



## High Kallikrein-Related Peptidase 6 in Non-Small Cell Lung Cancer Cells: an indicator of tumor proliferation and poor prognosis



Journal:	<i>Journal of Cellular and Molecular Medicine</i>
Manuscript ID:	JCMM-12-2008-061.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	
Complete List of Authors:	Heuzé-Vourc'h, Nathalie; Faculty of Medicine, INSERM U618 Planque, Chris; Faculty of Medicine, INSERM U618 Guyetant, Serge; Faculty of Medicine, INSERM U618 Coco, Catherine; Faculty of Medicine, INSERM U618 Brillet, Benjamin; Faculty of Medicine, INSERM U618 Bléchet, Claire; Faculty of Medicine, INSERM U618 Parent, Christelle; Faculty of Medicine, INSERM U618 Briollais, Laurent; Mount Sinai Hospital, Samuel Lunenfeld Research Institute Reverdiau, Pascale; Faculty of Medicine, INSERM U618 Jourdan, Marie-Lise; Faculty of Medicine, INSERM U921; CHRU de Tours Courty, Yves; Faculty of Medicine, INSERM U618
Keywords:	NSCLC, Kallikrein related peptidase, KLK6, overexpression, cell proliferation, cell cycle, c-Myc, cyclin, survival

1  
2  
3 Research Paper  
4

5 **High Kallikrein-Related Peptidase 6 in Non-Small Cell Lung Cancer Cells: an indicator**  
6 **of tumor proliferation and poor prognosis**  
7  
8  
9

10  
11  
12 Heuzé-Vourc'h Nathalie<sup>a, #, \*</sup>, Planque Chris<sup>a, #</sup>, Guyetant Serge<sup>a, b</sup>, Coco Catherine<sup>a, b</sup>, Brillet  
13 Benjamin<sup>a</sup>, Blechet Claire<sup>a, b</sup>, Parent Christelle<sup>a</sup>, Laurent Briollais<sup>c</sup>, Reverdiau Pascale<sup>a</sup>,  
14  
15 Jourdan Marie-Lise<sup>b, d</sup> and Courty Yves<sup>a</sup>.  
16  
17  
18

19  
20  
21  
22 Université François-Rabelais de Tours, France (a) INSERM U618 « Protéases et  
23  
24 Vectorisation Pulmonaires »; (b) CHRU de Tours; (d) INSERM U921 « Nutrition,  
25  
26 Croissance et Cancer », F-37000 Tours, France.  
27  
28

29 (c) Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada  
30  
31  
32  
33  
34  
35

36 Footnotes:  
37

38 # These authors contributed equally to this work.  
39  
40  
41  
42

43 \* Correspondance to: Nathalie Heuzé-Vourc'h, Ph D – INSERM U618 – Faculté de  
44 Médecine, 10 Boulevard Tonnellé, F-37032 Tours cedex 1, France. phone: + 33 2 47 36 62  
45  
46 37; Fax: +33 2 47 36 60 46. nathalie.heuze@univ-tours.fr  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

**ABSTRACT**

The human kallikrein-related peptidases (KLK) are serine proteases whose concentrations are often abnormal in common human malignancies and contribute to neoplastic progression through multifaceted roles. However, little attention has been paid to their synthesis and involvement in the development and dissemination of lung cancer, the leading cause of cancer mortality worldwide.

We have analyzed the production of KLK6 in normal lung and tumor tissues from patients with Non-Small Cell Lung Cancer (NSCLC). KLK6 immunoreactivity was restricted to epithelial cells of the normal bronchi, but most of the cancer samples were moderately or highly immunoreactive, regardless of the histological subtype. In contrast, little or no KLK6 was detected in NSCLC cells. We have developed NSCLC lines expressing wild type KLK6 in order to investigate the role of KLK6 in lung cancer biology, and analyzed its impact on proliferation. Ectopic KLK6 dramatically enhanced NSCLC cell growth and KLK6-producing NSCLC cells had accelerated cell cycles, between the G1 and S phases. This was accompanied by a marked increase in cyclin E and decrease in p21. KLK6 production was also associated with enhanced synthesis of c-Myc, which is known to promote cell-cycle progression. Finally, examination of specimens from patients with NSCLC revealed that KLK6 mRNA is overexpressed in tumor tissue, and high KLK6 concentrations were associated with lower survival rates. We conclude that a high concentration of KLK6 is an indicator of tumor proliferation and an independent predictive factor in NSCLC.

**KEY WORDS:** NSCLC, kallikrein related peptidase, KLK6, overexpression, cell proliferation, cell cycle, c-Myc, cyclin, survival

## INTRODUCTION

Human kallikrein-related peptidase 6 (KLK6) belongs to a family of secreted serine proteases encoded by 15 homologous genes (*KLK1-KLK15*) that are important factors in cancer proteolysis. KLK production is abnormal, due to disturbed gene transcription or protein synthesis, or both, in many common human malignancies, highlighting the clinical relevance of KLKs as markers for tumor diagnosis and follow-up [1].

KLK6 is present in a myriad of normal tissues including the brain, breast, salivary gland, colon, pancreas, skin, lung and biological fluids [3-6]. Several studies have found that KLK6 synthesis is abnormal in endocrine-related tumors such as ovarian and uterine carcinomas, and non-cancer diseases, suggesting its utility as a marker [1, 7-10]. The concentration of KLK6 is also increased in human skin diseases and this concentration is correlated with tumor progression [11]. The clinical use of KLK6 for diagnosing gastric and colon cancer has been discussed recently [11, 12]. While the biological role of KLK6 in these tissues has not yet been identified, several studies have shown how KLK6 contributes to various phases of cancer biology. KLK6 might promote tumor invasion by breaking down extracellular matrix components and stimulating cell proliferation [11, 13, 14]. However, the molecular mechanism by which it stimulates proliferation remains unclear. Despite these findings, little work has been done on KLK6 synthesis and function in lung cancer, the most common and the largest single cause of death from cancer worldwide [6].

We have analyzed the concentration and distribution of KLK6 in normal lung tissue and in 46 tissue samples from patients with a primary non-small cell lung cancer (NSCLC). KLK6 was abundant in both adenocarcinoma and squamous cell carcinoma NSCLC subtypes, but there was little or no KLK6 in a panel of NSCLC lines. We therefore investigated the role of KLK6 in lung cancer biology by transfecting NSCLC cells with the native form of KLK6. Ectopic

1  
2  
3 KLK6 production increased NSCLC cell proliferation, due to the acceleration of cell cycle  
4 progression through the G1-S transition via the induction of cyclin E and repression of p21.  
5  
6  
7  
8 KLK6 synthesis was also associated with increased c-Myc production, a key player in cell-  
9 cycle progression. Lastly, a quantitative study of matched tumor and non-tumor specimens  
10 from NSCLC patients revealed an overabundance of KLK6 transcripts that was correlated  
11 with an unfavorable patient outcome. Our findings indicate that KLK6 is involved in lung  
12 cancer proliferation and is an indicator of a poor prognosis.  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24

## 25 MATERIAL AND METHODS

26  
27 **Antibodies.** Polyclonal antibodies against KLK6, cyclin E and p21 were obtained from Santa  
28 Cruz Biotechnology, Inc. Monoclonal antibody against KLK6 was purchased from Serotec  
29 Immunological Excellence. Other antibodies were from Cell Signaling Technology Inc.  
30  
31  
32  
33  
34  
35

36 **Tissue samples and microarray.** Matched tumor and non-tumor tissue samples from forty-  
37 six patients with primary NSCLC (histological diagnosis and clinical history reported in 15  
38 and 16] were used in this study. All investigations were carried out according to Helsinki  
39 principles and French bioethical regulations. Non-necrotic tumor samples, identified by a  
40 pathologist from formalin fixed and paraffin embedded tumor blocks, were used to prepare  
41 tissue microarrays. Frozen tumor samples from the same cohort of patients and matched  
42 tumor and non-tumor tissues from eleven additional patients were used to analyze transcript  
43 production.  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55

56  
57 **Immunohistochemistry.** Immunohistochemical analysis of KLK6 used a monoclonal  
58 antibody and the DakoCytomation EnVision system suitable for rabbit or mouse primary  
59  
60

1  
2  
3 antibodies, according to the manufacturer's instructions. Staining was revealed with 3,3-  
4 diaminobenzidine (Dako Cytomation). Staining intensity and the percentage of stained cells  
5  
6 were graded semi-quantitatively by a pathologist using standard procedures.  
7  
8  
9

10  
11  
12 **Cell lines and culture conditions.** The following human NSCLC cell lines were obtained  
13 from the American Type Culture Collection (ATCC, LGC Promochem, France):  
14 bronchioloalveolar carcinoma cell line A549, squamous cell carcinoma cells H520, Calu-1,  
15 adenocarcinoma cell lines H23, H1838 and H522, and large cell carcinoma line H460. All the  
16 cells were grown in the recommended culture medium. The A549 Flp-In cell line was derived  
17 from A549 cells genetically modified in our laboratory. A549 Flp-In cells contain a unique  
18 recombinase-mediated DNA integration site (FLP Recombination Target (FRT)) at a  
19 transcriptionally active genomic locus and are resistant to zeocin (data not shown). A3-KLK6  
20 and A5-KLK6 are stable clones derived from A549 Flp-In, that express the native form of  
21 KLK6 and are resistant to hygromycin. A549 Flp-In parental or KLK6-expressing cells were  
22 grown at 37 °C in an atmosphere of 5% CO<sub>2</sub> in RPMI-1640 Glutamax I, except Calu-1 that  
23 was cultured in McCoy's 5a medium, containing 10% fetal bovine serum (FBS), 100U/mL  
24 penicillin, 100µg/mL streptomycin and if necessary supplemented with either 100µg/mL  
25 zeocin or 100µg/mL hygromycin.  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47

48 **Plasmids and stable transfections.** The complete coding sequence of KLK6 (144-1002 bp  
49 from NM\_002774) with Stop codon was amplified by PCR and integrated into the T/A  
50 cloning site of pcDNA5/FRT/V5-His TOPO (Invitrogen Corp.), which contains an FRT site.  
51 The sequences of the specific primers are available on request. The expression vector and the  
52 plasmid encoding the yeast Flp recombinase were co-transfected into A549 Flp-In using  
53 Lipofectamine 2000 (Invitrogen Corp.) according to the manufacturer's instructions. Stable  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 clones containing the gene of interest inserted into the genome at the FRT site were selected  
4  
5 and amplified. KLK6 secretion into the supernatants of clones was analyzed by Western  
6  
7 blotting.  
8  
9

10  
11  
12 **Cell proliferation assays.** Cells were seeded ( $2.5 \times 10^3$  cells/well) in 96-well plates and  
13  
14 allowed to grow for 24-96 hours. Cells were harvested at appropriate times and counted using  
15  
16 a Malassez hematimeter.  
17  
18

19  
20  
21  
22 **Cell cycle synchronization.** Cells were seeded ( $4 \times 10^5$  cells/well) in 6-well plated and grown  
23  
24 for 24 hours. They were then synchronized by starving them of serum in RPMI-1640 for 24  
25  
26 hours to arrest the cell cycle in G0-G1 [17]. The medium was then changed to RPMI-1640  
27  
28 containing 5% FCS and 3 mM hydroxyurea for another 24 hours so that the cells accumulated  
29  
30 at the end of G1. These cells were extensively washed and placed in fresh medium containing  
31  
32 5% FCS to continue dividing. Cells were harvested at various times after being released, fixed  
33  
34 in cold methanol and stored at  $-20^\circ\text{C}$ . Cell samples taken at the indicated times were also  
35  
36 processed for cyclin and other cell cycle regulator analysis by Western blotting.  
37  
38  
39  
40  
41  
42

43  
44 **Cell cycle analysis.** The methanol was removed from stored cells and they were treated with  
45  
46 the Coulter DNA-Prep reagents kit (Beckman Coulter). Cell cycle distribution was analyzed  
47  
48 with the Multicycle-AV software (Phoenix Flow Systems) [17].  
49  
50  
51

52  
53 **Protein extraction and Western blotting.** Cells were seeded in 6-well plates ( $5.10^5$   
54  
55 cells/well) or in  $75\text{cm}^2$  flasks and grown in appropriate maintenance cell culture medium for  
56  
57 24 hours. They were then harvested and proteins were extracted. The proteins were loaded on  
58  
59  
60

1  
2  
3 gels for SDS-PAGE and then electro-transferred to PVDF membranes [18]. Immunodetection  
4  
5 was performed according to the antibody manufacturer's instructions.  
6  
7  
8  
9

10 **RNA extraction, RT-PCR and quantitative real-time RT-PCR.** Total RNA was extracted  
11 from NSCLC cells using the RNeasy Minikit from Qiagen Inc. according to the  
12 manufacturer's instructions. Total RNA (2 µg) was reverse transcribed using 200 units of  
13 SuperScript III RNase H Reverse Transcriptase (Invitrogen Corp.) following the  
14 manufacturer's instructions. 1/10 of the cDNA was amplified by PCR using FastStart Taq  
15 DNA Polymerase (Roche Diagnostics) according to the manufacturer's instructions.  
16  
17 Reactions were performed under the following conditions for both KLK6 and β-actin: an  
18 initial denaturation step at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for  
19 30 s, annealing at 55.8°C for 30 s, and extension at 72°C for 30 s. The following primers were  
20 used: KLK6 For 5'-AAG ACA GCA GAT GGT GAT-3'; KLK6 Rev 5'-CTG GCT TCT CCT  
21 TTG AT-3' located on different exons; β-actin For 5'-GGC GGC ACC ACC ATG TAC CCT-  
22 3'; and β-actin Rev 5'-AGG GGC CGG ACT CGT CAT ACT-3'.  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37

38 Total RNA from tissues was extracted and reverse-transcribed, and KLK6 transcripts  
39 quantified by real-time PCR as previously described [15, 16]. The primer sets were designed  
40 to target two exons (KLK6 For 5'- tga tgg tgg tgc tga gt-3'; KLK6 Rev 5'-aca gtg gat gga taa  
41 gga c-3'). The cycling conditions were 95 °C for 5 min, followed by 50 cycles of 95 °C for  
42 10 s, 65 °C for 15 s, and 72 °C for 20 s. Fluorescent product was measured by a single  
43 acquisition mode for 15 s after each cycle. Gene expression was normalized to the amount of  
44 18S rRNA.  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57

58 **Statistical analysis.** The differences in the KLK6 in non-cancerous and tumor tissues were  
59 analyzed with the non-parametric Wilcoxon test for matched samples. The results of  
60



1  
2  
3 proliferation and synchronization assays are expressed as medians and clones and parental  
4 cells are compared using the Kruskal-Wallis non-parametric test. The survival curves for  
5 high-KLK6 and low-KLK6 cells were compared using a log-rank test. Cox proportional  
6 hazards models [19] were also used to assess the difference in the overall survival probability  
7 between groups with high-KLK6 and low-KLK6 after adjustment for clinical-pathological  
8 variables. These variables included gender, age, histological type, tumor size, nodal tumor  
9 status, stage and smoking status (packs/year). We used the Wald test to determine the  
10 statistical significance of the survival difference. A *P*-value below or equal to 0.05 was  
11 considered statistically significant.  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28

## 29 RESULTS

### 30 **KLK6 is present in both normal lungs and NSCLC tissues but not in NSCLC cell lines.**

31  
32  
33 We determined the KLK6 in normal lung tissues and in 46 tumor specimens from patients  
34 with primary NSCLC. There was moderate KLK6 immunoreactivity in the epithelium of the  
35 bronchial tree of normal lungs with the signal concentrated at the apical pole of cells, but type  
36 I and II pneumocytes were negative (figure 1A). There was diffuse cytoplasmic staining for  
37 KLK6 in most of the tumor cells of NSCLC. Semi-quantitative assessment of  
38 immunoperoxidase labeled KLK6 in a tissue micro-array containing 46 tumor samples from  
39 NSCLC patients showed two populations: 25% of cases were low-KLK6 and 75% of the  
40 cases were high-KLK6. Statistical analysis of KLK6 staining scores and clinical-pathological  
41 parameters revealed that KLK6 synthesis was not correlated with either the histological type,  
42 the tumor size, the nodal/tumor status, or tumor stage.  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 However analysis of the KLK6 mRNA in a panel of NSCLC cell lines by RT-PCR revealed  
4 few or no KLK6 transcripts (figure 1B). Similarly, KLK6 protein was not detected in either  
5 the cell lysate or the culture supernatants of those lines that were slightly positive by RT-PCR  
6  
7  
8  
9  
10 (data not shown). Thus, in contrast to tissue specimens, KLK6 was not produced in the cell  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

**Ectopic KLK6 increases the proliferation of NSCLC cells.** As there was little or no KLK6  
in the NSCLC cells we tested, we produced NSCLC lines synthesizing pre-proKLK6 in order  
to investigate further the role of KLK6 in lung cancer biology. The cDNA encoding pre-  
proKLK6 was inserted into the A549 Flp-In by homolog recombination to give stable  
isogenic clones [20]. Two clones that produced KLK6 and the control parental A549 Flp-In  
line were selected for further study (figure 2A). Because previous reports suggest that KLK6  
production influences cell proliferation [11, 12], we assessed the growth of both parental and  
KLK6-producing A549 cells grown at low density for 96 hours. The two clones producing  
KLK6 grew significantly faster than the A549 Flp-In cells after 72 hours in culture (Figure  
2B). The growth of a non-pulmonary line (HEK-293 Flp-In) genetically modified to produce  
KLK6 was similarly enhanced, but that of the same cells producing another KLK was not  
(data not shown).

**Ectopic KLK6 accelerates cell cycle progression in NSCLC lines.** We synchronized cells  
at the end of G1 phase and then released them in the cell cycle to analyze their progression.  
Starvation and hydroxyurea treatment resulted in the arrest of nearly 85% of the cells in G1  
phase (figure 3A). The cell cycle distribution of cells producing KLK6 5 and 8 hours after  
release from hydroxyurea was quite different from that of the control line. Significantly more  
KLK6-producing A549 cells accumulated in the S and G2 phases (Figure 3B), suggesting that

1  
2  
3 the KLK6-producing NSCLC cells passed more rapid through the S and next G2 phases.  
4  
5 Analysis of the cyclins regulating the G1 to S transition by Western blotting revealed that  
6  
7 there was considerably more cyclin E in KLK6-producing cells than in the parental line 5  
8  
9 hours after release of the cell cycle, while the amounts of cyclins D1 and D3 remained the  
10  
11 same in all the cell lines (Figure 3C). There was also a dramatic decrease in the amount of  
12  
13 p21, an inhibitor of cyclin-dependent kinases that limit cell cycle progression, in the KLK6-  
14  
15 producing cells both 5 and 8 hours after release from hydroxyurea.  
16  
17  
18  
19  
20  
21

### 22 **KLK6-dependent enhancement of cell cycle progression might be mediated by c-Myc.**

23  
24 The dramatic changes in the replication rate and cell cycle progression associated with ectopic  
25  
26 KLK6 production indicated that the cell proliferation regulators in KLK6-producing A549  
27  
28 cells were severely disrupted. Therefore, we analyzed the expression of *c-Myc*, a proto-  
29  
30 oncogene that is often constitutively overexpressed in lung cancers and promotes cell cycle  
31  
32 progression by inducing the genes encoding cyclins D2, -E and -A and repressing cell growth  
33  
34 arrest genes like *p21* [21-23]. KLK6-producing cells had markedly more c-Myc protein than  
35  
36 control cells, both when they were grown in complete medium (data not shown) and when  
37  
38 released from hydroxyurea in the cell cycle (figure 3C).  
39  
40  
41  
42  
43  
44  
45

### 46 **High KLK6 expression by NSCLC is associated with poor patient survival.**

47  
48 Because our findings suggest that KLK6 overproduction plays a dramatic role in lung cancer  
49  
50 development by promoting NSCLC cell proliferation, we compared *KLK6* gene expression in  
51  
52 samples of tumor and non-tumor tissues from 57 patients with primary NSCLC. There was  
53  
54 significantly more KLK6 mRNA (median value 23-fold more) in cancer tissue than in normal  
55  
56 lung tissue (Figure 4A). We analyzed the relationship between the amount of KLK6  
57  
58 transcripts and clinicalpathological parameters by univariate analysis. A high KLK6  
59  
60

1  
2  
3 expression was not correlated with any clinicopathological lung tumor variable (data not  
4  
5 shown).  
6

7  
8 We then studied the association between the KLK6 status and overall patient survival using  
9  
10 both Kaplan-Meier survival curves and the Cox proportional hazards regression model. There  
11  
12 was a significant correlation between NSCLC cells with a high concentration of KLK6  
13  
14 transcripts and poor patient survival (figure 4B and C). The hazard ratio measuring the effect  
15  
16 of KLK6 on the overall survival estimated from the Cox model was 5.79, which is highly  
17  
18 significant (p=0.001; 95% CI, 2.147-15.622). The overall survival at 40 months of patients  
19  
20 whose NSCLC cells were low-KLK6 was about 95%, while it was about 60% in the patients  
21  
22 with a high-KLK6 NSCLC. Because the Cox regression model was adjusted for the clinical-  
23  
24 pathological variables, the effect of KLK6 was significantly associated with the overall  
25  
26 survival independently of the other risk factors.  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38

## 39 DISCUSSION

40  
41  
42  
43 The human kallikrein-related peptidases, KLKs, have emerged as multifaceted players in  
44  
45 cancer proteolysis over the past decade. Numerous studies have shown that KLK synthesis  
46  
47 and proteolytic activity is altered in common types of human malignancies [1]. Although most  
48  
49 of the studies focused on hormone-dependent tumors, there is recent evidence that KLK  
50  
51 production is abnormal in other types of human malignancies [1, 11, 12]. We first  
52  
53 demonstrated that several KLKs are produced in the lung and that the synthesis of some of  
54  
55 them is deregulated in NSCLC [15, 16, 24]. However, little is known on the involvement of  
56  
57 the KLK-related peptidases in this disease. Sher et al. reported that KLK8 suppresses tumor  
58  
59  
60

1  
2  
3 growth and invasion in vitro and in vivo, and confers a favorable clinical outcome for patients  
4  
5 with lung cancer [25]. Herein, we examined the KLK6 status and implication in NSCLC. Our  
6  
7 immunohistochemical study on a sizeable cohort of patients with lung cancers shows that  
8  
9 KLK6 is often present in NSCLC, and that its concentration may be moderate or high. In  
10  
11 contrast to Singh et al [6], we detected KLK6 in both adenocarcinoma and squamous cell  
12  
13 carcinomas, the major histological subtypes of NSCLC. This discrepancy might be attributed  
14  
15 to the different anti-KLK6 antibodies used for IHC, or a rare phenotype encountered in the  
16  
17 single adenocarcinoma sample examined by these authors.  
18  
19  
20  
21

22 Strikingly, we did not detect KLK6 in a panel of NSCLC cell lines, suggesting that there is  
23  
24 little or no KLK6 in the lines derived from NSCLC tumors. Deregulation of KLK6 in NSCLC  
25  
26 cell lines might be attributed to multiple mechanisms including genetic alterations  
27  
28 (polymorphisms), hypermethylation in permanent cancer cell lines compared to their primary  
29  
30 counterpart due to the culture conditions, or lack of regulatory factors in the growing medium  
31  
32 as previously described [1, 26, 27]. Recently, hypermethylation of the KLK6 promoter  
33  
34 leading to *KLK6* gene silencing has been described in metastatic breast cancer cell lines  
35  
36 [28]. Moreover, accumulating evidences highlighted the importance of the interactions  
37  
38 between cancer cells and the dynamic microenvironment in which they live (containing  
39  
40 soluble and non soluble components of the extracellular matrix and “activated” non neoplastic  
41  
42 cells) in the regulation of gene expression in cancer cells [26, 29]. Further studies are now  
43  
44 required to elucidate exactly how *KLK6* gene expression is controlled in NSCLC tissues and  
45  
46 cell lines.  
47  
48  
49  
50  
51  
52  
53  
54

55 KLKs have recently been implicated in various phases of cancer. They may act alone or as  
56  
57 part of a proteolytic cascade in which KLKs collaborate with other KLKs and cancer-  
58  
59 associated proteases, to stimulate or inhibit tumor development and dissemination [1, 7, 14,  
60

1  
2  
3 25, 30]. Recent studies on the overexpression of the mouse orthologue *Klk6* gene in  
4  
5 keratinocytes, and the silencing of *KLK6* gene expression in a gastric cancer line, indicate that  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

KLK6 is involved in cell proliferation. In the light of these findings and our results showing the abundance of KLK6 in NSCLC, we postulate that KLK6 contributes to lung cancer development by promoting cell proliferation [11, 12]. This agrees well with our finding that ectopic KLK6 dramatically accelerates NSCLC cell growth. The results from our group and others indicate that both the human and mouse kallikrein 6 orthologues promote cell growth, and this KLK6-mediated proliferation is cell-type independent.

Because the molecular mechanisms underlying the KLK6-mediated enhancement of cell growth is unknown, we investigated the impact of KLK6 synthesis on the progression of NSCLC cell cycle. We found that KLK6-producing cells passed through the S and the next G2 phases faster than the controls, and that they produced more cyclin E, that regulates the G1-S phase transition of the cell-cycle. And the production of ectopic KLK6 by NSCLC was accompanied by a significant decrease in p21, an inhibitor of cyclin-dependent kinases. These kinases are key regulatory enzymes connected to cyclins. Thus, the promotion of cell growth by KLK6 is due to an acceleration of the G1-S transition following the induction of cyclin E and repression of p21. We also found that the synthesis of KLK6 is associated with an increase in c-Myc synthesis by both synchronized cells and cells cultured in standard conditions (data not shown). The *c-Myc* gene belongs to the *MYC* family of oncogenes that are often overexpressed in lung cancers [21]. One of the key functions of c-Myc is to regulate cell-cycle progression by activating cyclin D and E complexes and repressing p21, which governs cell cycle/growth arrest [21-23, 31]. Based on our results, we speculated that KLK6-mediated c-Myc enhancement leads to acceleration of cell cycle progression in lung tumor cells. Further experiments are required to validate this hypothesis, and determine the molecular mechanism associated with higher c-Myc expression.

1  
2  
3 Lung cancer is the leading cause of cancer mortality worldwide, with over one million deaths  
4 each year [32]. Most patients are diagnosed with advanced NSCLC, and the overall 5-year  
5 survival remains dismal (15% all stages included), despite recent advances in multi-modal  
6 therapy. Although a minority of patients present with stage I disease and undergo surgery  
7 alone, 35-50% relapse within 5 years. Therefore, early diagnosis and identification of specific  
8 high-risk patients are crucial for improving the management of patients with NSCLC.  
9 Because the synthesis of KLKs is often abnormal in common types of human malignancies,  
10 numerous studies have focused on the clinical value of KLKs for tumor diagnosis and follow-  
11 up [1]. Several KLKs are approved (like KLK3 or PSA) or emerging markers of endocrine  
12 tumors [1]. Recently, a multiparametric panel of serological KLKs has been proposed for lung  
13 cancer diagnosis [33]. The present report indicates that the *KLK6* gene is markedly  
14 overexpressed in lung cancer tissue, and that high *KLK6* mRNA expression is significantly  
15 correlated with a poor patient outcome, according to both univariate and multivariate analysis.  
16 Moreover, the fact that the effect of *KLK6* remains significant after adjustment for other  
17 clinical-pathological variables indicates that it is an independent prognostic factor. Elevated  
18 *KLK6* expression, both at the mRNA and protein levels, has been previously described as  
19 independent adverse prognostic marker in a subgroup of ovarian carcinomas and in colorectal  
20 and gastric cancers [8, 12].

21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48 In conclusion, we show that *KLK6* is a proliferative factor in NSCLC that might act by  
49 inducing c-Myc synthesis, which in turn modulates specific components of the cell cycle  
50 machinery to promote cell cycle progression. Our present results, together with those from  
51 previous reports showing the influence of *KLK6* on cancer cell migration and invasion [11,  
52 13], suggest that *KLK6* plays a central role in NSCLC development and progression. Finally,  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 high concentrations of KLK6 might be an unfavourable prognosis factor because a high  
4  
5 KLK6 concentration is associated with a poor clinical outcome of patients with NSCLC.  
6  
7  
8  
9  
10  
11

## 12 **ACKNOWLEDGMENTS**

13  
14  
15 We thank Audrey Lorthiois for technical assistance, Dr Sandra Régina for clinical information  
16  
17 support. The English text was edited by Dr Owen Parkes.  
18

19  
20 Financial Supports: INSERM ; Ligue Contre le Cancer Région Centre ; IFR 135 « Imagerie  
21  
22 Fonctionnelle » ; Association pour la Recherche sur le Cancer, grant n° 7935 ; Région Centre,  
23  
24 grant KalliCap. Chris Planque benefited a fellowship from the Région Centre and INSERM.  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60



## REFERENCES

1. **Borgoño CA, Diamandis EP.** The emerging roles of human tissue kallikreins in cancer. *Nat Rev Cancer.* 2004; 4: 876-90
2. **Hutchinson S, Luo LY, Yousef GM, Soosaipillai A, Diamandis EP.** Purification of human kallikrein 6 from biological fluids and identification of its complex with alpha(1)-antichymotrypsin. *Clin Chem.* 2003; 49: 746-51.
3. **Petraki CD, Karavana VN, Skoufogiannis PT, Little SP, Howarth DJ, Yousef GM, Diamandis EP.** The spectrum of human kallikrein 6 (zyme/protease M/neurosin) expression in human tissues as assessed by immunohistochemistry. *J Histochem Cytochem.* 2001; 49: 1431-41.
4. **Petraki CD, Papanastasiou PA, Karavana VN, Diamandis EP.** Cellular distribution of human tissue kallikreins: immunohistochemical localization. *Biol Chem.* 2006; 387: 653-63.
5. **Shaw JL, Diamandis EP.** Distribution of 15 human kallikreins in tissues and biological fluids. *Clin Chem.* 2007; 53: 1423-32.
6. **Singh J, Naran A, Misso NL, Rigby PJ, Thompson PJ, Bhoola KD.** Expression of kallikrein-related peptidases (KRP/hK5, 7, 6, 8) in subtypes of human lung carcinoma. *Int Immunopharmacol.* 2008; 8: 300-6.
7. **Paliouras M, Diamandis EP.** The kallikrein world: an update on the human tissue kallikreins. *Biol Chem.* 2006; 387: 643-52.
8. **Hoffman BR, Katsaros D, Scorilas A, Diamandis P, Fracchioli S, Rigault de la Longrais IA, Colgan T, Puopolo M, Giardina G, Massobrio M, Diamandis EP.** Immunofluorometric quantitation and histochemical localisation of kallikrein 6 protein in ovarian cancer tissue: a new independent unfavourable prognostic biomarker. *Br J Cancer.* 2002; 87: 763-71.
9. **Santin AD, Diamandis EP, Bellone S, Soosaipillai A, Cane S, Palmieri M, Burnett A, Roman JJ, Pecorelli S.** Human kallikrein 6: a new potential serum biomarker for uterine serous papillary cancer. *Clin Cancer Res.* 2005 ; 11: 3320-5.
10. **Zarghooni M, Soosaipillai A, Grass L, Scorilas A, Mirazimi N, Diamandis EP.** Decreased concentration of human kallikrein 6 in brain extracts of Alzheimer's disease patients. *Clin Biochem.* 2002; 35: 225-31.
11. **Klucky B, Mueller R, Vogt I, Teurich S, Hartenstein B, Breuhahn K, Flechtenmacher C, Angel P, Hess J.** Kallikrein 6 induces E-cadherin shedding and promotes cell proliferation, migration, and invasion. *Cancer Res.* 2007; 67: 8198-206.
12. **Nagahara H, Mimori K, Utsunomiya T, Barnard GF, Ohira M, Hirakawa K, Mori M.** Clinicopathologic and biological significance of kallikrein 6 overexpression in human gastric cancer. *Clin Cancer Res.* 2005; 11: 6800-6.

- 1  
2  
3 13. **Ghosh MC, Grass L, Soosaipillai A, Sotiropoulou G, Diamandis EP.** Human kallikrein  
4 6 degrades extracellular matrix proteins and may enhance the metastatic potential of tumour  
5 cells. *Tumour Biol.* 2004; 25: 193-9.
- 6  
7  
8 14. **Klokk TI, Kilander A, Xi Z, Waehre H, Risberg B, Danielsen HE, Saatcioglu F.**  
9 Kallikrein 4 is a proliferative factor that is overexpressed in prostate cancer. *Cancer Res.*  
10 2007; 67: 5221-30.
- 11  
12  
13 15. **Planque C, de Monte M, Guyétant S, Rollin J, Desmazes C, Panel V, Lemarié E,**  
14 **Courty Y.** KLK5 and KLK7, two members of the human tissue kallikrein family, are  
15 differentially expressed in lung cancer. *Biochem Biophys Res Commun.* 2005; 329: 1260-6.
- 16  
17  
18 16. **Planque C, Aïnciburu M, Heuzé-Vourc'h N, Régina S, de Monte M, Courty Y.**  
19 Expression of the human kallikrein genes 10 (KLK10) and 11 (KLK11) in cancerous and non-  
20 cancerous lung tissues. *Biol Chem.* 2006; 387: 783-8.
- 21  
22  
23 17. **Barascu A, Besson P, Le Floch O, Bougnoux P, Jourdan ML.** CDK1-cyclin B1  
24 mediates the inhibition of proliferation induced by omega-3 fatty acids in MDA-MB-231  
25 breast cancer cells. *Int J Biochem Cell Biol.* 2006; 38: 196-208.
- 26  
27  
28 18. **Heuzé-Vourc'h N, Liu M, Dalwadi H, Baratelli FE, Zhu L, Goodglick L, Pöld M,**  
29 **Sharma S, Ramirez RD, Shay JW, Minna JD, Strieter RM, Dubinett SM.** IL-20, an anti-  
30 angiogenic cytokine that inhibits COX-2 expression. *Biochem Biophys Res Commun.* 2005;  
31 333: 470-5.
- 32  
33  
34 19. **Cox DR.** Regression models with life tables. *J of the Royal Statistical Society.* 1972; 34:  
35 187-220.
- 36  
37  
38 20. **Heuzé-Vourc'h N, Aïnciburu M, Planque C, Brillard-Bourdet M, Ott C, Jolivet-**  
39 **Reynaud C, Courty Y.** Recombinant kallikrein expression: site-specific integration for hK6  
40 production in human cells. *Biol Chem.* 2006; 387: 687-95.
- 41  
42  
43 21. **Zajac-Kaye M.** Myc oncogene: a key component in cell cycle regulation and its  
44 implication for lung cancer. *Lung Cancer.* 2001; 34: S43-6.
- 45  
46  
47 22. **Pelengaris S, Khan M, Evan G.** c-MYC: more than just a matter of life and death. *Nat*  
48 *Rev Cancer.* 2002; 2: 764-76.
- 49  
50  
51 23. **Gartel AL, Shchors K.** Mechanisms of c-myc-mediated transcriptional repression of  
52 growth arrest genes. *Exp Cell Res.* 2003; 283: 17-21.
- 53  
54  
55 24. **Planque C, Bléchet C, Ayadi-Kaddour A, Heuzé-Vourc'h N, Dumont P, Guyétant S,**  
56 **Diamandis EP, El Mezni F, Courty Y.** Quantitative RT-PCR analysis and  
57 immunohistochemical localization of the kallikrein-related peptidases 13 and 14 in lung. *Biol*  
58 *Chem.* 2008; 389: 781-6
- 59  
60  
61 25. **Sher YP, Chou CC, Chou RH, Wu HM, Wayne Chang WS, Chen CH, Yang PC, Wu**  
62 **CW, Yu CL, Peck K.** Human kallikrein 8 protease confers a favorable clinical outcome in  
63 non-small cell lung cancer by suppressing tumor cell invasiveness. *Cancer Res.* 2006; 66:  
64 11763-70.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60
26. **Li B, Goyal J, Dhar S, Dimri G, Evron E, Sukumar S, Wazer DE, Band V.** CpG methylation as a basis for breast tumor-specific loss of NES1/kallikrein 10 expression. *Cancer Res.* 2001; 61: 8014-21.
27. **Smiraglia DJ, Rush LJ, Frühwald MC, Dai Z, Held WA, Costello JF, Lang JC, Eng C, Li B, Wright FA, Caligiuri MA, Plass C.** Excessive CpG island hypermethylation in cancer cell lines versus primary human malignancies. *Hum Mol Genet.* 2001;10:1413-9.
28. **Pampalakis G, Sotiropoulou G.** Multiple mechanisms underlie the aberrant expression of the human kallikrein 6 gene in breast cancer. *Biol Chem.* 2006; 387: 773-82.
29. **Tlsty TD, Coussens LM.** Tumor stroma and regulation of cancer development. *Annu Rev Pathol.* 2006;1:119-50.
30. **Pampalakis G, Sotiropoulou G.** Tissue kallikrein proteolytic cascade pathways in normal physiology and cancer. *Biochim Biophys Acta.* 2007; 1776: 22-31.
31. **Blagosklonny MV, Pardee AB.** The restriction point of the cell cycle. *Cell Cycle.* 2002; 1: 103-10.
32. **Parkin DM, Bray F, Ferlay J, Pisani P.** Global cancer statistics, 2002. *CA Cancer J Clin.* 2005 ; 55: 74-108.
33. **Planque C, Li L, Zheng Y, Soosaipillai A, Reckamp K, Chia D, Diamandis EP, Goodglick L.** A multiparametric serum kallikrein panel for diagnosis of non-small cell lung carcinoma. *Clin Cancer Res.* 2008; 14: 1355-62.

**FIGURE LEGENDS**

**Figure 1. KLK6 in tissues from patients with NSCLC but not in NSCLC-derived cell lines.** *A*, Immunohistochemistry of KLK6 in normal and malignant lung from 46 patients with primary NSCLC using a monoclonal antibody (original magnification: X200; hematoxylin counterstain). KLK6 immunostaining (dark) in the apical region of normal bronchial epithelial cells (thin arrow) and in the cytoplasm of most cancer cells (large arrow). *B*, KLK6 production in a panel of NSCLC lines by RT-PCR. Total RNA from NSCLC cell lines was extracted and reverse-transcribed. KLK6 and  $\beta$ -actin (internal control) mRNAs were amplified from cDNA by PCR and the amplification products were separated on a 1% agarose gel containing ethidium bromide. 1. A549; 2. Calu-1; 3. H23; 4. H460; 5. H520; 6. H522; 7. H1838; +: plasmid containing KLK6 cDNA.

**Figure 2. Ectopic KLK6 in NSCLC promotes proliferation.** *A*, Secretion of KLK6 by A549 Flp-In and stable isogenic clones (A3- and A5-KLK6) assessed by Western blotting using a polyclonal anti-KLK6 antibody. Equal volumes of cell culture supernatant were loaded onto 12% SDS-PAGE gels. *B*, Control A549 Flp-In (dashed line) and A3-KLK6 (black full line) and A5-KLK6 (grey full line) ( $2.5 \times 10^3$  cells of each) were seeded and cells were counted at the indicated times. *Points*, median value of triplicate determinations from one representative experiment (of three). *Bars*, lower and upper quartiles. *P* values were determined using a Kruskal & Wallis test. \*  $P < 0.05$ .

**Figure 3. Effect of KLK6 on NSCLC cell growth and regulation of cell cycle progression.** *A*, Distribution of control A549 Flp-In and A3-KLK6 and A5-KLK6 cells within the G1 (white), S (grey) and G2 (black) phases of the cell cycle 0, 5 or 8 hours after release from hydroxyurea. Results are expressed as the median values of five independent experiments. *B*,

1  
2  
3 Box plot of control A549 Flp-In and A3-KLK6 and A5-KLK6 cells passed from G1 into  
4  
5 G2+S phases 5 hours and 8 hours after release from hydroxyurea. Results are representative  
6  
7 of five independent experiments. *P* values were determined using a Kruskal & Wallis test. \*  
8  
9 *P* < 0.05, \*\* *P* < 0.025. *C*, Assay of cell-cycle proteins and c-Myc by Western blotting of  
10  
11 whole-cell extracts (50µg) from the control A549 Flp-In, A3-KLK6 and A5-KLK6 lines 5 and  
12  
13 8 hours after release from hydroxyurea. Actin protein was used to check for equal loading.  
14  
15 Results are from one of three experiments.  
16  
17  
18  
19  
20  
21

22 **Figure 4. KLK6 overexpression in tumor tissues from patients with NSCLC, and patient**  
23 **survival.** *A*, Box plot of KLK6 transcript concentration by quantitative real-time RT-PCR in  
24  
25 57 tumors and matched non tumor samples from patients with primary NSCLC undergoing an  
26  
27 initial surgical resection of the lung tumor (aged 44–83 years, median: 65). Gene expression  
28  
29 was normalized to the amount of 18S rRNA and is reported in arbitrary units. Median values  
30  
31 are indicated in italics. The difference between the concentration of KLK6 transcripts in non-  
32  
33 tumor and tumor tissues was determined by a Wilcoxon test for matched pairs. *B*, Kaplan-  
34  
35 Meier curves for overall survival of patients with high and low concentrations of KLK6  
36  
37 mRNA in lung tumor tissues. The optimal cut-off value for survival analysis was identified by  
38  
39 the  $\chi^2$  test, based on the ability of KLK6 to predict the overall survival of the population  
40  
41 studied. Using this cutoff, KLK6 mRNA concentration was categorized as high or low. A  
42  
43 cutoff of 600 (in arbitrary units) for KLK6 mRNA was equal to the 56th percentile. *C*,  
44  
45 Survival curves obtained from Cox regression models after adjusting for gender, age,  
46  
47 histological type, tumor size, nodal tumor status, stage and smoking status (packs/ year).  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60



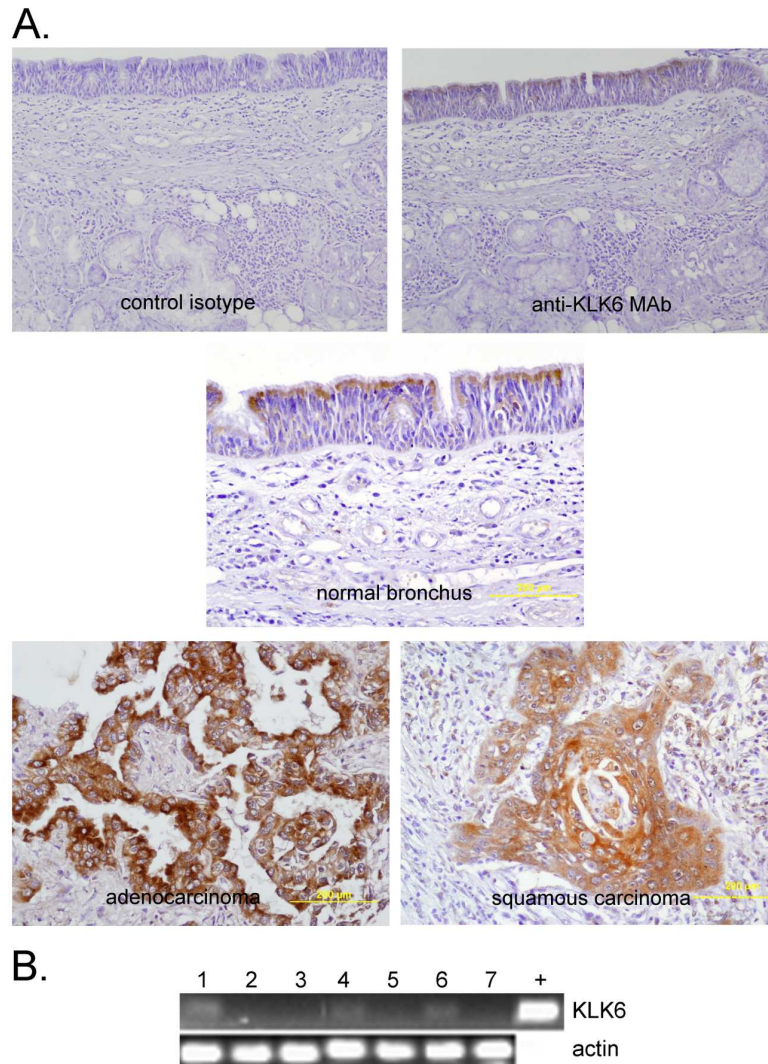
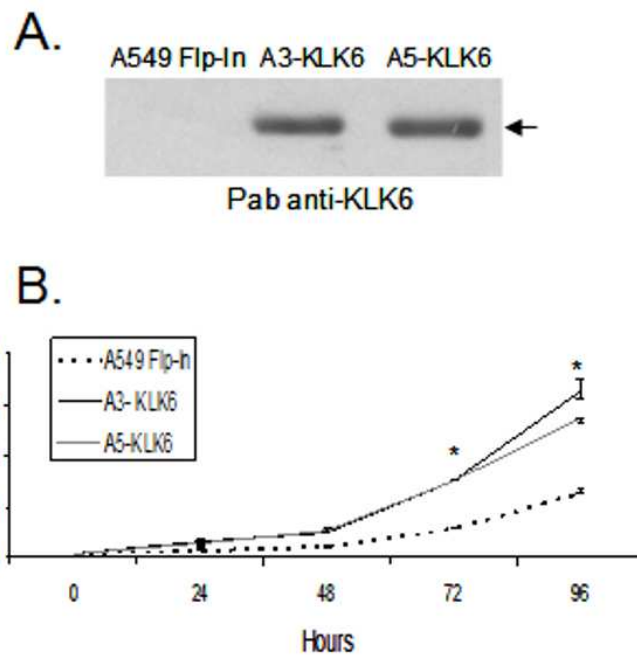


Figure 1. KLK6 in tissues from patients with NSCLC but not in NSCLC-derived cell lines. A, Immunohistochemistry of KLK6 in normal and malignant lung from 46 patients with primary NSCLC using a monoclonal antibody (original magnification: X200; hematoxylin counterstain). KLK6 immunostaining (dark) in the apical region of normal bronchial epithelial cells (thin arrow) and in the cytoplasm of most cancer cells (large arrow). B, KLK6 production in a panel of NSCLC lines by RT-PCR. Total RNA from NSCLC cell lines was extracted and reverse-transcribed. KLK6 and  $\beta$ -actin (internal control) mRNAs were amplified from cDNA by PCR and the amplification products were separated on a 1% agarose gel containing ethidium bromide. 1. A549; 2. Calu-1; 3. H23; 4. H460; 5. H520; 6. H522; 7. H1838; +: plasmid containing KLK6 cDNA.

109x170mm (300 x 300 DPI)



33 Figure 2. Ectopic KLK6 in NSCLC promotes proliferation. A, Secretion of KLK6 by A549 Flp-In and  
 34 stable isogenic clones (A3- and A5-KLK6) assessed by Western blotting using a polyclonal anti-KLK6  
 35 antibody. Equal volumes of cell culture supernatant were loaded onto 12% SDS-PAGE gels. B,  
 36 Control A549 Flp-In (dashed line) and A3-KLK6 (black full line) and A5-KLK6 (grey full line)  
 37 ( $2.5 \times 10^3$  cells of each) were seeded and cells were counted at the indicated times. Points, median  
 38 value of triplicate determinations from one representative experiment (of three). Bars, lower and  
 39 upper quartiles. P values were determined using a Kruskal & Wallis test. \*  $P < 0.05$ .  
 40 72x52mm (300 x 300 DPI)

41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

NEW

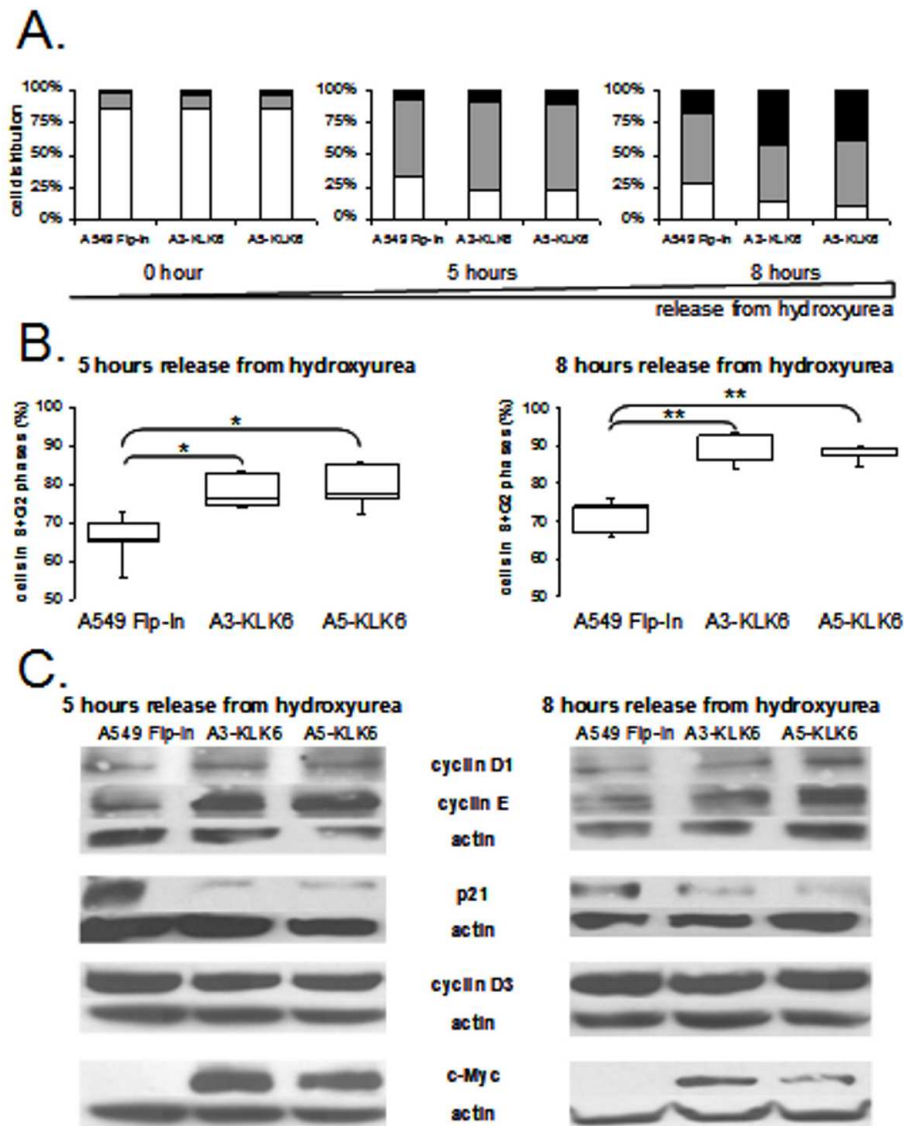


Figure 3. Effect of KLK6 on NSCLC cell growth and regulation of cell cycle progression. A, Distribution of control A549 Flp-In and A3-KLK6 and A5-KLK6 cells within the G1 (white), S (grey) and G2 (black) phases of the cell cycle 0, 5 or 8 hours after release from hydroxyurea. Results are expressed as the median values of five independent experiments. B, Box plot of control A549 Flp-In and A3-KLK6 and A5-KLK6 cells passed from G1 into G2+S phases 5 hours and 8 hours after release from hydroxyurea. Results are representative of five independent experiments. P values were determined using a Kruskal & Wallis test. \*  $P < 0.05$ , \*\*  $P < 0.025$ . C, Assay of cell-cycle proteins and c-Myc by Western blotting of whole-cell extracts (50 $\mu$ g) from the control A549 Flp-In, A3-KLK6 and A5-KLK6 lines 5 and 8 hours after release from hydroxyurea. Actin protein was used to check for equal loading. Results are from one of three experiments.

109x129mm (300 x 300 DPI)



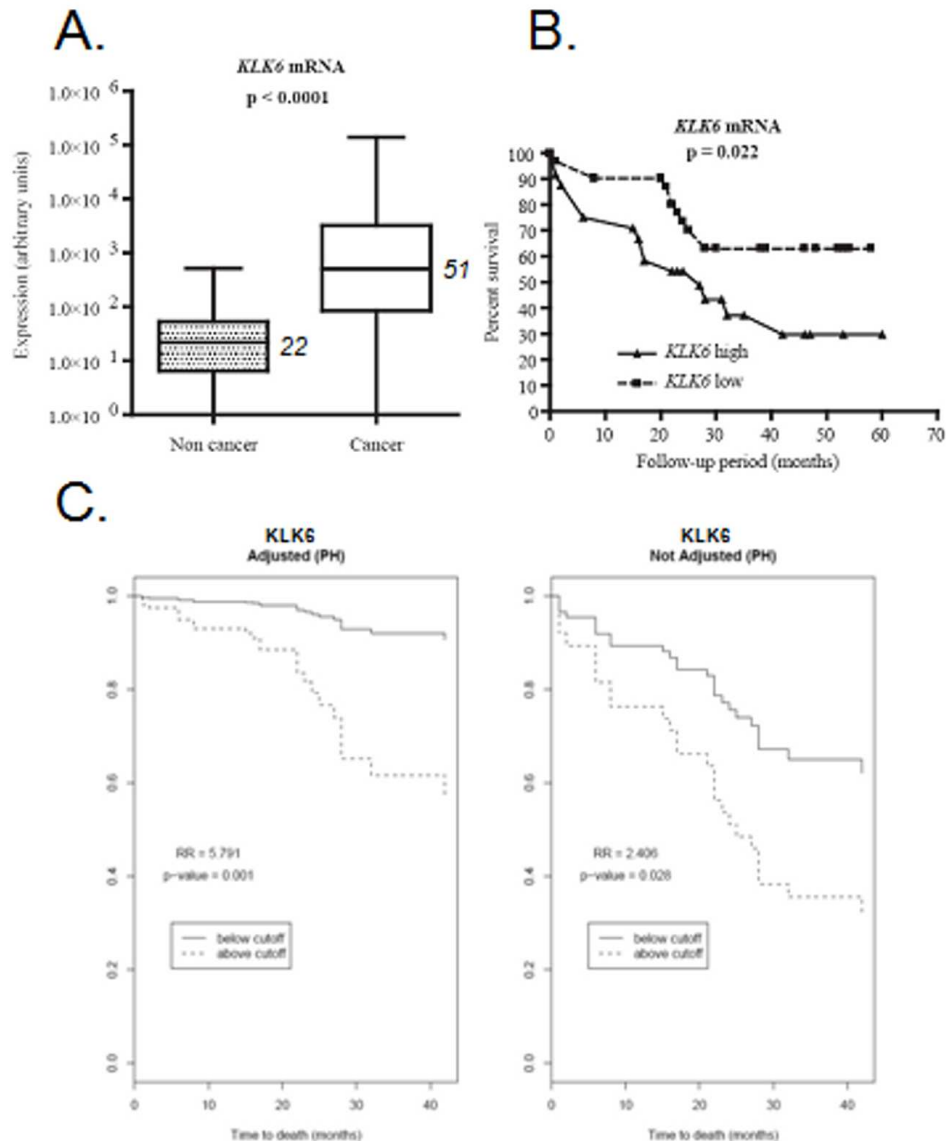


Figure 4. KLK6 overexpression in tumor tissues from patients with NSCLC, and patient survival. A, Box plot of KLK6 transcript concentration by quantitative real-time RT-PCR in 57 tumors and matched non tumor samples from patients with primary NSCLC undergoing an initial surgical resection of the lung tumor (aged 44–83 years, median: 65). Gene expression was normalized to the amount of 18S rRNA and is reported in arbitrary units. Median values are indicated in italics. The difference between the concentration of KLK6 transcripts in non-tumor and tumor tissues was determined by a Wilcoxon test for matched pairs. B, Kaplan-Meier curves for overall survival of patients with high and low concentrations of KLK6 mRNA in lung tumor tissues. The optimal cut-off value for survival analysis was identified by the  $\chi^2$  test, based on the ability of KLK6 to predict the overall survival of the population studied. Using this cutoff, KLK6 mRNA concentration was categorized as high or low. A cutoff of 600 (in arbitrary units) for KLK6 mRNA was equal to the 56th percentile. C, Survival curves obtained from Cox regression models after adjusting for gender, age,

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

histological type, tumor size, nodal tumor status, stage and smoking status (packs/ year).  
109x140mm (300 x 300 DPI)

For Peer Review