hours in RPMI containing 10% fetal bovine serum and then selected in 0.4 μg/mL puromycin (Sigma, St. Louis, MO).

***In situ* fibronectin degradation and cell adhesion assays.** Glass coverslips were coated with 15 μg/mL of FITC-conjugated human plasma fibronectin (Invitrogen) in 0.1 mol/L carbonate-bicarbonate buffer (pH 9.5) for 2 hours at 37°C and blocked with 1% bovine serum albumin (BSA) for 1 hour at 37°C. Cells were cultured on the coverslips for 17 hours at 37°C, and then fixed with 3% paraformaldehyde in PBS. Fluorescence images were taken with a confocal fluorescence microscope (MRC1000; Bio-Rad, Hercules, CA). Image quantification was done using the ImageJ program.[7]

To test the effect of hK8s on cell adhesion, cells were incubated for 40 minutes at 37°C on tissue culture plates coated with 10 ng/μL fibronectin or 1% BSA in PBS. Loosely bound cells were removed by washing with PBS, and the bound cells were stained as previously described (12). The percentage of adhesion was calculated by using the following formula: adhesion (%) = 100% × (number of cells adhering under the test condition) / (number of untreated cells adhering after 3 hours).

Microarray gene expression profile analysis. We prepared 150mer gene-specific DNA microarrays containing 13,440 unique human genes and 768 control genes as previously described (13). Cytoplasmic total RNA from cells was reverse-transcribed to cDNA and indirectly labeled with fluorescent dyes using the SuperScript Indirect cDNA labeling system (Invitrogen). The cDNA derived from CL1-5/Vector was labeled with Cy5, whereas the cDNAs from CL1-5/K8-2, CL1-5/K8-R, and CL1-0 cells were labeled with Cy3. Cells cultured without fibronectin were used as the negative controls. The labeled cDNA was then hybridized to the microarrays at 42°C for 16 to 18 hours in the Pronto! Universal Microarray Reagent System (Corning, NY).

To calculate log ratios of expression, the background-corrected intensities for the CL1-0, CL1-5/K8-2, or CL1-5/K8-R cells were divided by those for the CL1-5/Vector cells. The log ratio values (M) were calculated from the base 2 logarithm of the ratios normalized within and between chips by using the marrayNorm package from the Bioconductor project (14). The color gradation image displays positive M values in red, negative values in green, and no difference in expression in black. Kendall's τ rank correlation coefficient (15) was used to search for genes whose expression patterns most agreed with the expected profile.

Immunofluorescence imaging of actin filaments and filopodia. The cells were seeded onto fibronectin-coated coverslips, cultured overnight, and fixed with 3% paraformaldehyde for 30 minutes at room temperature. The cells were blocked with PBS containing 0.1% Triton X-100 and 5% BSA for 1 hour at 37°C, and then stained with FITC-phalloidin (Invitrogen). Fluorescence images were taken with a fluorescence microscope (Axiovert 200; Carl Zeiss, Gottingen, Germany). Image analysis was done using Meta Morph V 6.21 software (Universal Imaging Corporation, Downingtown, PA).

[7] http://rsb.info.nih.gov/nih-image/.

Protein expression assays. The expression of hK8 protein in the tumor mass was assayed by ELISA and Western blotting. The materials and methods for hK8 protein assays as well as for measurement of vascular endothelial growth factor (VEGF) and CD31 in the tumor mass are described in the Supplementary Data.

Analysis of tumor growth rate affected by hK8 expression. Three groups (four mice each) of 8-week-old male nonobese diabetic-severe combined immunodeficiency (SCID) mice were injected s.c. with 3×10^6 CL1-5/Vector, CL1-5/K8-2, or CL1-5/K8-R cells. The tumor volume (in cubic millimeters) was estimated using the ellipsoidal formula: length (mm) × width (mm) × height (mm) × 0.52 (16). The mice were monitored until the tumor size approached 2,000 mm^3 or until it appeared to be suffering or moribund. Mice were euthanized according to the institutional regulations for animal studies.

***In vivo* assay of cellular invasiveness in the mouse model.** The invasiveness of CL1-5 cells transfected with each *KLK8* splice variant was measured in the mouse model by measuring the level of circulating tumor cells. Peripheral blood samples were taken from mice in heparinized microhematocrit tubes (Assistant, Sondheim, Germany), and genomic DNA was extracted from the blood samples using a QIAamp mini DNA kit (Qiagen, Hilden, Germany). The level of circulating tumor cells was measured by quantitative PCR (qPCR) for the human *Alu* sequence (17).

Lung cancer patients and tissue specimens. Cancer tissue specimens from 88 patients with NSCLC who underwent surgical resection at the Taichung Veterans General Hospital between November 1999 and December 2004 were included in this study. The clinicopathologic features of the patients are given in Table 1. Written informed consent was obtained from all patients. The materials and methods for qPCR analysis of *KLK8* expression in clinical specimens are described in the Supplementary Data.

Statistical analyses. Where appropriate, the data are presented as the means ± SD. All statistical analyses were done using the Statistical Program for Social Sciences package, version 10.0 (Chicago, IL). Disease-free curves between groups with low and high *KLK8* expression were obtained by the Kaplan-Meier method. All statistical tests having two-sided $P < 0.05$ were considered to be statistically significant.

Results

High expression of *KLK8* transcripts correlates with low invasiveness in cancer cell lines. Using 19 cancer cell lines with different degrees of invasiveness, we examined the *KLK8* gene expression and cancer cell invasiveness in a Matrigel assay system.

Table 1. Clinicopathologic characteristics and their correlation with *KLK8* expression in patients with NSCLC

Characteristics	Low *KLK8*	High *KLK8*	*P*
Age (median ± SD)	64 ± 12.1	69 ± 6.9	0.053*
Gender (no. patients)			
Male	41	31	0.42
Female	11	5	
Stage †			
I–II	33	22	0.83
III	19	14	
Histology (no. of patients)			
Squamous cell carcinoma	20	20	0.13
Adenocarcinoma	32	16	

*Derived from the Mann-Whitney test; other P values were derived using Fisher's exact test. All statistical tests were two-sided.
†Tumor stage was classified according to the International System for Staging of Lung Cancer.

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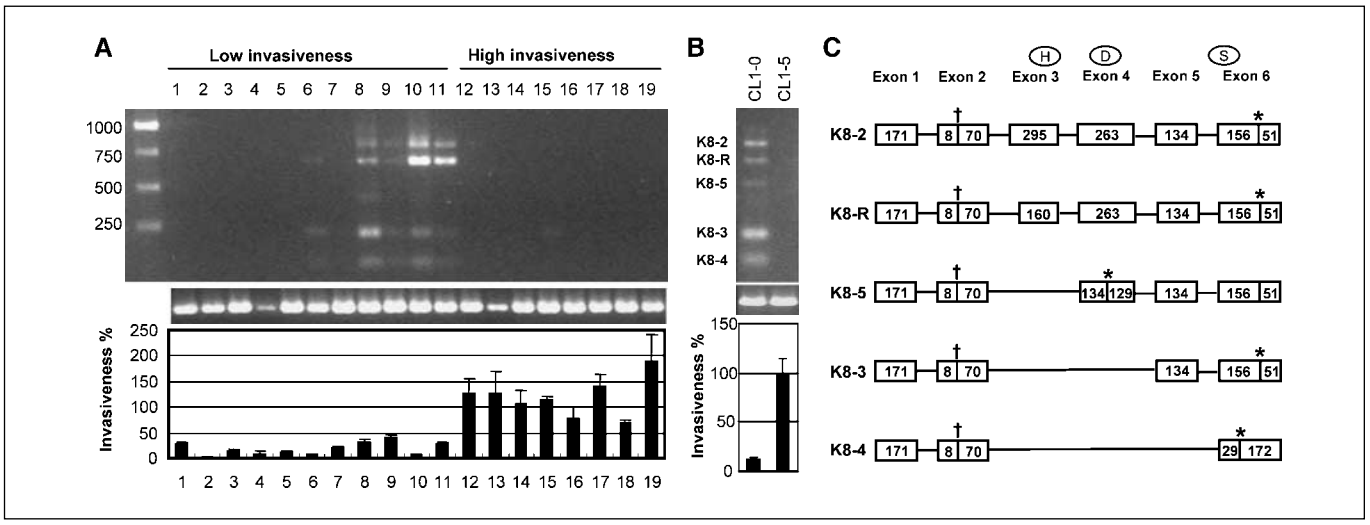

Figure 1. *KLK8* expression profiles in different cancer cell types with different degrees of invasiveness. *A, KLK8* expression was examined in the following cell lines: *1,* SK-BR3; *2,* T47D; *3,* ZR-75-1; *4,* Colo320DM; *5,* Colo320HSR; *6,* WiDr; *7,* LoVo; *8,* OVCAR-3; *9,* A59; *10,* HT1197; *11,* HT1197-4; *12,* Hs578T; *13,* ES2; *14,* PA1; *15,* A59-4; *16,* EJ; *17,* NTUB1; *18,* A549; and *19,* H928. The invasiveness of these cell lines was normalized by setting the invasion rate of the highly invasive CL1-5 lung adenocarcinoma cell line to 100%. This separated the 19 cell lines into two groups: weakly invasive, with an invasiveness of <50% (*1–11*); and highly invasive, with an invasiveness of >50% (*12–19*). *B, KLK8* expression was examined in two lung adenocarcinoma cell lines with different degrees of invasiveness (*CL1-0,* weakly invasive; *CL1-5,* highly invasive). *Top,* expression profile of *KLK8. Middle,* expression of *GAPDH* (internal control). *Bottom,* percentage of invasiveness of the cell lines determined by Matrigel analysis and compared with CL1-5 as 100%. Cell lines 1 to 3 and 12 are breast cancer cell lines; 4 to 7 are colon cancer cell lines; 8, 9, and 13 to 15 are ovarian cancer cell lines; 10, 11, 16, and 17 are bladder cancer cell lines; and 18 and 19 are lung cancer cell lines. *C,* genomic structure of the different *KLK8* splice variants. The exons and the number of nucleotides in each exon are indicated for each splice variant. The *KLK8* gene is composed of six exons and five introns, and the first exon is noncoding. †, location of the start codon; *, location of the stop codon; *H, D,* and *S,* approximate amino acid locations of the characteristic catalytic triad of serine proteases.

These cell lines can be categorized as weakly or highly invasive (Fig. 1*A, bottom*). RT-PCR analysis showed that five cell lines (nos. 6 and 8–11) in the weakly invasive group had relatively high levels of *KLK8* transcripts, whereas only one cell line (no. 15) in the highly invasive group exhibited a residual *KLK8* band (Fig. 1*A, top*). Based on these results, the positive detection rate of *KLK8* in the weakly invasive group was 45% (5 of 11), whereas it was 13% (1 of 8) in the highly invasive group. The quantitative data for *KLK8* expression are shown in Supplementary Table S1.

***KLK8* is overexpressed in weakly invasive lung cancer cells.** To further investigate the association of *KLK8* expression and cancer cell invasion, we examined the production of *KLK8* transcripts in two lung adenocarcinoma cell lines with different degrees of invasiveness: CL1-0 cells, which are weakly invasive, and CL1-5 cells, which are highly invasive. To avoid interference by incompletely spliced RNAs in the nucleus, we isolated cytoplasmic RNA. RT-PCR analysis of the cytoplasmic *KLK8* transcripts revealed five bands in the CL1-0 cell line, but only one faint band in the CL1-5 cell line (Fig. 1*B*). Further sequence analysis revealed that these five bands represent alternative splice variants of the *KLK8* gene (Fig. 1*C*). A detailed description of these five splice variants is provided in the Supplementary Data. Four of these five splice variants of the *KLK8* gene have been detected in ovarian cancer (18). Because the three catalytic residues essential for serine protease activity are located in exons 3, 4, and 6 (19), we chose to examine the function of the K8-2 and K8-R splice variants because they are expected to be catalytically active.

Overexpression of hK8 decreases the invasiveness of lung cancer cells. We examined the function of the K8-2 and K8-R isoforms by overexpressing them in CL1-5 cells. These two isoforms were detected on the Western blot at the expected molecular weights of 34 and 29 kDa, respectively (Supplementary Fig. S1*A*). CL1-5 cells expressing either K8-2 (CL1-5/K8-2) or K8-R (CL1-5/K8-R) had markedly lower invasiveness than CL1-5 cells transfected with an empty vector (CL1-5/Vector) ($P < 0.01$; Fig. 2*A*). We further investigated *KLK8* function using a shRNA targeting *KLK8* (shK8) to inhibit endogenous hK8 expression by CL1-0 cells (Supplementary Fig. S1*B*). As a negative control, the cells were also treated with a luciferase shRNA (shLuc). We found that the invasiveness of CL1-0/shK8 cells was significantly increased compared with that of CL1-0/shLuc cells or CL1-0 cells ($P < 0.01$; Fig. 2*B*). The reciprocal effects, i.e., that hK8 overexpression in CL1-5 cells reduces their invasiveness and that inhibition of hK8 expression in CL1-0 by shK8 increases their invasiveness, shows that hK8 plays a role in the suppression of tumor cell invasion.

hK8 degrades fibronectin and decreases cell adherence. The mouse homologue of hK8, neuropsin, has been reported to have strong proteolytic activity against fibronectin but no or only weak activity against gelatin and collagen types I, III, IV, and VI (20). We suspected that, like neuropsin, hK8 could remodel the extracellular microenvironment by degrading fibronectin. We assayed the protease activity by measuring the degradation of FITC-labeled fibronectin by cells transfected with or without *KLK8* transcripts. To emulate the extracellular degradation of fibronectin, we cultured CL1-5/*KLK8* splice variant cells on coverslips coated with FITC-conjugated human plasma fibronectin and examined them by confocal fluorescence microscopy. We found that CL1-0, CL1-5/K8-2, and CL1-5/K8-R cells degraded FITC-conjugated fibronectin to different extents, as shown by the formation of a dark region under the cells (Fig. 2*C*). Fibronectin was not degraded by the CL1-5/Vector cells. The extent of degradation, quantified by dividing the dark area in the fluorescence image by the area of the cell in the phase contrast image, was 61% for K8-2, 68% for K8-R, and 95% for CL1-0. These results were further confirmed using a cell adhesion assay. Specifically, we found that CL1-5/K8-2 and CL1-5/K8-R cells displayed weaker adherence to fibronectin-coated

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plates than the CL1-5/Vector cells ($P < 0.01$; Fig. 2*D*). The lower degree of adherence by the CL1-5/K8-2 and CL1-5/K8-R cells can be attributed to the degradation of fibronectin.

Gene expression profiling in *KLK8*-transfected cells. We next compared the gene expression profiles of a CL1-5/*KLK8* splice variant and CL1-5/Vector cells using DNA microarray analysis to further investigate the pathways that are affected by hK8-mediated degradation of fibronectin, and which lead to the suppression of cell invasion. We used Kendall's τ correlation coefficient to search for genes with expression profiles most concordant with the relative degree of invasiveness. A theoretical profile was created (Fig. 3*A, top left*) based on the relative invasiveness of the cells (CL1-0 = 0, CL1-5/K8-2 = CL1-5/K8-R = 1, CL1-5/Vector = 2). The expression profiles of the genes (Fig. 3*A, left*) were then sorted according to their concordance between this profile and their Kendall's τ correlation coefficients.

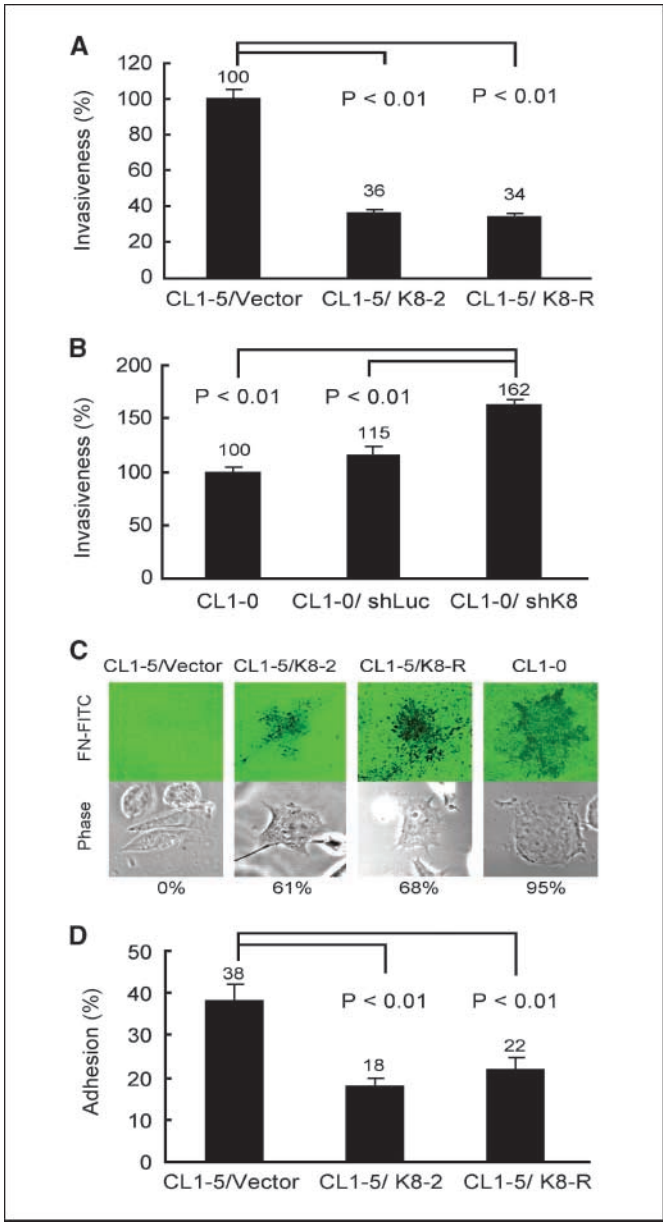

Figure 2. Association of *KLK8* with cancer cell invasiveness. *A, in vitro* Matrigel invasion assay for CL1-5 cells transfected with different splice variants. The invasiveness of the different cell lines was normalized by that of CL1-5/Vector cells. *B, in vitro* Matrigel invasion assay for CL1-0 cells transfected with shLuc or shK8. The invasiveness was normalized by that of CL1-0 cells. *C,* degradation of FITC-conjugated fibronectin coated on coverslips by *KLK8*-transfected cells. The fluorescence images were taken with a confocal microscope. Degraded FITC-conjugated fibronectin (*dark region under the cell*). *D,* CL1-5 cells overexpressing *KLK8* splice variants were assayed for adhesion to a fibronectin-coated substrate.

The genes in the most concordant cluster are listed in Supplementary Table S2, and the probable pathways involved were analyzed using the Ingenuity Pathway Analysis program.[8] This analysis indicated that several signaling pathways, such as those for integrin, phosphatidylinositol 3-kinase/AKT, transforming growth factor-β, extracellular signal-regulated kinase/mitogen-activated protein kinase, VEGF, and Wnt/β-catenin, were down-regulated in the CL1-0, CL1-5/K8-2, and CL1-5/K8-R cells compared with the CL1-5/Vector cells (Supplementary Table S3).

The gene expression profiles related to fibronectin-activated integrin signaling (21) and cell migration (22) were selected from the microarray data (Fig. 3*B*). Close examination of these genes revealed that they were grouped in the most concordant cluster (C1). In the absence of fibronectin, the expression of these genes did not differ from that in CL1-5/Vector cells (Fig. 3*B*). These genes can be grouped into three categories related to cell migration (I, cell polarization; II, protrusion and adhesion formation; and III, rear retraction; ref. 22). We selected a few genes from each of these three categories to verify the microarray results by real-time qPCR. The results were similar to the microarray results shown in Fig. 3*B*, with high Pearson's correlation coefficients (Supplementary Table S4).

To verify whether hK8 is involved in modulating integrin signaling, we examined the phosphorylation of Src, a downstream signaling target in the fibronectin-integrin pathway. The ratio of activated Src [i.e., phosphorylated Src protein (p-Src)] versus total Src protein was lower in CL1-5/K8-2 and CL1-5/K8-R cells than in CL1-5/Vector cells (ratio = 0.59, 0.51, and 0.74, respectively; Fig. 3*C*). Conversely, the p-Src/Src ratio was higher in CL1-0/shK8 cells than in CL1-0 cells (ratio = 0.57 and 0.23, respectively). Both the microarray and the Src protein assay data indicate that hK8 expression blocked fibronectin-activated integrin signaling in cancer cells.

Tumor invasion is associated with dynamic changes in actin polymerization, which is known to play a key role in cell motility (23). To verify that cytoskeletal reorganization signaling pathways are suppressed in K8-expressing cells, we examined the distribution of actin filaments (F-actin) by staining with FITC-conjugated phalloidin, which binds tightly to F-actin but not to free actin monomers (24). As shown in Fig. 3*D*, confocal fluorescence microscopy revealed highly visible filopodia (long, thin, needle-like projections protruding from the cell membrane) along the cell membrane in CL1-5/Vector cells. CL1-5/K8-2 and CL1-5/K8-R cells, however, had few filopodia. These experimental results show that hK8 proteins interfere with the fibronectin-integrin signaling pathways, altering the actin cytoskeleton so that fewer filopodia are produced. This, in turn, reduces the motility of the cells.

***KLK8* overexpression suppresses tumor growth and cancer cell invasion *in vivo*.** To investigate whether hK8 can suppress

[8] http://www.ingenuity.com.

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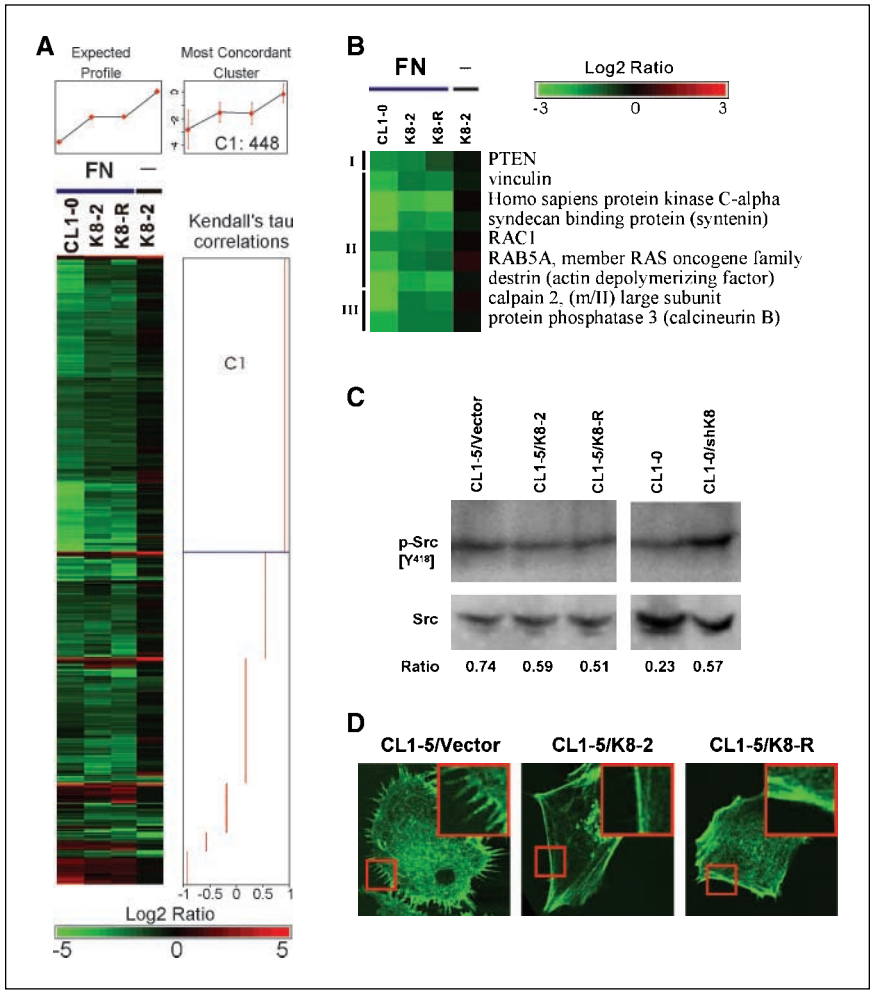

Figure 3. *A,* gene expression profiles clustered by correlation analysis to the expected profile using Kendall's τ correlation coefficients. *Top left,* the expected profile. The most concordant cluster (*C1*) with the expected profile was formed by a cutoff value of 0.91 and resulted in 448 genes. *Top right,* most concordant cluster. *Points,* mean; *bars,* 1 SE. *B,* expression patterns of genes related to fibronectin matrix assembly and cell migration. *Green,* genes with suppressed expression in CL1-0 or CL1-5/*KLK8* splice variant–transfected cells compared with the CL1-5/Vector control cells. The genes can be grouped into three categories: *I,* cell polarization; *II,* protrusion and adhesion formation; and *III,* rear retraction. *C,* effect of hK8 on integrin signaling. Cells were plated on fibronectin for 40 minutes and then lysed. p-Src was detected by Western blotting with antibody to Src phosphorylated on tyrosine. The relative level of p-Src was determined using the ImageJ program by dividing the level of p-Src by the level of total Src protein. *D,* analysis of F-actin and filopodia in cells overexpressing hK8. F-actin was detected with phalloidin-FITC. *Insets,* enlarged images of filopodia along the cell membrane.

cancer cell invasion *in vivo*, we injected SCID mice s.c. with CL1-5/Vector, CL1-5/K8-2, or CL1-5/K8-R cells. The expression of hK8 in s.c. tumors in SCID mice was also confirmed by ELISA and Western blotting (Supplementary Fig. S2*A* and *B*). The mice were monitored for tumor growth every 3 to 4 days (Fig. 4*A*). Over a 17-day period, the tumors produced by CL1-5/Vector cells were significantly larger than those produced by CL1-5/K8-2 or CL1-5/K8-R cells.

We also monitored the intravasation of the s.c. implanted CL1-5/Vector, CL1-5/K8-2, and CL1-5/K8-R cells in the peripheral blood of the mice by the circulating tumor cell assay method (25, 26) using human *Alu* as a marker. Real-time qPCR for human *Alu* showed that circulating tumor cells were not present on day 10; however, on days 13 and 17, more circulating tumor cells were detected in SCID mice bearing CL1-5/Vector tumor cells than mice bearing CL1-5/K8-2 or CL1-5/K8-R tumor cells. After normalizing by the tumor size, the number of circulating tumor cells on the 17th day after implantation was greater in mice implanted with CL1-5/Vector cells than in mice implanted with *KLK8*-expressing tumor cells. These results indicate that hK8 proteins suppress the growth and invasiveness of tumor cells *in vivo*.

Early-stage NSCLC patients with high *KLK8* expression had a lower recurrence rate. We hypothesized that the suppression of invasion by *KLK8* expression retards metastasis and results in a favorable prognosis in cancer. To examine the validity of this hypothesis, we studied *KLK8* expression in specimens from 88 patients with NSCLC using real-time qPCR. The ΔC_T value for the 88 tumor samples ranged from −18.2 to −5.4, with a mean of −13.8. We arbitrarily used the mean value of the ΔC_T to classify patients into low- and high-expression groups. There was no statistically significant association between *KLK8* expression and clinicopathologic variables, such as age, gender, stage, and histologic cell type (Table 1). The time to postoperative recurrence was longer for early-stage patients (stages I and II) with high *KLK8* expression (mean, 49.9 months; 95% confidence interval, 41.4–58.5 months) than for patients with low *KLK8* expression (mean, 22.9 months; 95% confidence interval, 18.6–27.3 months) as shown by the Kaplan-Meier analysis graph in Fig. 5*A*. The same analysis for the late-stage patients (stage III) did not achieve statistical significance.

Further analysis of the percentage of postoperative recurrence showed that the early-stage NSCLC patients with high *KLK8* expression had a significantly lower rate of recurrence than patients in the low *KLK8* expression group at two different follow-ups 6 months apart (P = 0.049 and 0.026 by Fisher's exact test; Fig. 5*B*). For the stage III patients, however, the rate of recurrence at the last follow-up for the high *KLK8* expression group (71%) was not significantly lower than for the low *KLK8* expression group (84%; P = 0.42). The difference in the recurrence rate was marginally significant for the high *KLK8* expression group at 6 months before the last follow-up (74% versus 43%; P = 0.148).

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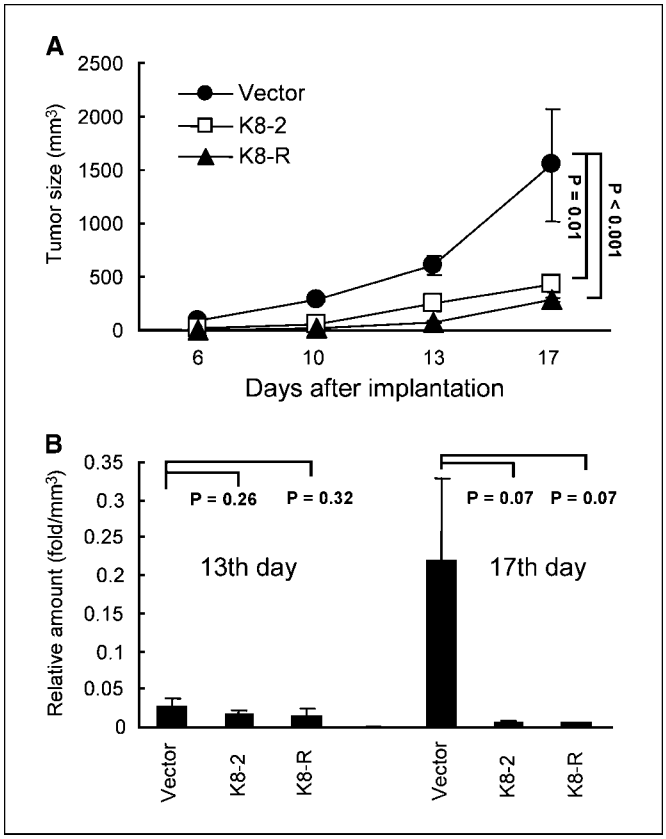

Figure 4. Functional assay of hK8 in an animal model. *A,* tumor volume was measured after s.c. implantation of tumor cells. SCID mice ($n = 4$ for each group) were s.c. injected with 3×10^6 human CL1-5 tumor cells on day 0. *B,* circulating CL1-5 tumor cells were detected by real-time qPCR for human *Alu*. The relative amount of circulating tumor cells was calculated by dividing the qPCR results by those from control mice that were not implanted with tumor cells. The relative amount of circulating tumor cells was normalized by the tumor size. A high amount of circulating tumor cells was detected in SCID mice injected with CL1-5/Vector cells, but a low amount was detected in mice injected with CL1-5/K8-2 or CL1-5/K8-R cells. *Columns,* means of assays performed on the 13th and 17th days after s.c. implantation of tumor cells; *bars,* SD.

These results suggest that *KLK8* expression retards recurrence and that *KLK8* can be used as a prognostic marker in early-stage NSCLC patients.

Discussion

The proteases involved in tumor progression are generally thought to act by degrading ECM proteins to promote tumor invasion; however, some serine proteases have been shown to function as negative regulators of invasion, for example, prostasin, NES1, hepsin, and thrombomodulin (27–29). In this study, we found that hK8 suppresses invasion by degrading fibronectin, thereby remodeling the ECM and modulating tumor cell behavior.

We detected multiple splice variants of *KLK8* in weakly invasive cancer cell lines derived from different tissues (Fig. 1*A*). To test the correlation between paired samples, we used the Wilcoxon test, which is the nonparametric equivalent of the paired sample *t* test. The results of the Wilcoxon test ($P < 0.01$) showed that the expression of *KLK8* is significantly different between the weakly and highly invasive cell lines. On the other hand, the linear correlation coefficient calculation yielded a value of −0.4. These statistical results show that *KLK8* gene expression levels inversely correlate with cancer cell line invasiveness, although the correlation is not linear. The lack of a linear correlation between expression levels and invasiveness is not surprising because these cell lines originate from different tissues and are highly heterogeneous. Therefore, we examined the linearity between hK8 protein expression and invasiveness in a single cell line (CL1-5) transfected with or without *KLK8* (Fig. 2*A*). We found a linear correlation coefficient of −0.92, indicating a strong ($|r| > 0.8$) linear correlation.

Although multiple *KLK8* splice variants were present in the weakly invasive cell lines, we found only two splice variants with the complete catalytic triad (K8-2 and K8-R) to have sufficient protease activity to suppress the invasiveness of lung cancer cells. We also overexpressed the K8-3 splice variant, which contains only one residue of the catalytic triad, in CL1-5 cells. The level of protein expression, however, was low and its ability to suppress invasion

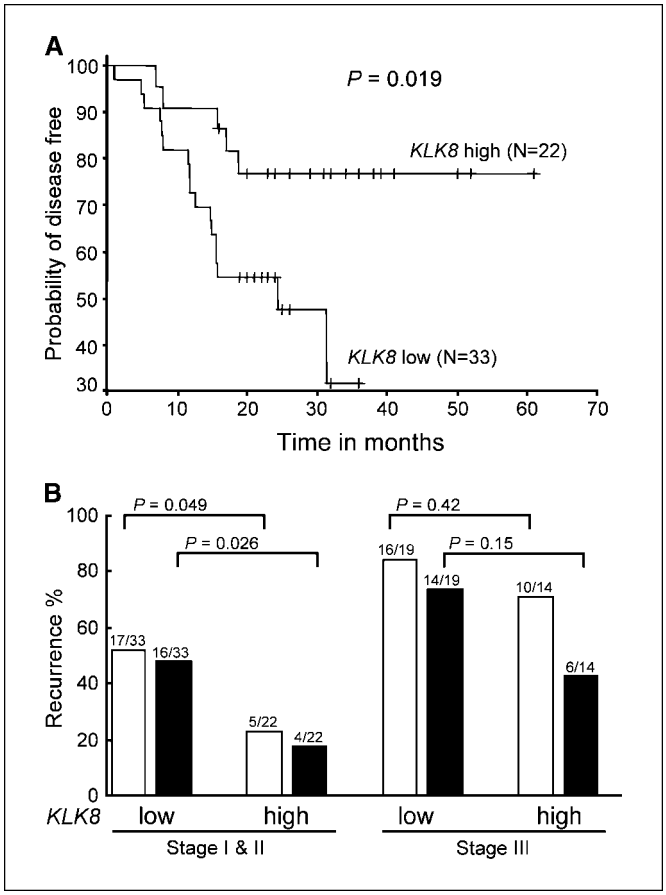

Figure 5. Expression of *KLK8* in tumor tissue specimens from 88 patients with NSCLC. *A,* there is a statistically significant difference in the probability of disease-free survival between the patients with high and low *KLK8* early-stage expression (stages I and II; $P = 0.019$). *Tick marks,* all patients who remained in complete remission as of their last follow-up. *B,* the recurrence rate was monitored at two follow-up times. *Open columns,* recurrence rate calculated at the last follow-up; *filled columns,* recurrence rate 6 months before the last follow-up. For the last follow-up, the recurrence rate for early-stage patients (stages I and II) was 52% (17 of 33) for patients with low *KLK8* expression and 23% (5 of 22) for patients with high *KLK8* expression, whereas the recurrence rate for late-stage patients (stage III) was 84% (16 of 19) for patients with low *KLK8* expression and 71% (10 of 14) for patients with high *KLK8* expression. For the follow-up 6 months earlier, the recurrence for stage I and II patients was 48% (16 of 33) for patients with low *KLK8* expression and 18% (4 of 22) for patients with high *KLK8* expression, whereas the recurrence for stage III patients was 74% (14 of 19) for patients with low *KLK8* expression and 43% (6 of 14) for patients with high *KLK8* expression.

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was negligible (data not shown). Thus, the protease activity of hK8 plays an important role in suppressing cancer cell invasiveness.

Multiple splice variants of *KLK8* were also found in tumor tissues from patients with lung cancer. The mRNA expression profiles were similar, and K8-2 and K8-R were the major splice variants in both tissues and cell lines from patients with lung cancer. Other splice variants of *KLK8* either generated no protein or lacked noticeable protease activity. The finding that patients with stage I and II lung cancer with high *KLK8* expression had a better outcome should be attributed to the expression of splice variants K8-2 and K8-R. Why multiple splice variants are simultaneously expressed in weakly invasive tumor cells and tumor tissues remains to be determined. Regardless, the availability of multiple *KLK8* splice variants allowed us to design *in vitro* and *in vivo* studies to gain insight into how *KLK8* suppresses cancer cell invasion.

Actin plays a key role in various cell motility processes, including the formation of large, broad lamellipodia or spike-like filopodia (30). Filopodia are the first locomotor structures to appear in stimulated migratory cells and act as motors to pull the leading edge of the cell forward. We found that fewer filopodia were present in weakly invasive, *KLK8*-overexpressing cells than in highly invasive, vector-transfected cells. On the basis of the microarray results, the Src protein assay, and the known mechanisms of cytoskeletal reorganization, we propose that *KLK8* splice variants reduce cancer cell invasion by preventing the binding of fibronectin to integrin.

Angiogenesis provides the nutrients and oxygen required for tumor cell growth and is essential for cancer development and growth (31). A key inducer of angiogenesis is VEGF. Our microarray analysis showed that VEGF signaling was down-regulated in cells overexpressing *KLK8*. Furthermore, in the mouse model studies, the *KLK8*-overexpressing tumor cells formed smaller tumors than cells lacking *KLK8* expression (Fig. 4*A*). Because the replication times for CL1-5/Vector, CL1-5/K8-2, and CL1-5/K8-R cells in culture were the same (data not shown) and because cell death was not observed when *KLK8*-overexpressing CL1-5 and CL1-0 cells were cultured in fibronectin, it is plausible that angiogenesis was suppressed in mice bearing CL1-5/K8-2 or CL1-5/K8-R tumor cells. The protein levels of VEGF and the endothelial cell marker CD31 were lower in *KLK8*-overexpressing tumor lysate than in the lysate of CL1-5/Vector tumors (Supplementary Fig. S3). Recently, several ECM protein fragments with potent antiangiogenic properties have been isolated. These antiangiogenic properties were apparent only after proteolytic cleavage of their parental molecules (32). One study (33) showed that fragments of fibronectin were potent inhibitors of endothelial cell growth. It is therefore possible that hK8 inhibits angiogenesis by degrading fibronectin into antiangiogenic fragments.

Phosphatase and tensin homology deleted on chromosome 10 (*PTEN*) has been identified as a tumor-suppressor gene that inhibits cell migration and invasion (34). Mutations of *PTEN* have been identified in a variety of malignancies, and a loss of *PTEN* activity is associated with the invasive and metastatic potential of tumors (35). The highly invasive cell line CL1-5 expressed a *PTEN* transcript with a deletion in exon 5, which is located within the putative phosphatase domain, and encodes a truncated protein. On the other hand, the weakly invasive cell line CL1-0 expressed the wild-type *PTEN* transcript (36). Therefore, we expected that weakly invasive cells would express less mutated *PTEN* than highly invasive cells. The microarray study showed no difference in the expression of *PTEN* in CL1-5 cells with or without *KLK8* expression when they were cultured without fibronectin. In contrast, in cells cultured on fibronectin, those with a higher *KLK8* expression had a lower *PTEN* expression. This indicates that cellular interaction with fibronectin is involved in *PTEN* expression. Fibronectin is known to interact with integrin receptors $\alpha_4\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_1$ (37), and different fibronectin-binding integrins have opposite effects on cell migration/invasion and PTEN expression (38–41). These findings raise new questions about which integrins are involved, and to what extent they participate, in the suppression of invasion by *KLK8*. Addressing these questions will require further studies.

In our study, we found that early-stage (stages I and II) NSCLC patients with higher expression of *KLK8* in their tumor cells have significantly longer remission times and lower rates of recurrence (Fig. 5*A*). Our results also provide a good explanation of why ovarian cancer patients who have detectable levels of *KLK8* mRNA in their cancer tissue (8, 18), or higher concentrations of hK8 in their ascites fluid (42), have a better prognosis.

Figure 5*B* shows that the rate of recurrence was lower for patients with high *KLK8* expression at both early and late stages. Although the Fisher's exact *t* test shows that the *P* values are lower at the earlier follow-up (*filled* versus *open columns*), the results are statistically significant only for the early-stage patients. Table 1 shows that *KLK8* expression does not inversely correlate with the tumor stage in the NSCLC patients. A similar observation was reported in patients with ovarian cancer (18). These observations raise the long-standing question of whether metastasis arises from rare highly metastatic cell variants within the primary tumor or is due to a generic predisposition of the primary tumor (43, 44).

The experiments on clinical specimens were carried out using the bulk population of cells from the primary tumors. It has been shown that cells isolated from metastases are frequently more highly metastatic than the bulk population of cells from primary tumors (45). This may account for the results in Fig. 5*B*, showing that the recurrence was due to previously metastasized cells in stage III patients and that the outcome had less to do with the cancer cells in the primary tumor.

On the other hand, the expression of diagnostic or prognostic markers derived from the predisposition signature of primary tumors did not change with tumor progression (43). This may account for the fact that *KLK8* expression is not lower in later-stage patients than in early-stage patients and that *KLK8* expression did not correlate with tumor stage.

Accumulating evidence indicates that the *KLK* family is a rich source of tumor biomarkers, particularly for hormone-dependent malignancies. *KLK8* is no exception, and it has been reported to be differentially expressed in breast, cervical, and ovary cancer tissues compared with their normal tissue counterparts (7, 46, 47). Our experimental data showed that *KLK8* is also expressed in weakly invasive non–hormone-dependent tumor cells, including lung (CL1-0) and bladder (HT1197) cancer cells. In summary, we provide experimental results from cell line models, animal models, and clinical studies to show that human *KLK8* degrades fibronectin, thereby suppressing tumor cell invasion, which, in turn, retards cancer metastasis and results in a favorable prognosis in early-stage NSCLC.

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