Epigenetic activation of human kallikrein 13 enhances malignancy of lung adenocarcinoma by promoting N-cadherin expression and laminin degradation

Ruey-Hwang Chou [a,b,c,*], Sheng-Chieh Lin [a,d], Hui-Chin Wen [a,1], Cheng-Wen Wu [a,d,e], and Wun-Shaing Wayne Chang [a,d,*]

[a] *National Institute of Cancer Research, National Health Research Institutes, Miaoli 35053, Taiwan*

[b] *Center for Molecular Medicine, China Medical University Hospital, Taichung 40447, Taiwan*

[c] *Department of Biotechnology, Asia University, Taichung 41354, Taiwan*

[d] *College of Life Science, National Tsing Hua University, Hsinchu 30013, Taiwan*

[e] *Institute of Biochemistry and Molecular Biology, National Yang Ming University, Taipei 11221, Taiwan*

***** Corresponding authors' Address: National Institute of Cancer Research, National Health Research Institutes, No. 35, Keyan Road, Zhunan Town, Miaoli 35053, Taiwan. Fax: +886-37-586463 (W.S.W. Chang); Center for Molecular Medicine, China Medical University Hospital, No. 6, Hsueh-Shih Road, Taichung 40447, Taiwan. Fax: +886-4-22333496 (R.H. Chou)

E-mail: wayne@nhri.org.tw (W.S.W. Chang); rhchou@gmail.com (R.H. Chou)

[1] Present address: Institute of Cellular and System Medicine, National Health Research Institutes, Taiwan

Abbreviations: KLK: kallikrein; ECM: extracellular matrix; shRNA: small hairpin RNA

ABSTRACT

The tissue kallikrein (*KLK*) family contains 15 genes (*KLK1-KLK15*) tandemly arranged on chromosome 19q13.4 that forms the largest cluster of contiguous protease genes in the human genome. Here, we provide mechanistic evidence showing that the expression of *KLK13*, one of the most recently identified family members, is significantly up-regulated in metastatic lung adenocarcinoma. Whilst overexpression of *KLK13* resulted in an increase in malignant cell behavior; knockdown of its endogenous gene expression caused a significant decrease in cell migratory and invasive properties. Functional studies further demonstrated that *KLK13* is activated via demethylation of its upstream region. The elevated KLK13 protein then enhances the ability of tumor cells to degrade extracellular laminin that, subsequently, facilitates cell metastatic potential in the *in vivo* SCID mouse xenograft model. *KLK13* was also found to induce the expression of N-cadherin to help promote tumor cell motility. Together, these results reveal the enhancing effects of *KLK13* on tumor cell invasion and migration, and that it may serve as a diagnostic/prognostic marker and a potential therapeutic target for lung cancer.

Keywords: kallikrein, CpG methylation, invasion, migration, lung cancer

1. Introduction

Based on the currently available genome sequences, there are approximately 2-5% of the genes estimated to encode for proteolytic regulators, irrespectively of the organism source [1]. In the human genome alone, more than 500 genes are thought to function as proteolytic enzymes. Among these enzymes, the tissue kallikrein (*KLK*) family contains 15 genes (*KLK1-KLK15*) tandemly arranged on chromosome 19q13.4 that forms the largest cluster of contiguous protease genes in the genome. These *KLKs* are known to participate in a wide range of biological functions and pathological processes including inflammation, hypertension and pancreatitis [2]. In the disease of cancer, the differential expressions of some *KLK* family members have been detected and used as markers for the screening, diagnosis and management of hormone-dependent cancers, such as *KLK3* (commonly known as prostate specific antigen, PSA) and *KLK2* for prostate and breast cancers, *KLK6* and *KLK10* for ovarian cancer, *KLK11* for ovarian and prostate cancers [3], and *KLK13* for breast [4,5] and ovarian cancers [6,7,8]. However, much less is known about the roles and functions of kallikrein proteases in non-hormone-dependent cancers, such as lung cancer.

Lung cancer is the worldwide leading cause of cancer mortality, comprising of 23% and 11% of the total cancer death in males and females, respectively [9]. Its five-year survival rate is only ~16%, with more than half of patients diagnosed in advanced stages with distant metastasis and worse prognosis [10]. Therefore, early diagnosis and prompt treatment are of utmost importance in lung cancer research. To date, only a very few studies have attempted to characterize the differential expression patterns of *KLK* genes in lung cancer. For example, *KLK5* was shown up-regulated whereas *KLK7* was down-regulated in lung cancer tissues [11]. An alternate transcript variant of *KLK8* was also reported to be an independent predictor of favorable prognosis in lung cancer [12]. A more recent study further revealed that *KLK13*, one of the newly identified *KLK* members, was closely associated with the malignancy of

non-small-cell lung carcinoma [13]. Here, we provide mechanistic evidence showing that during tumor progression, *KLK13* is epigenetically up-regulated by aberrant hypomethylation. The elevated KLK13 protease then enhances the ability of malignant cells to degrade extracellular laminin and to promote N-cadherin expression that, together, facilitate the metastatic potentials of lung cancer cells.

2. Materials and methods

2.1 Cell culture, RNA extraction and RT-PCR

Human normal lung cell lines (IMR-90, WI-38 and BEAS-2B) and lung adenocarcinoma cell lines (CL1-0, CL1-3, and CL1-5) were cultured, respectively, in MEM and RPMI-1640 media supplemented with 10% fetal bovine serum (Thermo Scientific HyClone, Logan, UT, USA) at 37°C in a humidified 5% CO_2 atmosphere. Total RNA was extracted using Trizol regent and cDNA was generated by M-MLV reverse transcriptase and oligo(dT) 12-18 primers as described by the manufacturer (Invitrogen Life Technologies, Carlsbad, CA, USA). The PCR was carried out in a thermocycler (Biometra, Göttingen, Germany) and conducted in a 25-μl reaction volume containing 0.2 μM of each primer, 200 μM dNTP, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 1 U Taq DNA polymerase (TaKaRa, Kyoto, Japan), and 1 μl cDNA under the following conditions: 1 cycle of 94°C for 2 min; 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec; and 1 cycle of 72°C for 10 min. The primers were detailed in supplementary Table I.

2.2 Gene cloning and stable transfection

The full-length coding sequence of *KLK13* was amplified from human keratinocyte matchmaker cDNA library (BD Biosciences Clontech, Palo Alto, CA, USA) and subsequently cloned into pCDNA3-HA vector (Invitrogen Life Technologies) with HindIII

and KpnI. The *KLK13* construct was transfected into the desired cell lines with Lipofectamine 2000 (Invitrogen Life Technologies) and, after 24 h of transfection, cells were sub-cultured at a 1:10 dilution in growth medium. Culture medium containing 0.5 mg/ml G418 was then used to select stable transfectants.

2.3 Prediction and bisulfite sequencing of the CpG sites of the KLK13 promoter region

Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA). One μg of purified genomic DNA was then modified with sodium bisulfite by the CpGenome DNA modification kit (Millipore, Billerica, MA, USA) under alkali condition at 50°C for 16 h. The potential GC-rich region of *KLK13* promoter (from -2000 to +100 related to transcriptional start site) was predicted by MethPrimer program (www.urogene.org/methprimer) with the following criteria: window size = 100, Obs/Exp > 0.6, and GC% > 50. The predicted CpG island of *KLK13* was amplified from the bisulfite-treated genomic DNA by two-step PCR with 1 cycle of 95°C for 5 min, followed by 5 cycles of 95°C for 1 min, 50°C for 1 min, 72°C for 1 min, and then 35 cycles of 95°C for 1 min, 45°C for 1 min, 72°C for 1 min. The outer primer set for the first-step PCR are 5'-GAGTTTTAGGAGGTTGTATATTGTT-3' for the forward primer and 5'-CATTCTTACCTCCTAACAAAACCAA-3' for the reverse primer. The inner primer set for the second-step PCR are 5'-TTAGGGTTAGGGTTGTTAAGAGTGA-3' for the forward primer and 5'-CCTAACAAAACCAAAATCAAAAAAA-3' for the reverse primer. The PCR product was purified and cloned into pGEM-T vector (Promega, Madison, WI, USA). At least 10 clones were randomly selected and subjected to sequencing by the core facility of the National Health Research Institutes, Taiwan. The methylation status of each CpG site was determined by comparing with normal *KLK13* genomic sequence. The cytosine methylation percentage of each CpG site was calculated as the number of methylated CpGs/the number of

clones analyzed [14].

2.4 Construction of small hairpin RNA (shRNA) against KLK13

Based on the main configuration of antisense strand, loop, sense strand and termination signal, a total of 4 sets of shRNA oligonucleotides (sh286-306, sh628-648, sh710-730 and sh834-854) were designed against *KLK13* mRNAs (see supplementary Table II) by the siRNA target finder program (GenScript, Piscataway, NJ, USA). The oligonucleotides were cloned into the downstream of the U6 promoter of pRNA-U6.1/Neo vector (GenScript) with BamHI and HindIII, and each obtained construct was stably transfected into high-metastatic CL1-5 cells using Lipofectamine 2000 as aforementioned.

2.5 Extracellular matrix (ECM) degradation assay

The FITC-conjugated laminin or fibronectin was coated onto the sterilized cover slips overnight at 4°C. The cover slips were washed and cells were seeded onto the slips and incubated for another 16 h at 37°C. The test cells were then fixed with 3% paraformaldehyde and the images were observed under fluorescent microscope. To quantify the results, the total area where the coated FITC-laminin or -fibronectin disappeared was normalized to the total cell numbers (counting at least 100 cells per microscopic field) as the percentage of degrading cells relative to control conditions [15].

2.6 In vitro invasion/migration assays and in vivo experimental metastasis assay

The *in vitro* invasion assay was performed using a modified Boyden chamber as described previously [16]. The migration assay was similar to that of the invasion assay, except the inserted polycarbonate membrane was not coated with any extracellular matrix proteins and that test cells were seeded at a density of 2.5×10^4 cells/well instead. For the *in*

vivo experimental metastasis assay, approximate 4- to 6-week-old SCID mice were tail-vein injected with 1 x 10^6 test cells with or without the expression of *KLK13*. After 8 weeks, the mice were sacrificed and the numbers of nodules and the weight of lung were measured.

3. Results

3.1 The expression of KLK13 is regulated by epigenetic modification

In order to determine whether there exists a difference in *KLK13* expression between normal and cancerous lung cells, we performed RT-PCR in 3 lung normal cell lines (IMR-90, WI-38, and BEAS-2B) and 3 lung adenocarcinoma cell lines (CL1-0, CL1-3, and CL1-5 cells) with gradually increased invasive potentials. As shown in Fig. 1A, whilst *KLK13* was undetectable in any of normal cells, it was expressed in all 3 tumor cell lines and up-regulated in accordance with cell malignancy. More interestingly, by treating these control normal cell lines and the low-invasive CL1-0 lung tumor cell line with the demethylation agent, 5-aza-deoxycytocine (5-aza-dC), the expression of *KLK13* became significantly increased in a dose-dependent manner (Fig. 1B). This indicates that the observed differential expression of *KLK13* during tumor development is likely to be governed by epigenetic modification, such as cytosine methylation. To identify possible CpG islands responsible for this regulatory difference, the genomic sequence from positions -2,000 to +100 related to the *KLK13* transcriptional start site was analyzed by MethPrimer program. The predicted result revealed that there were 10 possible CpG sites at positions -78, -45, -43, -34, -28, +9, +13, +31, +37, and +67 (Fig. 1C and 1D). By performing bisulfite sequencing, many of the identified CpG sites were found hypermethylated in normal control cells. The same sites, however, became moderately methylated in low-invasive CL1-0 cell line and hypomethylated (≥80-100%) in high-invasive CL1-3 and CL1-5 cell lines (Fig. 1D and 1E). These data suggest that the promoter region of *KLK13* was imprinted by hypermethylation in normal cells, but became

demethylated during lung cancer progression.

3.2 KLK13 modulates the invasiveness and motility of lung cancer cells

After the expression of *KLK13* was found positively correlated with the malignancy of lung cancer, its consequential effects on cell motility and invasiveness were analyzed. By transfecting and over-expressing *KLK13* in both the low-invasive CL1-0 and high-invasive CL1-5 cell lines, two stable clones (*KLK13*/CL1-0 clone#10 and *KLK13*/CL1-5 clone#1) with the highest *KLK13* expression (Fig. 2A) were selected for subsequent invasion and migration assays. As shown in Fig. 2, while forced expression of *KLK13* gave rise to a drastic increase in cellular invasion and migration (Fig. 2B), knockdown of the *KLK13* expression by shRNAs resulted in a notable decrease in cell motility and invasiveness (Fig. 2C). These results indicate that *KLK13* may serve as a novel metastasis-enhancing gene to regulate both the invasiveness and motility of lung cancer cells.

3.3 Enhanced laminin degradation and up-regulation of N-cadherin by KLK13

To clarify how *KLK13* could enhance the invasiveness of malignant cells, the ECM degradation assay was performed in both the control and *KLK13*-overexpressed cell lines. As laminin and fibronectin are two main components of the ECM, we examined whether *KLK13* cloud degrade these extracellular proteins during the process of cell invasion. As represented in Fig. 3, when compared to control cells, overexpression of *KLK13* caused a higher degree of ECM degradation (Fig. 3A), with approximately 2-3 folds of proteolytic cleavage against FITC-labeled laminin in both low-invasive CL1-0 and high-invasive CL1-5 cells (Fig. 3B). The same overexpression, however, failed to increase the ability of tumor cells to hydrolyze FITC-labeled fibronectin (Fig. 3C). These results suggest that laminin, but not fibronectin, is the major substrate of KLK13 proteolytic enzyme.

To further understand how *KLK13* may promote tumor cell motility, we determined the expression levels of several adhesion molecules (i.e. N-cadherin, P-cadherin, plakophilin 4, and junction plakoglobin) in CL1-0 cells with or without overexpression of *KLK13*. As shown in Fig. 3D, whilst *KLK13* was overexpressed in cells, only N-cadherin but not other adhesion molecules became significantly increased. In addition, by blocking these augmented N-cadherin molecules with its monoclonal antibody, a notable decrease of tumor cell motility was detected (Fig. 3E). This indicates that *KLK13* is likely to enhance tumor cell motility by up-regulating the expression of N-cadherin.

To sum up, we proposed a model hypothesizing that the CpG island of the *KLK13* promoter region is imprinted in normal lung cells but can be reactivated by cytosine demethylation during lung cancer progression. The augmented KLK13 protein is then secreted into the extracellular microenvironment to reach and hydrolyze laminin that, subsequently, enhances the invasive and metastatic potentials of lung cancer cells. *KLK13* also can induce the expression of N-cadherin to increase cell motility and, as expected by theory, knockdown of *KLK13* by shRNAs would disrupt the migratory and invasive abilities of tumor cells (Fig. 3F).

3.4 KLK13 enhances the metastatic potential of tumor cells in SCID mice

Given the aforementioned *in vitro* and *in vivo* findings, we sought to determine the effect of modulating *KLK13* expression on the metastatic potential of tumor cells in SCID mouse xenograft model. We compared the experimental metastasis of low invasive CL1-0 cells in the presence or absence of *KLK13* overexpression (gain of function), and of high invasive CL1-5 cells with or without *KLK13* knockdown (loss of function). Fig. 4 showed that, when compared to parental CL1-0 cells-injected mice, an increased number of lung nodules (left, Fig. 4A and 4B) and lung weight gain (left, Fig. 4C) were observed in those mice injected

with the *KLK13*-overexpressed CL1-0 cells. On the contrary, knockdown of *KLK13* in CL1-5 cells significantly suppressed the formation of lung nodules (right, Fig. 4A and 4B) and also reduced the total lung weight (right, Fig. 4C). These results provide evidence that *KLK13* is likely to play an important role in regulating lung tumor invasion and metastasis.

4. Discussion

Epigenetic changes such as DNA methylation are known to play a critical role in the development and progression of cancer. For example, tumor suppressor genes such as Rb are often found to be hypermethylated and inactivated, whereas protooncogene such as c-jun and c-myc are frequently hypomethylated and reactivated that, together, contribute to cancer growth and spread [17]. Here, we report a novel invasion-enhancing protease gene, *KLK13*, which expression is differentially regulated and positively correlated with the malignancy of lung cancer (Fig. 1A). This phenomenon is likely to be a consequence of its CpG island being hypermethylated in normal cells, but become hypomethylated during lung cancer progression (Fig. 1B-1E).

It should be noted that, unlike our study in lung adenocarcinoma, several reports have suggested that *KLK13* may serve as an independent indicator of favorable prognosis in hormone-dependent cancers [5,6]. For instance, *KLK13* was found down-regulated at the mRNA level in breast cancer and that its expression can be stimulated by steroid hormones in BT-474 breast cancer cell line [4]. For patients with grade I-II breast cancer, a high degree of *KLK13* expression was shown to be associated with disease-free survival and overall survival than patients with lower *KLK13* expression [5]. *KLK13* was also reported to be a favorable prognostic marker in ovarian cancer, with *KLK13*-positive patients in stage I-II exhibited a longer progression-free survival than those *KLK13*-negative patients [6]. A more recent but controversial study, however, reported that *KLK13* is significantly expressed in invasive

ovarian cancers than normal controls, and that patients with high *KLK13* expression may have a shorter recurrence-free survival than patients with low *KLK13* [7]. Such an increased *KLK13* expression was also recently identified in late-stage non-small-cell lung carcinoma patients, in which *KLK13* expression was found positively correlated with the malignancy of the disease [13]. Consistent with this finding, here we demonstrate that the up-regulation of *KLK13* expression in non-hormone-dependent lung cancer is likely due to the loss of epigenetic control during the course of tumor progression (Fig. 1). We also provide the direct evidence clarifying that *KLK13* is an invasion-enhancing protease in lung adenocarcinoma cells (Fig. 2), which promotes the metastatic potential in the *in vivo* SCID mouse xenograft model (Fig. 4).

As evident in Fig. 3D, *KLK13* was shown to be capable of inducing the expression of N-cadherin. N-cadherin is a Ca^{2+}-dependent adhesion molecule essential for cell-cell interaction. This protein molecule has been reported to play a pivotal role in the extravasation of cancerous cells. By adhering to endothelial cells, N-cadherin is able to facilitate the transendothelial migration and invasion of malignant cells [18,19]. Such a regulation can be impaired by blocking N-cadherin with its own antibody [20] or with (-)-epigallocatechin-3-gallate, a green tea-derived polyphenol [21], thus leading to a decrease in tumor cell metastasis. In this study, we demonstrate that the increased expression of *KLK13* in tumor cells can promote cell motility either by a direct or indirect up-regulation of N-cadherin (Fig. 3D and 3E). We further disclosed that the elevated KLK13 protease can help hydrolyze extracellular laminin protein (Fig. 3A-3C) and thereby facilitate the invasive and metastatic behavior of lung tumor cells both *in vitro* (Fig. 2) and *in vivo* (Fig. 4). Together our data provide the first evidence that during lung tumor progression, *KLK13* is epigenetically activated by aberrant hypomethylation which contributes to a malignant phenotype by enhancing tumor cell migration and invasion. This newly identified

metastasis-related protease gene may thus serve as a diagnostic/prognostic marker and a potential therapeutic target for lung adenocarcinoma.

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Figure Legends

Fig. 1. Expression and epigenetic cytosine methylation of *KLK13* in normal and cancerous lung cell lines. A) The expression levels of endogenous *KLK13* in different cell lines were determined by RT-PCR. B) Cells were treated with indicated concentration of 5-aza-dC for 3 days. The expression of *KLK13* in each treated cell line was measured by RT-PCR. C) The potential CpG island (gray shadow) was predicted by MethPrimer program. D) The methylation status of each individual CpG site was determined by bisulfite sequencing of 10 randomly selected clones. Each row of circles represents the methylation pattern obtained from individual clones of the *KLK13* gene promoter. Open circles indicate unmethylated sites and black filled circles indicate methylated sites. E) The histogram of CpG methylation across the *KLK13* promoter region represents the percentage of each methylated CpG site in all cell lines. The x-axis is nucleotide position relative to the transcriptional start site, and the y-axis is the percentage of cytosine methylation.

Fig. 2. Enhancing effects of *KLK13* on the invasiveness and motility of lung cancer cells. A) The expressions of exogenous *KLK13* in stable transfectants were determined by Western

blotting with anti-HA antibody. B) Two clones (*KLK13*/CL1-0 clone#10 and *KLK13*/CL1-5 clone#1) with the highest *KLK13* expression were selected for subsequent invasion (upper panel) and migration (lower panel) assays. C) Four shRNAs against *KLK13* were individually transfected into high-invasive CL1-5 cells. The inhibitory effect of each shRNA on *KLK13* was determined by RT-PCR (bottom panel). The upper and middle panels represent the invasiveness and motility of each transfectant, respectively. The statistic analysis was performed by t-test. The symbols "*" and "**" indicate *p* value <0.05 and 0.01, respectively.

Fig. 3. The roles of *KLK13* in the regulation of invasiveness and motility in lung cancer. A) The representative results of FITC-laminin degradation in control and *KLK13*-overexpressed CL1-0 transfectants. The percentage of cells degrading the coated FITC-laminin and FITC-fibronectin in control and *KLK13*-overexpressed cells were shown in B) and C), respectively. D) The expression levels of *KLK13* and indicated adhesion molecules in control and *KLK13*-overexpressed CL1-0 transfectant were determined by RT-PCR. E) Control and *KLK13*-overexpressed CL1-0 cells were treated with or without 40 μg/ml anti-N-cadherin monoclonal antibody (Sigma) or mouse IgG, and their motility was examined. F) The proposed model of how *KLK13* enhances the invasiveness and motility of lung cancer cells. ***KLK13*:** kallikrein 13; *NCAD*: N-cadherin; *PCAD*: P-cadherin; *PKP4*: plakophilin 4; *JUP*: junction plakoglobin; *ACTB*: beta-actin.

Fig. 4. The effect of *KLK13* on the metastasis of lung cancer cells *in vivo*. SCID mice were injected with $1x10^6$ lung tumor cells via tail vein. After 8 weeks, mice were sacrificed and the numbers of lung nodules and lung weight were measured. A) The photographs of lung are representative results from indicated cells-injected mice. The numbers of lung nodules and lung weight from each group of CL1-0 (n=9), CL1-0-*KLK13* (n=7), CL1-5 (n=8), and

CL1-5-*KLK13* shRNA (n=9) cells-injected mice were shown in B) and C), respectively.