Kallikrein-related peptidase 13 expression and clinicopathological features in lung squamous cell carcinoma

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Abstract. Lung squamous cell carcinoma (LSCC) is associated with poor prognosis. Molecular targeting drugs have been demonstrated to be effective for lung adenocarcinoma; however, they are often not effective for LSCC. Kallikrein-related peptidase 13 (KLK13) expression enhances the malignancy of lung adenocarcinoma; however, its expression and crucial role in LSCC remain largely unknown. The present study examined the relationship between the KLK13 expression and clinicopathological features of LSCC. A total of 94 patients diagnosed with LSCC who underwent lobectomy, segmentectomy or wedge resection were selected. KLK13 expression was evaluated through immunostaining of formalin-fixed paraffin-embedded sections of surgical specimens. Of the 94 LSCC samples, 70 exhibited no KLK13 expression, while the remaining 24 exhibited ectopic expression. KLK13 expression in tumors was focal and restricted to the cytoplasm of keratinized cells. LSCC cases were classified into KLK13-negative and KLK13-positive groups, and KLK13 expression was positively associated with E-cadherin expression (P=0.0143). Associations between KLK13 expression and keratinization (P=0.0052) or absence of lymphatic vessel invasion (P=0.0603) were observed; however, these trends did not reach statistical significance. The present findings indicated that KLK13 expression in keratinized LSCC may have a protective role in lymphatic vessel invasion of LSCC, which suggests its significance for therapeutic applications against LSCC.

Introduction

Lung squamous cell carcinoma (LSCC) is a subtype of non-small cell lung carcinoma (NSCLC) and accounts for 20-30% of all lung cancers (1). LSCC is known to be associated with a poor prognosis; the 5-year overall survival rate of LSCC patients with clinical stages I and II is approximately 40%, while that of patients with clinical stages III and IV is less than 5% (2). Recent developments in genetic and molecular techniques has allowed the identification of driver mutations in epidermal growth factor receptors (EGFR), anaplastic lymphoma kinase (ALK), and c-ROS oncogene 1 (ROS1) with regards to NSCLC (3-6). Subsequent analyses revealed that these mutations were frequently observed in adenocarcinoma, another subtype of NSCLC, but were rarely detected in LSCC. For example, activating mutations in the EGFR gene were detected in 30% of adenocarcinomas, while less than 3 % of SCC patients were identified as having these mutations (7). Therefore, based on these findings, molecular-targeted drugs have been proven to be effective for lung adenocarcinoma. However, advances in the treatment of LSCC are lacking as compared to those of lung adenocarcinoma, and there is currently no definite clinical implication for LSCC (8). Furthermore, few regimens, including the VEGF-A-targeting monoclonal antibody bevacizumab, cannot be selected for LSCC. Based on the responses to these treatments and the results of clinical trials for immune checkpoint inhibitors, NSCLC is currently divided into SCC and non-SCC (9-11). Therefore, identification of LSCC-specific clinicopathological features is necessary to improve the cure and survival rates of LSCC patients.

Human tissue kallikreins (KLKs) is a group of 15 members of the serine-protease family. KLKs are present in a variety of healthy human tissues, including airway tissues, and play crucial roles in the pathophysiology of chronic, infectious, and tumor lung diseases (12). One of the 15 kallikrein subfamily members, KLK5, has been shown to contribute to the remodeling of the airway epithelium in patients with chronic obstructive pulmonary disease (COPD) (13), while KLK13 has been known to enhance the malignancy of lung adenocarcinoma (14). In contrast, a recent study showed that the downregulation of KLK13 is correlated with a poor prognosis in several carcinomas, such as bladder cancer (15), oral squamous cell carcinoma (16), breast cancer (17), and colorectal cancer (18). KLK13 has diverse physiological functions, including many cancer-related processes, and whether KLK13 expression promotes or suppresses tumor progression

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may depend on the context (19). However, the expression and clinicopathological features of KLK13 in LSCC remain unknown.

In this study, we immunohistochemically examined LSCC specimens for KLK13 expression, and found that the KLK13 expression was limited to keratinizing cells in LSCC. We further assessed 94 cases with detailed clinical information and retrospectively analyzed the relationship between KLK13 expression and the clinicopathological parameters of LSCC. Our results demonstrated that the KLK13 expression in LSCC correlated with the absence of lymphatic vessel invasion.

Materials and methods

Patients. We examined 94 patients diagnosed with LSCC who underwent lobectomy, segmentectomy, or wedge resection between January 2011 and September 2018 at the National Center for Global Health and Medicine (NCGM). This study was approved by the NCGM Research Ethics Committee (2417), and the requirement for consent was waived and a poster was displayed before the start of the study. Formalin-fixed paraffin-embedded sections of surgical specimens were used for immunohistochemical analyses.

Immunohistochemical analysis. Formalin-fixed paraffin-embedded sections of surgically resected LSCC specimens were deparaffinized and rehydrated. Target Retrieval Solution (Dako, Glostrup, Denmark) was used to retrieve the antigens. Sections were stained with anti-KLK13 antibody (Sigma-Aldrich, Inc., St. Louis, MO, USA) and an ImmPACTTM DAB peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA, USA), and counterstaining was performed using hematoxylin. Anti-human papillomavirus (HPV) type 16 L1 antibody (GeneTex Inc. CA, USA) was used to examine the HPV status of LSCC patients. For double staining with KLK13, the MACH 2 Double Stain 1 Kit (Biocare Medical, Concord, CA) and Warp Red Chromogen Kit (Biocare Medical) were used to detect the anti-p40 (Biocare Medical) or anti-E-cadherin antibody (Santa Cruz Biotechnology, Inc., Dallas, TX) signals. Regarding the expression of E-cadherin, we classified the LSCC cases into E-cadherin-positive and -negative groups based on the localization of E-cadherin at the membranes of tumor cells in dominant area. Two observers reviewed all slides, without any access to clinical or pathological data, exhibiting high inter-observer reliability with a clear difference between KLK13-positive/negative samples.

Statistical analysis. Each tumor was classified based on its location, size, pathology, condition of the lymph nodes, and degree of metastasis (pTNM, 8th edition, 2017) (20). The KLK13 staining results were compared using Fisher's exact tests for sex, pT status, pN status, pM status, cancer stage, vessel invasion, lymphatic vessel invasion, pathological differentiation, E-cadherin expression, and HPV expression. In using the Fisher's exact tests, we re-classified datasets of pT, pN, and stage into 2 groups. Unpaired t-test was used for comparison of age between the groups. Statistical analyses were performed using Prism 8 (GraphPad Software Inc., La Jolla, CA, USA). All tests were two-tailed, and P values <0.05 were considered statistically significant.

Results

KLK13 was limited in the keratinized tumor cells of LSCC. Although prior studies have previously reported the mRNA expression of KLK13 in normal lung tissues (21), its protein expression has never been reported before. Therefore, we first performed immunohistochemical analyses to investigate the protein expression and localization of KLK13 in normal lung tissue and LSCC tissue. The demographic features of the 94 patients are summarized in Table I. This study included 79 male and 15 female patients, all of whom had a smoking history. In normal lungs, the protein expression of KLK13 was detected in the connecting tissue between epithelium and lamina propria in the proximal airway (Fig. 1A and B), as well as in the bronchiole (Fig. 1C). In LSCC, 70 of the 94 samples did not express KLK13 proteins, whereas the remaining 24 tumor samples showed an obvious KLK13 expression. In KLK13-positive LSCC, the protein expression of KLK13 was focal and restricted to the cytoplasm and cellular membrane of keratinized cells, but was not detected in the nuclei of keratinized cells or fibrous and stromal cells in LSCC (Fig. 1D). The specificity of the staining signals observed in tumors was confirmed by their disappearance in the presence of blocking peptides (Fig. 1E).

Associations between KLK13 immunoreactivity and LSCC clinicopathological features. To analyze the relationship between KLK13 expression and the clinicopathological features of LSCC tumors, we classified LSCC cases into the KLK13-negative and KLK13-positive groups (Table II). There were no significant differences between the groups with respect to sex, pT, pN, pM, cancer stage, or vessel invasion. In contrast, KLK13 expression in tumors trended to associate with keratinization (P=0.052) and no lymphatic vessel invasion (P=0.0603). Because KLK13 inhibits the cell invasion and migration due to the upregulation of E-cadherin in oral squamous cell carcinoma (16), we examined the relation between the expression of KLK13 and of E-cadherin. Immunohistochemically, the membrane expression of E-cadherin was observed in KLK13-positive LSCC, whereas its expression levels was decreased in tumor cells without KLK13 expression (Fig. 2A). KLK13 expression was significantly associated with E-cadherin expression (P=0.0072, Table II). Furthermore, we examined the relationship between KLK13 expression and HPV status because well differentiated-LSCC had high prevalence of HPV (22). Although there was no significant difference between the KLK13-positive groups and KLK13-negative groups (34.21% vs. 19.64%, P=0.1488, Table II), cases of KLK13-positive tumors tended to have an HPV-positive status. We further conducted double staining for KLK13 with p40 (ΔNp63), which is widely used as an LSCC diagnostic marker and is seen in cells with stemness (23). Tumor cells expressing KLK13 were negative for p40 nuclear staining, but were surrounded by p40-expressing cells (Fig. 2B).

Discussion

In the present study, we observed an ectopic expression of KLK13 in keratinized LSCC cells. Although a previous study reported that KLK13 was associated with cell invasion and migration in lung adenocarcinoma (14), KLK13 expression in

Table I. Patient characteristics (n=94).

Characteristics	Value
Mean age ± SD, years	71.64±7.43
Sex, n (%)	
Male	79 (84)
Female	15 (16)
Smoking history, n (%)	
Positive[a]	94 (100)
Negative	0 (0)
pT classification, n (%)	
T1	43 (46)
T2	30 (32)
T3	14 (15)
T4	7 (7)
pN classification, n (%)	
N0	80 (85)
N1	8 (9)
N2	6 (6)
pM classification, n (%)	
M0	91 (97)
M1	3 (3)
Cancer stage[b], n (%)	
I	55 (58)
II	23 (25)
III	13 (14)
IV	3 (3)
Differentiation, n (%)	
Keratinizing	72 (77)
Non-keratinizing	22 (23)
Lymphatic vessel invasion, n (%) (n=93)	
Positive	11 (12)
Negative	82 (88)
Vessel invasion, n (%) (n=93)	
Positive	47 (51)
Negative	46 (49)

[a]Positive includes ex-smokers and current smokers. bBased on the Union for International Cancer Control guidelines, 8th edition. Lymphatic vessel invasion and vessel invasion were analyzed in 93 evaluable cases because there were no areas in the specimen that could be evaluated for vessel invasion or lymphatic vessel invasion in 1 case.

LSCC was associated with negative lymphatic vessel invasion. Invasion of cancer cells into the surrounding tissue and infiltration into the lymphatic and blood vessels are key features of cancer progression and metastasis. Lymphatic vessel invasion has been reported to be associated with tumor recurrence and prognosis in NSCLC (24); few reports have argued that NSCLC patients with lymphatic vessel invasion would require more aggressive treatment after surgery (25). In the present study, KLK13 expression was negatively correlated with lymphatic vessel invasion. Furthermore, cases of KLK13-positive tumors tended to have a better postoperative prognosis than those with a negative expression of KLK13, although this difference is not statistically significant (P=0.18) because of the specific feature of the cases examined in this retrospective study. All tumors were in the early stages, and the patients had absolute indications for surgical intervention of the (stage I + II) LSCC. Additional studies should include a higher number of cases and clarify the biological roles of KLK13 in established cancer cells to validate KLK13 as a clinical prognostic marker in LSCC. Planque *et al* (26) reported that NSCLC patients with high KLK13 expression at the mRNA trended to have lower overall survival, although the difference were marginally significant. Since their results were contrary to our present finding that cases of KLK13 immunoreactivity-positive tumors tended to have a better postoperative prognosis than those with a negative expression of KLK13 protein, we suspected that protein levels of KLK13 is post-transcriptionally regulated. Therefore, further study to clarify the molecular mechanisms regulating the protein levels of KLK13 may be required to explain this discrepancy. In 2015, the World Health Organization classified the subtyping of LSCC into keratinizing, non-keratinizing, and basaloid subtypes (27). Recent studies have revealed that there is no significant difference in the survival indications between keratinizing SCC and non-keratinizing SCC (28). Furthermore, Chen *et al* (29) reported that lymphatic vessel invasion was not affected by the keratinizing status in LSCC. We also analyzed the relationship between keratinization and lymphatic vessel invasion in the 94 LSCC cases in this study and found no significant association between them (P=0.29, data not shown). Therefore, we suspect that the expression of KLK13 may influence the lymphatic vessel invasion or the prognosis of LSCC not via the induction of keratinization, although KLK13 was expressed in a part of keratinized cells (Table II).

The results of the present study implied that KLK13 may exert a protective role in lymphatic vessel invasion and eventually cancer progression in LSCC. Although these results depict a diametrically opposed role of KLK13 than is observed for adenocarcinoma, prior research has reported that KLK13 has diverse physiological functions in carcinogenesis and cancer progression. Indeed, in the oral and the esophageal squamous cell lines, the overexpression of KLK13 inhibits the cell invasion and migration (16,30), possibly due to the upregulation of adhesion molecules such as E-cadherin, α-catenin, β-catenin, desmoglein3, and desmoplakin. In the present study, we found the significant association between the expression of KLK13 and of E-cadherin (Fig. 2A and Table II). Generally, E-cadherin is known as a marker of differentiated epithelial cells and is involved in cell–to–cell adhesion. The disruption of the E-cadherin-mediated cell–cell adhesion is often observed in malignant cancer progression such as tumor invasion and metastasis. Therefore, E-cadherin expression may explain the findings of our study, where KLK13 expression inhibits lymphatic vessel invasion in LSCC. Hural *et al* (31) reported that KLK4 has the potential to be useful as a vaccine for prostate cancer. Additionally, several kallikrein proteins, such as KLK3, KLK5, KLK6, KLK10, and KLK14, have been proposed as serum markers for diagnosis and prognosis of prostate and breast carcinomas (32); however, we did not assess the KLK13 levels using serum samples in the present study. Although additional studies to clarify the biological roles

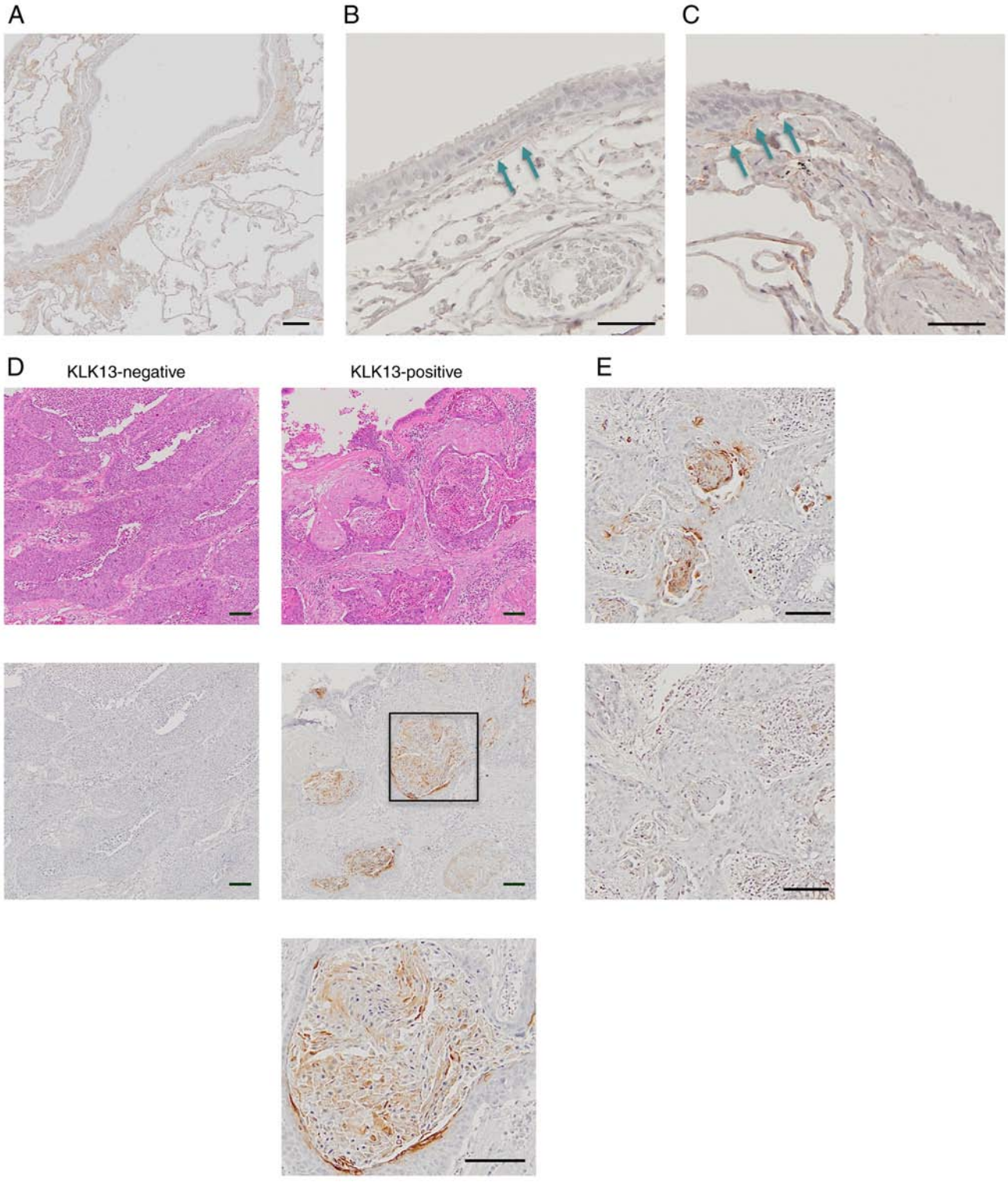

Figure 1. (A-C) Representative images of formalin-fixed, paraffin-embedded normal bronchial epithelia samples stained with anti-KLK13. (A) Overall view of bronchus with low magnification. The localization of KLK13-positive cells in (B) bronchus and (C) bronchiole. Arrows indicate KLK13-positive cells. Scale bar, (A) 100 μm or (B and C) 50 μm. (D) Representative images of formalin-fixed, paraffin-embedded LSCC samples stained with hematoxylin and eosin or anti-KLK13. Scale bar, 100 μm. Upper panels showing LSCC samples stained with hematoxylin and eosin. Middle panels showing LSCC samples stained with anti-KLK13. Right-lower panel showing a higher magnification view of the KLK13-positive area in the right middle panel (box). KLK13 was focal and restricted to the cytoplasm and cellular membrane of keratinized cells. (E) Representative images of formalin-fixed, paraffin-embedded LSCC samples stained with anti-KLK13 with (upper panel) or without (lower panel) blocking peptide. Scale bar, 100 μm. KLK13, kallikrein-related peptidase 13; LSCC, lung squamous cell carcinoma.

of KLK13 in established cancer cells as well as in sera will refine the value of KLK13 expression as a molecular target in LSCC, the previous study findings, as well as ours, encourage us to further investigate the therapeutic application of KLK13 as a molecular-target drug or peptide vaccine against LSCC. Because KLK13 has diverse functions, including the promotion

Table II. Clinicopathological features of patients with KLK13-positive and KLK13-negative lung squamous cell carcinoma.

Characteristics	Total (n=94)	KLK13-negative (n=70)	KLK13-positive (n=24)	P-value
Mean age ± SD, years	72.00±7.40	71.64±7.43	70.29±7.05	0.3054
Sex, n (%)				0.3395
Male	79 (84.0)	57 (81.4)	22 (91.7)	
Female	15 (16.0)	13 (18.6)	2 (8.3)	
pT classification, n (%)				0.4393
T1-2	73 (77.7)	53 (75.7)	20 (83.3)	
T3-4	21 (22.3)	17 (24.3)	4 (16.7)	
pN classification, n (%)				0.7072
N0	80 (85.1)	59 (84.3)	21 (87.5)	
N1-2	14 (14.9)	11 (15.7)	3 (12.5)	
pM classification, n (%)				0.1592
M0	91 (96.8)	69 (98.6)	22 (91.7)	
M1	3 (3.2)	1 (1.4)	2 (8.3)	
Cancer stage[a], n (%)				0.9573
I-II	78 (83.0)	58 (82.9)	20 (83.3)	
III-IV	16 (17.0)	12 (17.1)	4 (16.7)	
Differentiation, n (%)				0.0521
Keratinizing	72 (76.6)	50 (71.4)	22 (91.7)	
Non-keratinizing	22 (23.4)	20 (28.6)	2 (8.3)	
Lymphatic vessel invasion, n (%) (n=93)				0.0603
Positive	11 (11.8)	11 (15.9)	0 (0.0)	
Negative	82 (87.2)	58 (84.1)	24 (100)	
Vessel invasion, n (%) (n=93)				>0.9999
Positive	47 (50.5)	35 (50.7)	12 (50.0)	
Negative	46 (49.5)	34 (49.3)	12 (50.0)	
E-cadherin expression, n (%)				0.0143[b]
Positive	37 (39.4)	22 (31.4)	15 (62.5)	
Negative	57 (60.6)	48 (68.6)	9 (37.5)	
HPV, n (%)				0.1488
Positive	38 (40.4)	25 (35.7)	13 (54.2)	
Negative	56 (59.6)	45 (64.3)	11 (45.8)	

[a]Based on the Union for International Cancer Control guidelines, 8th edition. [b]P<0.05. Lymphatic vessel invasion and vessel invasion were analyzed in 93 evaluable cases because there were no areas in the specimen that could be evaluated for vessel invasion or lymphatic vessel invasion in 1 case. HPV, human papillomavirus; KLK13, kallikrein-related peptidase 13.

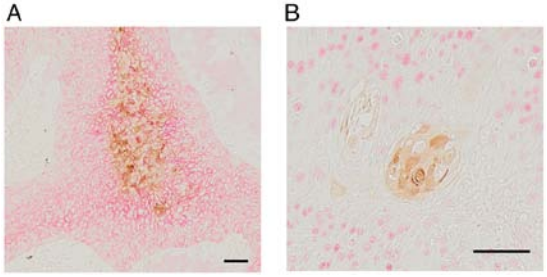

Figure 2. (A) Representative image of formalin-fixed, paraffin-embedded LSCC tissue samples double-stained with anti-KLK13 (dark brown) and anti-E-cadherin (pink) antibodies. Scale bar, 50 μm. (B) Representative image of formalin-fixed, paraffin-embedded LSCC tissue samples double-stained with anti-KLK13 (dark brown) and anti-p40 (pink) antibodies. Scale bar, 100 μm. KLK13, kallikrein-related peptidase 13; LSCC, lung squamous cell carcinoma.

of tumor cell motility via the induction of N-cadherin and vimentin in lung adenocarcinoma (14), it may be necessary to assess side effects of molecular targeted drugs modulating the expression of KLK13 for clinical applications in LSCC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YIK conceived and designed the study. RS, TH and YIK acquired data. RS, TH, KY and YIK analyzed and interpreted the data. RS, SN, HM, TI and KY provided clinical material support and analyzed clinicopathological data. RS and YIK drafted the manuscript. RS and YIK confirm the authenticity of all the raw data. RS, SN, KY and YIK obtained funding. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved (2417) by the Research Ethics Committee of National Center for Global Health and Medicine (Tokyo, Japan). The requirement for written informed consent was waived by the Institutional Review Board of National Center for Global Health and Medicine (Tokyo, Japan) because the present study was retrospective and non-interventional in nature. Therefore, the poster was displayed before this study had started in accordance with our hospital's ethics. In addition, it has been described in the poster that all the patients can refuse to participate in this study at any time.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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