Abstract. The aim of this study was to clarify the association between expression of human leukocyte antigen (HLA) class I in non-small-cell lung cancer (NSCLC) cells and patient survival. To address this, immunohistochemical staining for HLA class I was performed on specimens from 111 patients with NSCLC, and overall survival curves were compared using the log-rank test. In addition, multivariate analyses were performed using Cox's proportional hazard model. The cases were divided into 5 classes based on the expression of HLA class I heavy chain and β2-microglobulin. The overall survival rate for patients with tumors lacking HLA class I heavy chain (30 cases; 27.0%) was significantly decreased. The multivariate analysis demonstrated that the absence of HLA class I heavy chain was an independent predictor of poor prognosis. There was a trend towards an unfavorable prognosis for patients whose tumors did not express β2-microglobulin (57 cases; 51.4%). Downregulation of HLA class I heavy chain expression was significantly associated with the downregulation of β2-microglobulin. Cases lacking HLA class I heavy chain as well as β2-microglobulin expression (23 cases; 20.7%) had a statistically significant unfavorable prognosis compared with other cases. The present findings demonstrate that the lack of HLA class I heavy chain expression in tumor cells is an independent prognostic factor for poor NSCLC survival, and is likely to exert an important influence on immune surveillance in patients.

Introduction

The most effective treatment for early stage non-small-cell lung cancer (NSCLC) is surgical resection. However, >60% of patients with NSCLC relapse following surgery (1-3). The immune system can not only suppress tumor growth by destroying cancer cells or inhibiting their proliferation, but can also promote tumor progression by selecting for tumor cells that can survive in an immunocompetent host or by establishing conditions within the tumor microenvironment that facilitate tumor growth (4,5). The anti-tumor arsenal of the immune system includes cluster of differentiation (CD)4+ T cells that kill tumor cells in a major histocompatibility complex (MHC) class II expression-dependent manner, and natural killer cells/macrophages that mediate attacks on cells by antibody-dependent cellular cytotoxicity. In addition, CD8+ T cells are hypothesized to serve an important role in the elimination of tumor cells by identifying human leukocyte antigen (HLA) class I expression on their surface. The association between HLA class I expression on tumor cells and CD8+ T cell infiltration has been reported in laryngeal squamous (6), pancreatic (7) and ovarian carcinoma (8).

HLA class I molecules are transmembrane glycoproteins comprising a polymorphic 45-kDa heavy chain and a non-polymorphic 12-kDa β2-microglobulin light chain. There are 3 HLA class I heavy chains, HLA-A, -B and -C, encoded by 3 separate genes on chromosome 6p21. Loss or downregulation of HLA class I has been reported in various types of cancer, including pancreatic, ovarian and breast carcinoma (7-13), and a number of studies have described HLA class I downregulation in NSCLC (14-19). However, in these previous studies, there were discrepant associations between HLA class I downregulation and prognosis (15-17,19), which may be due to the use of different antibodies, control methods and tumor classification schemes. Attempts to standardize methods have included the use of the EMR8-5 antibody, which recognizes HLA-A, -B and -C (20), and the establishment of a novel control method for staining, using severe combined immune deficiency (SCID) mouse xenografts to examine the expression of CD40 and CD154 in NSCLC (21).
However, a classification system based on immunostaining for loss or downregulation of HLA class I has not been established.

In the present study, the influence of downregulation of HLA class I in tumors on the survival of patients with NSCLC was assessed using EMR8-5 immunostaining. SCID mouse xenografts as quantitative controls and novel classification schemes based on tumor heterogeneity, and the association between loss or downregulation of HLA class I expression and NSCLC prognosis was reviewed.

Materials and methods

Cell lines. Human lung cancer cell lines were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). A549, H226, LC-1, LK2, PC3, PC10 and RERF-LC-MS cells were cultured in RPMI-1640 medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) with 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA) and 1% penicillin/streptomycin. ABC-1 and VMRC-LCD cells were maintained in Eagle's Minimum Essential Medium (Sigma-Aldrich; Merck KGaA) with 10% FBS and 1% penicillin/streptomycin. All cell lines were maintained at 37°C in a humidified incubator with 5% CO₂. Peripheral blood cells were taken from a healthy volunteer and human peripheral blood mononuclear cells (PBMCs) were obtained using the Ficoll separation method.

Mice and xenograft models. Formalin-fixed paraffin-embedded tissue specimens were obtained from mice xenograft models established in a previous study (22). CB17/SCID mice were obtained from Charles River Laboratories, Inc. (Wilmington, MA, USA). All mice were 4-6-week-old females mice were obtained from Charles River Laboratories, Inc. (Wilmington, MA, USA) was used as the loading control of western blot analysis.

 Patients and tissue specimens. Whole surgical specimens of NSCLC obtained between December 1992 and January 2001 and relevant patient data were used in the present study. All patients lacked any evidence of metastases to secondary sites and had not received prior anticancer treatment. Adjacent matching normal tissue >5 cm away from the tumor was additionally obtained and stored at -80°C. A section of the normal tissue was stained with hematoxylin & eosin (as described in the immunohistochemistry section) to verify there were no cancerous cells present. Cases of in-hospital and non-cancer-associated mortalities were excluded. Patients provided informed consent, and the study was conducted in accordance with the guidelines of The Hokkaido University Institutional Review Board (Sapporo, Japan). The study included a total of 111 NSCLC surgical specimens from patients meeting these criteria who had undergone curative resection of the primary tumor, including systematic lymph node dissection, at The Hokkaido University Hospital. The surgical specimens were obtained from 72 men and 39 women with a mean age of 62.7 years (range, 36-80 years). The median duration of follow-up was 60.9 months (range, 4.0-100.7 months), and 43 patients (38.7%) succumbed during the follow-up period.

The clinical patient data were analyzed according to the expression results of their tissue samples. According to the Western blot analysis. Western blotting was used to analyze the expression of HLA class I heavy chain and β2-microglobulin in lung cancer cells. Each frozen tissue was crushed to powder in liquid nitrogen using a CRYO-PRESS (Microtec Co., Ltd., Chiba, Japan) and resuspended in radioimmunoprecipitation assay lysis buffer (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) with the addition of protease inhibitors (Promega Corporation, Madison, WI, USA) and homogenized two times by Bioruptor (Cosmo Bio, Tokyo, Japan) for 30 sec on ice. Lysates were incubated at 4°C for 20 min and centrifuged at 12,000 x g at 4°C for 20 min to remove cellular debris. Supernatants were recovered and protein concentrations were determined by a Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Lysates (10 µg protein/lane) were resolved using 15% SDS-PAGE, and then transferred to nitrocellulose membranes (GE Healthcare, Chicago, IL, USA). After blocking in TBS-Tween-20 (TBS-T, 50 mM Tris, 150 mM NaCl, 0.01% Tween-20) with 5% skimmed milk at room temperature for 1 h, membranes were incubated with EMR8-5 (1:1,000 dilution) or EMR-B6 (1:400 dilution) antibodies at room temperature for 1 h. The membrane was washed three times with TBS-T, 5 min each, and then incubated with the peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:10,000 dilution) at room temperature for 1 h. The bound antibodies were detected using enhanced chemiluminescence western blotting detection reagent (GE Healthcare Life Sciences, Shanghai, China) and ChemiDoc XRS+ (Bio-Rad Laboratories, Inc.). Membranes were stripped of EMR-8-5, or EMR-B6 antibodies using Restore™ PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific, Inc.), and probed with the anti-β-actin antibody (1:10,000 dilution) at room temperature for 1 h. Lysate from normal PBMCs was used as the positive control for HLA class I heavy chain and β2-microglobulin expression.
World Health Organization classification system (23), the specimens consisted of 56 adenocarcinomas, 39 squamous carcinomas, 3 adenosquamous carcinomas, 12 large-cell carcinomas and 1 carcinosarcoma. The site and size of the lesion were assessed on the gross specimen. The clinicopathological stage of the tumor was determined according to the standard Tumor-Node-Metastasis (TNM) classification system (24). The results indicated that 50 samples were T1 and 61 were T2-4. The lymph nodes were negative in 79 patients and positive in 32. The stage of the surgical specimen was defined according to the 7th International Union Against Cancer Classification (UICC) (23). Diagnosis was performed independently by ≥2 pathologists.

Immunohistochemistry. The surgical specimens were fixed in 10% formalin solution and kept at room temperature for 2-4 days. Subsequently, the specimens were embedded in paraffin for sectioning at 4 µm. The sections were then deparaffinized in xylene, dehydrated through a graded ethanol series and autoclaved for 5 min. Endogenous peroxidase activity was blocked by a 10-min incubation with hydrogen peroxide at room temperature. Following 3 washes in PBS containing 0.1% Tween 20, the sections were incubated in 10% normal goat serum (Histofine SAB-PO kit; Nichirei Biosciences, Inc., Tokyo, Japan) for 30 min at room temperature. The samples were then incubated at room temperature for 1 h with the EMR8-5 anti-human HLA class I heavy chain mouse monoclonal antibody (1:400 dilution). In addition, serial tissue sections of each sample were individually incubated at room temperature for 1 h with the EMR-B6 anti-β2-microglobulin mouse monoclonal antibody (1:400 dilution). Following 3 additional washes, the sections were incubated with Histofine Simple Stain MAX-PO (Multi; Nichirei Biosciences, Inc.) for 30 min at room temperature and the reaction products were visualized by incubation for ~10 min with 3,3'-diaminobenzidine tetrahydrochloride (Nichirei Biosciences, Inc.), followed by washing in distilled water. The sections were counterstained in hematoxylin for 1.5 min at room temperature, and mounted in Permount (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) for 2-4 days. Subsequently, the specimens were embedded in 10% formalin solution and kept at room temperature for 7 days. The lymph nodes were negative in 79 patients and positive in 32. The stage of the surgical specimen was defined according to the 7th International Union Against Cancer Classification (UICC) (23). Diagnosis was performed independently by ≥2 pathologists.

Statistical analysis. The statistical significance of associations between HLA class I heavy chain and β2-microglobulin expression in cancer cells was evaluated using the Mann-Whitney U test. The χ² test (or extended Fisher's exact test, where appropriate) was used to analyze the comparisons between HLA class I heavy chain and β2-microglobulin expression in cancer cells and patient parameters, including histopathological findings. The cumulative survival rate was calculated using the Kaplan-Meier method and statistical significance was analyzed with the log-rank test. Cancer-specific survival rates were calculated from date of surgery to date of cancer-associated mortality. Univariate and multivariate analyses were performed using the Cox proportional hazard regression model. P<0.05 was considered to indicate a statistically significant difference. All analyses were performed using StatView-J version 5.0 software (SAS Institute, Inc., Cary, NC, USA).

Results

Expression of HLA class I heavy chain and β2-microglobulin in NSCLC cell lines and their derived xenografts. Western blot analysis revealed that HLA class I heavy chain (45 kDa) was detected most strongly in the H226 cell line (Fig. 1A; top panel), and expression was lowest in the VMRC-LCD and LK2-derived xenografts (Fig. 1A; lower panel). β2-microglobulin (12 kDa) was strongly expressed in 4 cell lines: PC3, RERF-LC-MS, VMRC-LCD and H226 (Fig. 1B; top panel), and downregulated in 4 xenografts: ABC-1, VMRC-LCD, LC-1 and LK2 (Fig. 1B; bottom panel). HLA class I heavy chain was expressed in normal human lung tissue at levels similar to those detected in the PBMC (Fig. 1A; top panels). Of the 9 cell lines, A549 and PC10 demonstrated downregulated HLA class I heavy chain and β2-microglobulin expression, although the xenografts from these cells exhibited higher expression of these molecules. PC10-derived xenografts were used as positive tissue controls for HLA class I heavy chain and β2-microglobulin expression because the proteins were highly expressed and clearly detected in these samples (Figs. 1A, 2A and B). LK2-derived xenografts, which exhibited downregulated HLA class I heavy chain and β2-microglobulin expression by western blotting, were used as negative tissue controls (Figs. 1B, 2C and D). From these results, the xenografts derived from PC10 and LK2 cells were used as positive and negative controls, respectively, for HLA class I expression. Isotype-matched negative control antibodies were used to control for non-specific staining.

Establishment of a quantitative evaluation for tumor HLA class I and β2-microglobulin expression. Immunostaining intensities were grouped into 3 categories: Strongly positive (equivalent to staining of alveolar epithelium), weakly positive relative to alveolar epithelium, and absent (no detectable staining) (Fig. 2).

Expression of HLA class I heavy chain and β2-microglobulin in tumors of patients with NSCLC. The mean of five independent areas occupied by each of these three immunostaining intensity categories (strongly positive, weakly positive, or absent) of HLA class I heavy chain and β2-microglobulin in each of the cases was calculated and graded in 10% steps. The cases were further divided into 5 categories: Homogeneous strong staining (Group A), heterogeneous strong/weak staining (Group B), homogeneous weak staining (Group C), heterogeneous strong/weak/absent staining (Group D) and heterogeneous weak/absent staining (Group E).

Regarding the HLA class I heavy chain staining, of the 111 cases, 31 (27.9%) were classified into Group A, 38 (34.2%) into Group B, 12 (10.8%) into Group C, 21 (18.9%) into Group D and 9 (8.1%) into Group E (Fig. 3A). With regards to β2-microglobulin staining, 12 tumors (10.8%) were classified into Group A, 37 (33.3%) into Group B, 5 (4.5%) into Group C, 48 (43.2%) into Group D and 9 (8.1%) into Group E (Fig. 3B).

Survival analysis and HLA class I heavy chain and β2-microglobulin expression. The 5-year disease-specific survival rate was 62.2% for all patients (data not shown). Kaplan-Meier survival curves for each HLA class I heavy chain
Figure 1. Representative images of western blot analyses for (A) HLA and (B) β2m in human lung cancer cell lines (top panel) and lysates from mouse xenograft models constructed using the lung cancer cell lines (bottom panel). Lysates from homogenized NHLT and human PBMCs were used as non-tumor control and PC, respectively. HLA, human leukocyte antigen class I heavy chain; β2m, β2-microglobulin; LCMS, RERF-LC-MS; LCD, VMRC-LCD; AC, adenocarcinoma; SCC, squamous cell carcinoma; NHLT, normal human lung tissue; PBMCs, peripheral mononuclear cells; PC, positive control.

Figure 2. Immunohistochemical staining for HLA and β2m. The top panel includes images of HLA and β2m staining in mouse xenograft specimens. Expression of (A) HLA or (B) β2m in PC10-derived tumor. Absence of (C) HLA expression or (D) β2m expression in LK2-derived tumor. Magnification, x400. The bottom two panels are images of non-small-cell lung cancer samples in which the cell membranes are stained for HLA or β2m. (E) Strong, (F) weak and (G) no staining for HLA. (H) Strong, (I) weak and (J) no staining for β2m. Larger panel magnification, x100; smaller panel magnification, x400. HLA, human leukocyte antigen class I heavy chain; β2m, β2-microglobulin.
expression group and for intergroup comparisons are plotted in Fig. 4A. The overall prognosis of groups without an absent part of HLA class I heavy chain expression in tumors (Group A-C) was favorable compared with the groups with absent parts of HLA class I heavy chain expression (Group D-E; \( P=0.039 \); Fig. 4B and C). When divided according to UICC 7th cancer
stage, in 71 cases with stage I, 51 cases in Group A-C had a favorable prognosis compared with 20 cases in Group D-E (P=0.048; Fig. 4D), but no significant difference in survival rate was observed between the groups of stages II-III (P=0.104; data not shown).

The survival curves according to β2-microglobulin expression groups are plotted in Fig. 5A. No significant difference in survival rate was observed between the groups overall (Fig. 5B).

Associations between HLA class I heavy chain and β2-microglobulin according to immunostaining expression patterns. From the results of survival analysis of HLA class I heavy chain expressions, Group A-C was defined as the ‘positive’ group and Group D-E as the ‘negative’ group in both of HLA class I heavy chain and β2-microglobulin in the present study (Table I). When HLA class I heavy chain and β2-microglobulin were analyzed together, 46 tumors (41.4%) were double positive, 35 (31.5%) were positive only for HLA class I heavy chain, 8 (7.2%) were positive only for the β2-microglobulin antigen and 22 (19.8%) were double negative. Additionally, a significant association between the expression of HLA class I heavy chain and β2-microglobulin was observed (P=0.005).

Table I. Tumor HLA class I heavy chain expression and clinicopathological variables.

| Variables                  | Negative (n=30) | Positive (n=81) | P-value |
|----------------------------|-----------------|-----------------|---------|
| Age, years                 |                 |                 |         |
| ≤62                        | 11              | 47              | 0.045   |
| >62                        | 19              | 34              |         |
| Sex                        |                 |                 | 0.255   |
| Male                       | 22              | 50              |         |
| Female                     | 8               | 31              |         |
| Histology                  |                 |                 | 0.312†  |
| AC                         | 13              | 43              |         |
| SQCC                       | 13              | 25              |         |
| LCC                        | 2               | 11              |         |
| Other                      | 2               | 2               |         |
| T classification           |                 |                 | 0.825   |
| pT1                        | 13              | 37              |         |
| pT2-4                      | 17              | 44              |         |
| Lymph node metastasis      |                 |                 | 0.760   |
| Absence                    | 22              | 57              |         |
| Presence                   | 8               | 24              |         |
| Histopathological grading  |                 |                 | 0.835   |
| G1                         | 9               | 22              |         |
| G2-3                       | 17              | 46              |         |
| TNM stage                  |                 |                 | 0.718   |
| I                          | 20              | 51              |         |
| II-III                     | 10              | 30              |         |
| β2-microglobulin staining  |                 |                 | 0.005   |
| Positive                   | 8               | 46              |         |
| Negative                   | 22              | 35              |         |

†Calculated using Fisher’s exact test. AC, adenocarcinoma; SCC, squamous cell carcinoma; LCC, large-cell carcinoma; Other, adenosquamous carcinoma (3 cases) and carcinosarcoma (1 case); TNM, Tumor-Node-Metastasis.

Tumor HLA class I heavy chain expression and clinicopathological features. The tumor HLA class I heavy chain expression results were analyzed against the clinicopathological data of the patient and no significant associations were detected between the expression levels and sex, histology, T classification, N classification, histopathological grading or TNM stage (Table I).

Univariate and multivariate analyses. Univariate analysis for overall survival using a Cox regression model included age, sex, T classification, N classification, TNM stage, histopathological grade and tumor expression of HLA class I heavy chain for the patient cohort included in this study. Multivariate analysis on the same set of patients was performed for T classification, N classification, and tumor expression of HLA class I heavy chain. The results indicated that absence of the HLA class I heavy chain is an independent prognostic factor.
Table II. Univariate and multivariate analysis of clinicopathological factors affecting overall survival of patients.

| Variables                                                                 | Univariate analysis | Multivariate analysis |
|---------------------------------------------------------------------------|---------------------|-----------------------|
|                                                                           | HR                  | 95% CI                | P-value | HR                  | 95% CI                | P-value |
| Age (>62 vs. ≤62), years                                                 | 1.77                | 0.97-3.25             | 0.065   | 3.48                | 1.70-7.14             | 0.001   |
| Sex                                                                       | 1.98                | 0.99-3.94             | 0.051   | 3.72                | 1.99-6.94             | <0.001  |
| T-classification (1 vs. 2-4)                                              | 4.08                | 2.00-8.33             | <0.001  | 3.94                | 2.25-7.69             | <0.001  |
| N-classification                                                          | 4.17                | 2.25-7.69             | <0.001  | 3.72                | 1.99-6.94             | <0.001  |
| TNM stage (I vs. II-III)                                                 | 4.61                | 2.45-8.62             | <0.001  | 3.72                | 1.99-6.94             | <0.001  |
| Histopathological grading (1 vs. 2-3)                                     | 1.31                | 0.67-2.54             | 0.433   | 1.96                | 1.06-3.62             | 0.032   |
| HLA class I heavy chain staining (negative vs. positive)                 | 1.88                | 1.02-3.47             | 0.043   | 1.96                | 1.06-3.62             | 0.032   |

HR, hazard ratio; CI, confidence interval; TNM, Tumor-Node-Metastasis.

(P=0.032). Additionally, T and N classification had independent prognostic value, with hazard ratios of 3.48 (P=0.001) and 3.72 (P<0.001), respectively (Table II).

**Discussion**

The aim of the present study was to develop a quantitative immunohistochemistry protocol to investigate whether expression of HLA class I in NSCLC was associated with patient survival. Previously reported associations are controversial and difficult to compare because of differences in antibodies, positive controls and classification systems (Table III). A reproducible and carefully controlled quantitative immunohistochemistry method was developed, providing a robust approach for accurate classification of NSCLC specimens into categories based on HLA class I expression levels and characteristics. One of the most important issues in previous studies was a lack of consideration of tumor heterogeneity in immunohistochemistry evaluation. Therefore, in the present study, HLA class I downregulation was classified in consideration of tumor heterogeneity, assessed using EMR8-5 and SCID mouse xenografts. This methodology may be useful for others working in the field, and may provide a means to improve the consistency and comparability of similar studies in the future.

Whereas strong expression of HLA class I in the carcinoma cell membrane was easily distinguished from lack of expression, the categorization of tumors with weak expression was more challenging. To address this, the NSCLC cases were divided into 5 groups according to the HLA class I expression pattern. Furthermore, these 5 groups were classified into different combinations of 2 groups primarily based on strong or absent expression of HLA class I. The correlations between prognosis and the different combinations of groups were investigated. Groups of patients with absent expression of parts of HLA class I heavy chain demonstrated a significantly unfavorable prognosis compared with no absence of HLA class I. The present data may reflect the result of emerging immune-escaping variants, indicating the intra-tumor heterogeneity for HLA class I. However, although there were notable associations in immunostaining expressions with HLA class I heavy chain, β2-microglobulin was not associated with the prognosis of patients with lung cancer in our cohort.

In the present study, the absence of HLA class I heavy chain expression on at least a part of the tumors (in Group D or E) was revealed to be an independent factor predicting poor prognosis, although expression of this factor was not associated with the tested clinicopathological variables. These results suggest that NSCLC would progress regardless of the expression of HLA class I heavy chain, and host immunity serves a limited role in influencing tumor growth prior to surgery. However, following surgery, relapse occurred in a higher number of cases with absent parts of HLA class I compared with cases with detectable levels of expression. Furthermore, the prognosis was particularly favorable for patients with cancer stage I and HLA class I heavy chain-positive cases, but in patients with advanced stage (II or III) cancer, no association was observed between prognosis and HLA class I heavy chain expression, in agreement with previous studies (17,19). Therefore, the most suitable individuals for treatment with immune therapy for NSCLC may be patients with earlier stages of cancer.

It is well accepted that inflammation or an inflammatory gene expression in tumors is associated with anti-tumor immune responses (25,26). MHC class I expression is predominantly upregulated by interferon-γ in the tumor microenvironment. In addition, interferon-γ is essential for eliciting the immune response to tumor cells (27). Therefore, the findings of the present study, which link HLA class I heavy chain expression with prognosis, may involve interferon-γ signaling. Certain active immunotherapies, including novel agents capable of generating anti-tumor immunity, have been reported to exert clinical efficacy against lung cancer (28), and may act by boosting the interferon-γ signature in the tumor microenvironment. Notably, immunological responses were independent of stage and prior therapy in a study of NSCLC dendritic cell vaccines (29), consistent with the possibility that the effectiveness of immunotherapy may be associated with expression of HLA class I heavy chain in tumor cells.

Currently, recurrent or surgically non-resectable tumors are often treated with immunotherapy, although the immune system of the patients has a limited capacity to deal with the heavy tumor burden. The results of the present study suggest that immunotherapy would be beneficial as postoperative adjuvant therapy in NSCLC.
| Study                  | Patients, Histology | Loss or downregulation, Tissue Antibody | Definition of downregulation | Positive control | Association between prognosis and expression (Ref.s.) |
|-----------------------|--------------------|-----------------------------------------|-----------------------------|------------------|------------------------------------------------------|
| Redondo et al, 1991   | AC 59 (100%)       | Frozen W6/32 HC-10                     | <5% staining            | Not described    | Not examined                                         |
|                       | SCC 15 (25%)       |                                         |                            |                  |                                                       |
|                       |                    |                                         |                            |                  |                                                       |
| Korkolopoulou et al,  | AC 93 (100%)       | Frozen W6/32 HC-10                     | ≤75% staining            | Normal lung tissue| Epithelium and lymphocytes                            |
| 1996                  | SCC 29 (31%)       |                                         |                            |                  |                                                      |
|                       |                    |                                         |                            |                  |                                                      |
| Redondo et al, 1997   | AC 123 (100%)      | Frozen W6/32 HC-10                     | <20% staining            | Stromal cells    | Not examined                                         |
|                       | SCC 38 (31%)       |                                         |                            |                  |                                                      |
|                       |                    |                                         |                            |                  |                                                      |
| Ramnath et al, 2006   | AC 190 (100%)      | FFPE HC-10                             | ≤75% staining            | Non-neoplastic lung tissue                           | No association                                       |
|                       | SCC 109 (57%)      |                                         |                            |                  |                                                      |
|                       |                    |                                         |                            |                  |                                                      |
| Kikuchi et al, 2007   | AC 161 (100%)      | FFPE EMR8-5                            | <80% staining            | Endothelial cells                                      | Loss/downregulation associated with poorer prognosis |
|                       | SCC 64 (34%)       |                                         |                            |                  | in early stages                                       |
|                       |                    |                                         |                            |                  |                                                      |
| Torigoe et al, 2012   | AC 83 (52%)        | FFPE EMR8-5                            | <75% staining            | Endothelial cells and stromal lymphocytes             | Not examined                                         |
|                       | SCC 68 (42%)       |                                         |                            |                  |                                                      |
|                       |                    |                                         |                            |                  |                                                      |
| Torigoe et al, 2012   | AC 35 (100%)       | FFPE EMR8-5                            | <75% staining            | Endothelial cells and stromal lymphocytes             | Not examined                                         |
|                       | SCC 64 (34%)       |                                         |                            |                  |                                                      |
|                       |                    |                                         |                            |                  |                                                      |
| Ichinokawa et al, pre| AC 111             | FFPE EMR8-5                            | No staining              | SCID mouse xenograft                                    | Loss/downregulation associated with a poorer         |
| sent study            | SCC 38             |                                         |                            |                  | prognosis                                             |
|                       |                    |                                         |                            |                  |                                                      |

AC, adenocarcinoma; SCC, squamous cell carcinoma; N/A, not applicable; FFPE, formalin fixed paraffin embedded.
The authors would like to thank Mr Hiraku Shida and Ms Naomi Saito, The Affiliated Department of Gastroenterological Surgery II, Hokkaido University Graduate School of Medicine (Sapporo, Japan), for their technical assistance.

Funding

The present study was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan (grant no. 18591534).

Acknowledgements

The present study was supported by a Grant-in-Aid from Hokkaido University Graduate School of Medicine (Sapporo, Japan), for their technical assistance.

Surgery II, Hokkaido University Graduate School of Medicine

Naomi Saito, The Affiliated Department of Gastroenterological Surgery II, Hokkaido University Graduate School of Medicine

Acknowledgements

The authors would like to thank Mr Hiraku Shida and Ms Naomi Saito, The Affiliated Department of Gastroenterological Surgery II, Hokkaido University Graduate School of Medicine (Sapporo, Japan), for their technical assistance.

Funding

The present study was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan (grant no. 18591534).

Availability of data and materials

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

Authors' contributions

KI designed the present study, analyzed and interpreted the patient data, performed the histological examination, and was a major contributor in writing the manuscript. YN interpreted the data and drafted the manuscript. YH, TK and KK performed the surgical procedures and treated the specimens. TT interpreted the data and revised the manuscript. TI performed the histological examinations and evaluated the immunohistochemical specimens. MK performed the operations and treated the specimens. SH interpreted the data and drafted the manuscript.

Ethics approval and consent to participate

Patients provided informed consent to participate in the present study, which was approved by The Hokkaido University Institutional Review Board (approval no. 017-0476). Mice specimens used in this study were obtained from a previous study which was conducted in accordance with the guidelines of The Hokkaido University Institutional Animal Care and Use Committee using an approval protocol (approval no. 04157).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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