

Downregulated expression of human leukocyte antigen class I heavy chain is associated with poor prognosis in non-small-cell lung cancer

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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 β 2-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 β 2-microglobulin (57 cases; 51.4%). Downregulation of HLA class I heavy chain expression was significantly associated with the downregulation of β 2-microglobulin. Cases lacking HLA class I heavy chain as well as β 2-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 β 2-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).

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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 and housed with food and water *ad libitum* under specific pathogen free conditions. The room was kept on a 12/12-h light/dark cycle, at 22.0-24.0°C. Humidity was not regulated. All animal procedures were conducted in accordance with the guidelines of the Hokkaido University Institutional Animal Care and Use Committee using an approval protocol.

A total of 6 mice per one cell line (54 in total) were used. Xenografts from the lung cancer cell lines were generated by injecting 5×10⁶ cells in 200 µl PBS subcutaneously into the flanks of the CB17/SCID mice. When the tumor diameter exceeded 10 mm, the mice were sacrificed and the tumors were separated into 2 portions. One portion was snap frozen using liquid nitrogen to extract proteins for western blot analysis, and the other was immersed in 10% formalin and incubated at 4°C for 24 h for immunohistological analysis.

Reagents and antibodies. The pan anti-human HLA-A, -B, and -C antigen mouse EMR8-5 monoclonal antibody (cat. no. FA01) and anti-β2-microglobulin mouse EMRB6-12 monoclonal antibody (cat. no. GB-01B) were purchased from Hokudo Co., Ltd. (Sapporo, Japan). Negative control mouse IgG1 (cat. no. X0931) was purchased from Dako (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA). Peroxidase-conjugated goat anti-mouse IgG (cat. no. 115-035-003) was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Anti β-actin mouse antibody (cat. no. MAB 150-1R; Chemicon International; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used as the loading control of western blot analysis.

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 × 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.

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

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 μ m. 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- $\beta 2$ -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 (Micro Slides; Muto Pure Chemicals Co., Ltd., Tokyo, Japan). The categories and percentages of immunohistochemical stained cells were assessed in five independent high-power (magnification, $\times 400$) microscopic fields for each tissue sample using a light microscope.

Statistical analysis. The statistical significance of associations between HLA class I heavy chain and $\beta 2$ -microglobulin expression in cancer cells was evaluated using the Mann-Whitney U test. The χ^2 test (or extended Fisher's exact test, where appropriate) was used to analyze the comparisons between HLA class I heavy chain or $\beta 2$ -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 $\beta 2$ -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). $\beta 2$ -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 $\beta 2$ -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 $\beta 2$ -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 $\beta 2$ -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 $\beta 2$ -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 $\beta 2$ -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 $\beta 2$ -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 $\beta 2$ -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 $\beta 2$ -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

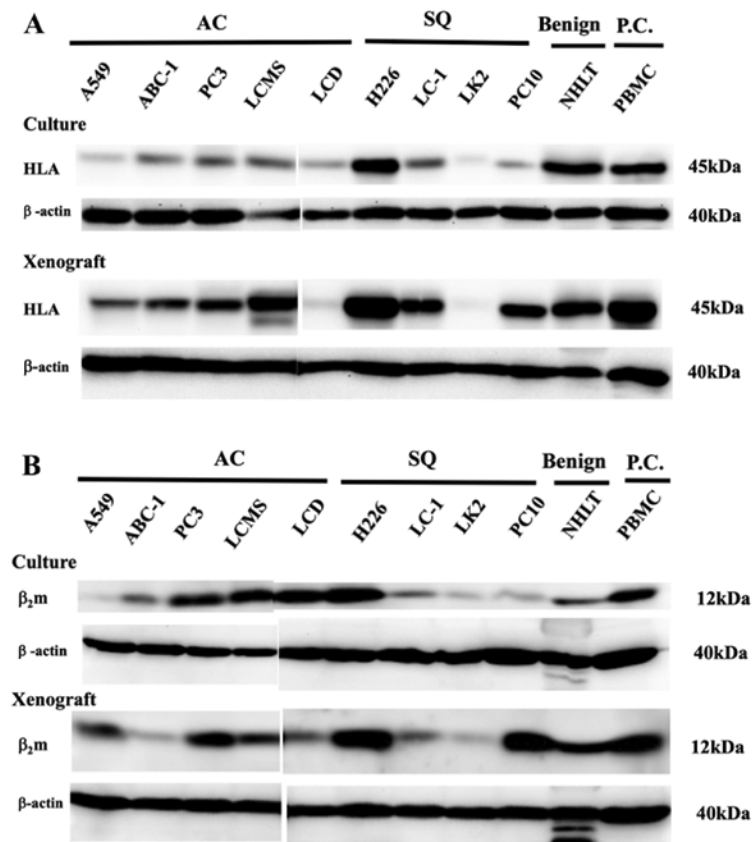


Figure 1. Representative images of western blot analyses for (A) HLA and (B) β₂m in human lung cancer cell lines (top panel) and lysates from mouse xenograft models constructed using the lung cancer cell lines (bottom panel). Lysates from homogenized NHLT and human PBMCs were used as non-tumor control 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.

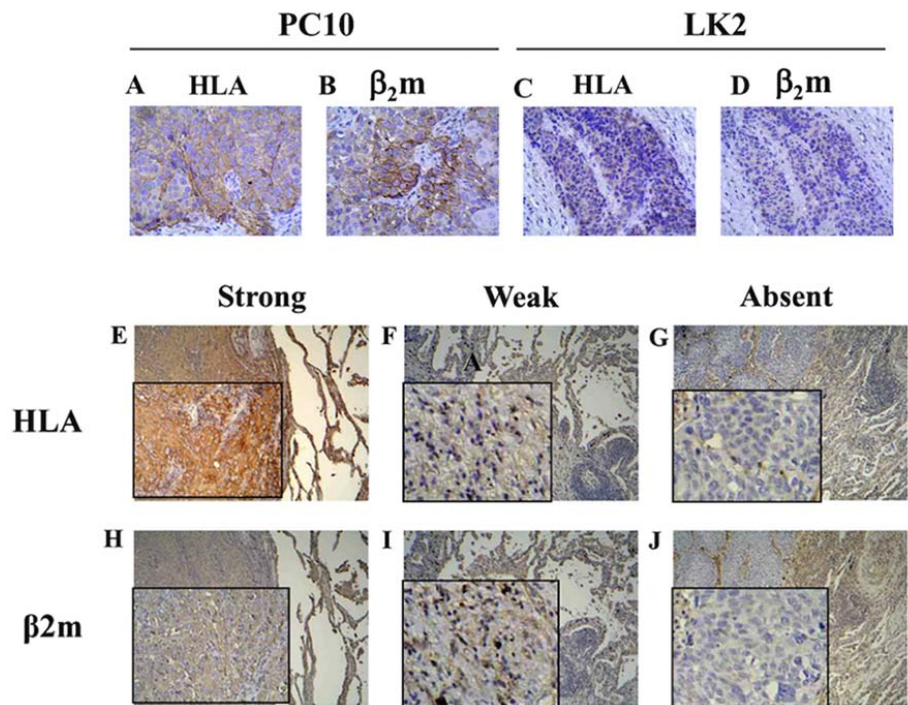


Figure 2. Immunohistochemical staining for HLA and β₂m. The top panel includes images of HLA and β₂m staining in mouse xenograft specimens. Expression of (A) HLA or (B) β₂m in PC10-derived tumor. Absence of (C) HLA expression or (D) β₂m expression in LK2-derived tumor. Magnification, x400. The bottom 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.

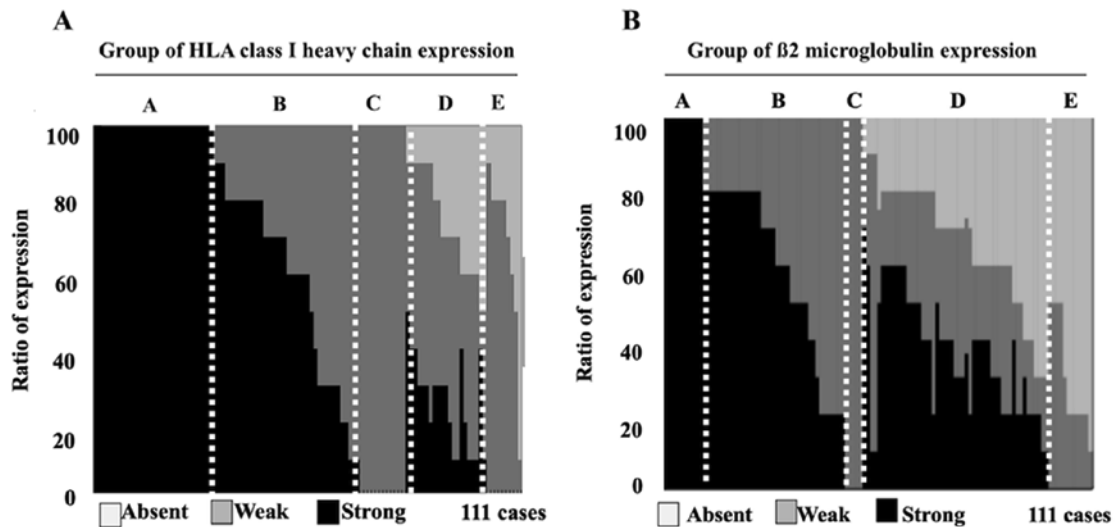


Figure 3. HLA class I heavy chain and β 2-microglobulin expressions. (A) HLA class I heavy chain expression in NSCLC. Group A, n=31; group B, n=38; group C, n=12; group D, n=21; and group E, n=9. (B) β 2-microglobulin expression in NSCLC. Group A, n=12; group B, n=37; group C, n=5; group D, n=48; and group E, n=9. HLA, human leukocyte antigen; NSCLC, non-small-cell lung cancer; group A, homogeneous strong staining; group B, heterogeneous strong/weak staining; group C, homogeneous weak staining; group D, heterogeneous strong/weak/absent staining; group E, heterogeneous weak/absent staining.

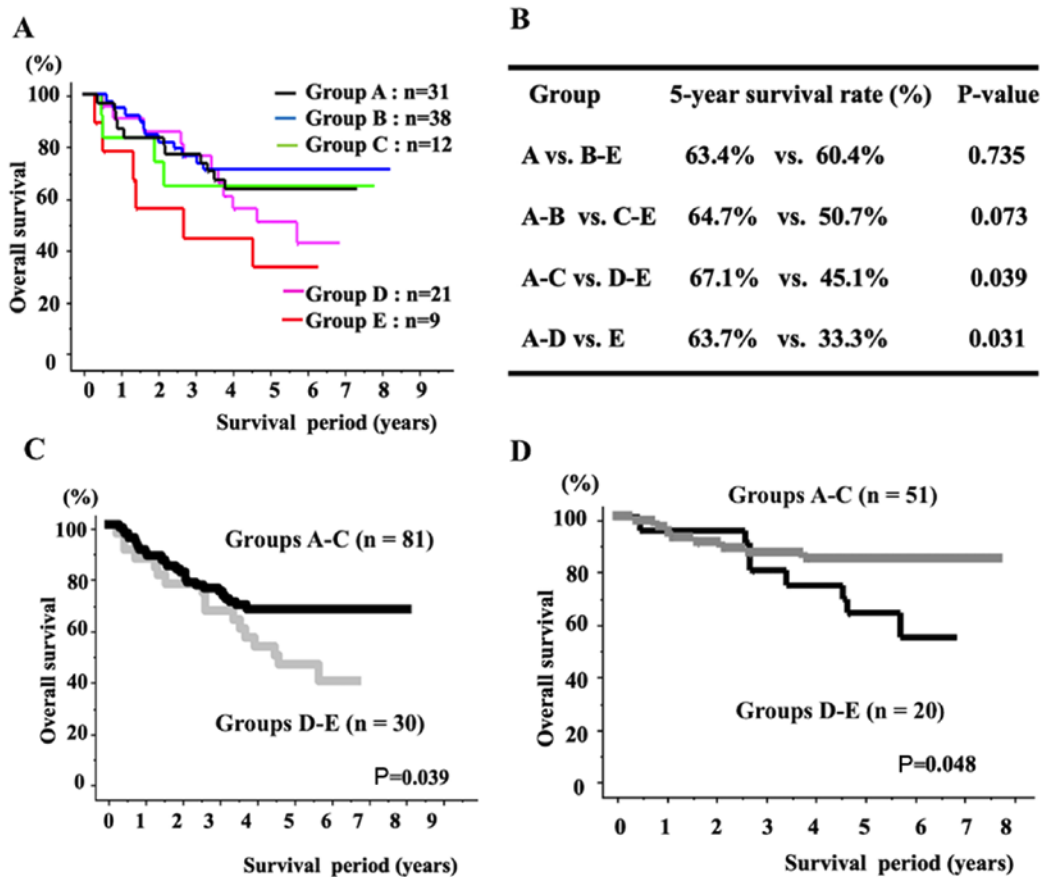


Figure 4. Survival analysis of HLA class I heavy chain. (A) Survival curves of patients with NSCLC divided into 5 groups according to the tumor HLA class I heavy chain expression. No significant differences in prognosis were observed among the groups. (B) Survival analyses where patients were divided into 2 groups according to expression of HLA class I heavy chain. (C) Survival curves of patients with NSCLC divided into 2 groups according to the HLA class I heavy chain expression, positive (n=81) and negative (n=30). (D) Survival curves of patients with stage I NSCLC according to their HLA class I heavy chain expression, positive (n=67) and negative (n=24). HLA, human leukocyte antigen; NSCLC, non-small-cell lung cancer; group A, homogeneous strong staining; group B, heterogeneous strong/weak staining; group C, homogeneous weak staining; group D, heterogeneous strong/weak/absent staining; group E, heterogeneous weak/absent staining.

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

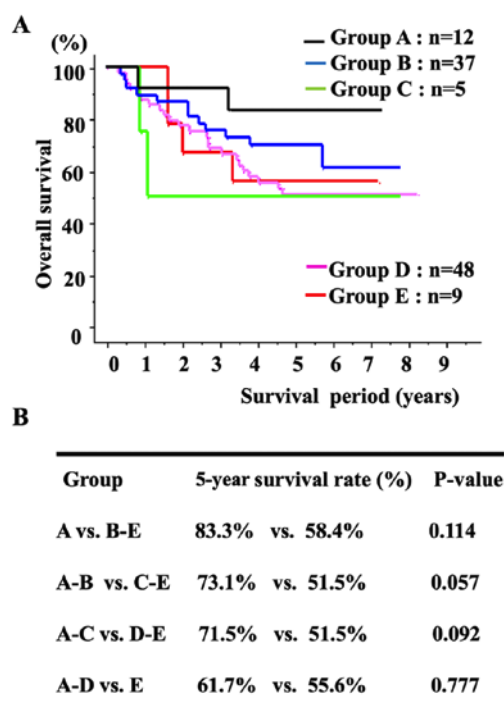


Figure 5. Survival analysis of β 2-microglobulin expression. (A) Survival curves of patients with NSCLC divided into 5 groups according to tumor β 2-microglobulin expression. No significant differences in prognosis were observed among the groups. (B) Survival analyses where patients were divided into 2 groups according to expression of β 2-microglobulin. NSCLC, non-small-cell lung cancer; group A, homogeneous strong staining; group B, heterogeneous strong/weak staining; group C, homogeneous weak staining; group D, heterogeneous strong/weak/absent staining; group E, heterogeneous weak/absent staining.

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 β 2-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 β 2-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 β 2-microglobulin in the present study (Table I). When HLA class I heavy chain and β 2-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 β 2-microglobulin antigen and 22 (19.8%) were double negative. Additionally, a significant association between the expression of HLA class I heavy chain and β 2-microglobulin was observed ($P=0.005$).

Tumor HLA class I heavy chain expression and clinicopathological features. The tumor HLA class I heavy chain

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

Variables	HLA class I heavy chain staining, n		P-value
	Negative (n=30)	Positive (n=81)	
Age, years			0.045
≤62	11	47	
>62	19	34	
Sex			0.255
Male	22	50	
Female	8	31	
Histology			0.312 ^a
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	
β 2-microglobulin staining			0.005
Positive	8	46	
Negative	22	35	

^aCalculated using Fisher's exact test. AC, adenocarcinoma; SCC, squamous cell carcinoma; LCC, large-cell carcinoma; Other, adeno-squamous carcinoma (3 cases) and carcinosarcoma (1 case); TNM, Tumor-Node-Metastasis.

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			
Sex	1.98	0.99-3.94	0.051			
T-classification (1 vs. 2-4)	4.08	2.00-8.33	<0.001	3.48	1.70-7.14	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			
Histopathological grading (1 vs. 2-3)	1.31	0.67-2.54	0.433			
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, β_2 -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.

Table III. Studies describing human leukocyte antigen class I heavy chain expression in non-small-cell lung cancer.

Study	Histology	Patients, n (%)	Loss or downregulation, n (%)	Tissue	Antibody	Definition of downregulation	Positive control	Association between prognosis and expression (Refs.)
Redondo <i>et al</i> , 1991	AC	59 (100%)	16 (27%)	Frozen	W6/32	<5% staining	Not described	Not examined (14)
	SCC	15 (25%) 40 (68%)	-		HC-10			
Korkolopoulou <i>et al</i> , 1996	AC	93 (100%)	37 (40%)	Frozen	W6/32	≤75% staining	Normal lung tissue	No association (15)
		29 (31%)	10 (35%)		HCA2		Epithelium and lymphocytes	
Redondo <i>et al</i> , 1997	SCC	57 (61%)	27 (47%)		MA2.1			Not examined (18)
		123 (100%)	36 (29%)	Frozen	W6/32	<20% staining	Stromal cells	
	AC	38 (31%)	-		HC-10			
	SCC	30 (24%)	-					
Ramnath <i>et al</i> , 2006		190 (100%)	178 (94%)	FFPE	HC-10	≤75% staining	Non-neoplastic lung tissue	No association (16)
Kikuchi <i>et al</i> , 2007	AC	109 (57%)	104 (95%)					Loss/downregulation associated with poorer prognosis in early stages (17)
	SCC	64 (34%)	58 (91%)					
Torigoe <i>et al</i> , 2012		161 (100%)	111 (69%)	FFPE	EMR8-5	<80% staining	Endothelial cells	Not examined (20)
	AC	83 (52%)	47 (57%)				Stromal lymphocytes	
	SCC	68 (42%)	57 (84%)				Endothelial cells and stromal lymphocytes	
Hanagiri <i>et al</i> , 2013		35 (100%)	7 (20%)	FFPE	EMR8-5	<75% staining	Endothelial cells	Loss/downregulation associated with poorer prognosis in early stages (19)
		136 (100%)	87 (64%)	FFPE	EMR8-5	<80% staining	Endothelial cells	
Ichinokawa <i>et al</i> , present study	AC	96 (71%)	56 (58%)				Stromal lymphocytes	Loss/downregulation associated with a poorer prognosis N/A
	SCC	33 (24%)	25 (76%)					
		111	30 (27%)	FFPE	EMR8-5	No staining	SCID mouse xenograft	
							Endothelial cells	
	AC	56	13 (23%)					
	SCC	38	13 (34%)					

AC, adenocarcinoma; SCC, squamous cell carcinoma; N/A, not applicable; FFPE, formalin fixed paraffin embedded.

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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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