HLA class I antigen expression is associated with a favorable prognosis in early stage non-small cell lung cancer

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Human leukocyte antigen (HLA) class I displays a repertoire of endogenously processed peptides to CD8$^+$ T lymphocytes. The present study assessed correlations between HLA class I expression, clinicopathologic factors, and tumor-infiltrating immune cells in human non-small cell lung cancers (NSCLC). Expression of HLA class I was assessed in 161 resected primary NSCLC by immunohistochemistry using EMR8–5, a novel monoclonal anti-pan HLA class I heavy chain antibody. Expression of HLA class I was classified into three categories: strongly positive, weakly positive, or negative. Tumor-infiltrating CD8$^+$ lymphocytes and CD56$^+$ natural killer cells within cancer nests and stroma were also counted. Expression of HLA class I was strongly positive in 50 tumors, weakly positive in 57 tumors, and negative in 54 tumors. Down-regulation of HLA class I was significantly correlated with male sex, history of smoking, non-adenocarcinoma histology, and moderate-/low-grade differentiation. The density of cancer nest-infiltrating CD8$^+$ cells in HLA class I-negative tumors was significantly decreased compared to that in HLA class I strongly positive tumors ($P < 0.01$). Kaplan–Meier analysis revealed a significant favorable influence on overall survival for patients displaying tumors with strongly positive expression of HLA class I ($P < 0.01$). Multivariate analysis revealed down-regulation of HLA class I as an independent factor of poor prognosis in pathological stage I patients, but not in late-stage patients. These results suggest that down-regulation of HLA class I expression in NSCLC is a marker of poor prognosis, and this may play a critical role in immune surveillance of patients with NSCLC. (*Cancer Sci* 2007; 98: 1424–1430)

Human leukocyte antigen (HLA) class I antigens are transmembrane glycoproteins comprising a polymorphic 45-kDa heavy chain and a non-polymorphic 12-kDa β_2-microglobulin light chain. These antigens are expressed on the surface of most nucleated cells in the human body, and display a repertoire of endogenously processed peptides, such as viral or tumor antigens, to CD8$^+$ T lymphocytes.[1] Loss or down-regulation of HLA class I expression has been reported at 16–50% among various types of cancer.[2–4] In addition, the frequency of HLA class I defects is reportedly higher in metastatic lesions than in primary or premalignant lesions. Down-regulation of HLA class I expression is thus thought to be one of the mechanism that allows tumor cells to escape immunosurveillance.[2,5] Down-regulation of HLA class I expression reportedly correlated with tumor stage in ovarian cancer,[6] and low-grade differentiation in breast cancer and non-small cell lung cancer (NSCLC),[7,8] and also reportedly was a poor prognostic factor in esophageal cancer[9] and colon cancer.[10] However, no correlations with prognosis have been found for NSCLC.[11–13]

Torigoe *et al*. recently established a novel monoclonal anti-pan HLA class I heavy chain antibody named EMR8-5, allowing detection of HLA-A, -B, and -C antigens in formalin-fixed paraffin-embedded tissue.[14] In analysis using this antibody, expression of HLA class I on tumor cells contributed significantly to the therapeutic effects of Bacillus Calmette–Guérin (BCG) immunotherapy for bladder cancer,[15] and correlated with a favorable prognosis in osteosarcoma[16] and clear cell renal cancer.[17] The present study assessed the expression of HLA class I in NSCLC by immunohistochemistry using EMR8-5 antibody, and analyzed correlations with clinicopathologic factors and tumor-infiltrating immune cells.

Materials and Methods

Study population. A retrospective analysis of patients diagnosed with NSCLC who underwent surgery as initial treatment at the Hokkaido University Medical Hospital was conducted between 1982 and 1994. The present study included 161 patients for whom adequate archival primary tissues were available for analysis. Of these, 131 patients overlapped with the patients for whom we have previously reported about tumor-infiltrating CD8$^+$ and CD4$^+$ T cells in NSCLC.[18] We cut paraffin blocks into new sections and updated survival data in May 2006. Histological diagnosis and grade of differentiation were determined in accordance with the World Health Organization criteria for histopathologic classification.[19] The pathologic stage was based on the American Joint Committee on Cancer guidelines for postoperative tumor–node–metastasis (TNM).[20] Survival data were available for 150 of the 161 patients who survived for >3 months after surgery. The median follow-up time among patients who did not die was 3349 days.

Immunohistochemistry. Resected primary tumor specimens fixed in formalin and embedded in paraffin were cut into sequential 5-μm-thick sections and deparaffinized. Antigen retrieval was performed by autoclave heating at 121°C for 20 min in 10 mM citrate buffer (pH 6.0). After incubation in 0.3% hydrogen peroxide for 30 min, tissue slides were stained by immunohistochemistry using streptavidin–biotin complex (SimpleStain MAX-PO kit, Nichirei, Tokyo, Japan), followed by reaction with 3,3′-diaminobenzidine tetrahydrochloride-hydrogen peroxide as a chromogen and counterstaining using hematoxylin solution. The following clones of mouse monoclonal antibodies were used: EMR8-5 (anti-HLA class I heavy chain);[14] EMR-B6 (anti-β_2-microglobulin);[14] clone C8/144b (anti-CD8, DAKO, Glostrup, Denmark), clone 1B6 (anti-CD56, NovoCastra, Newcastle, UK).

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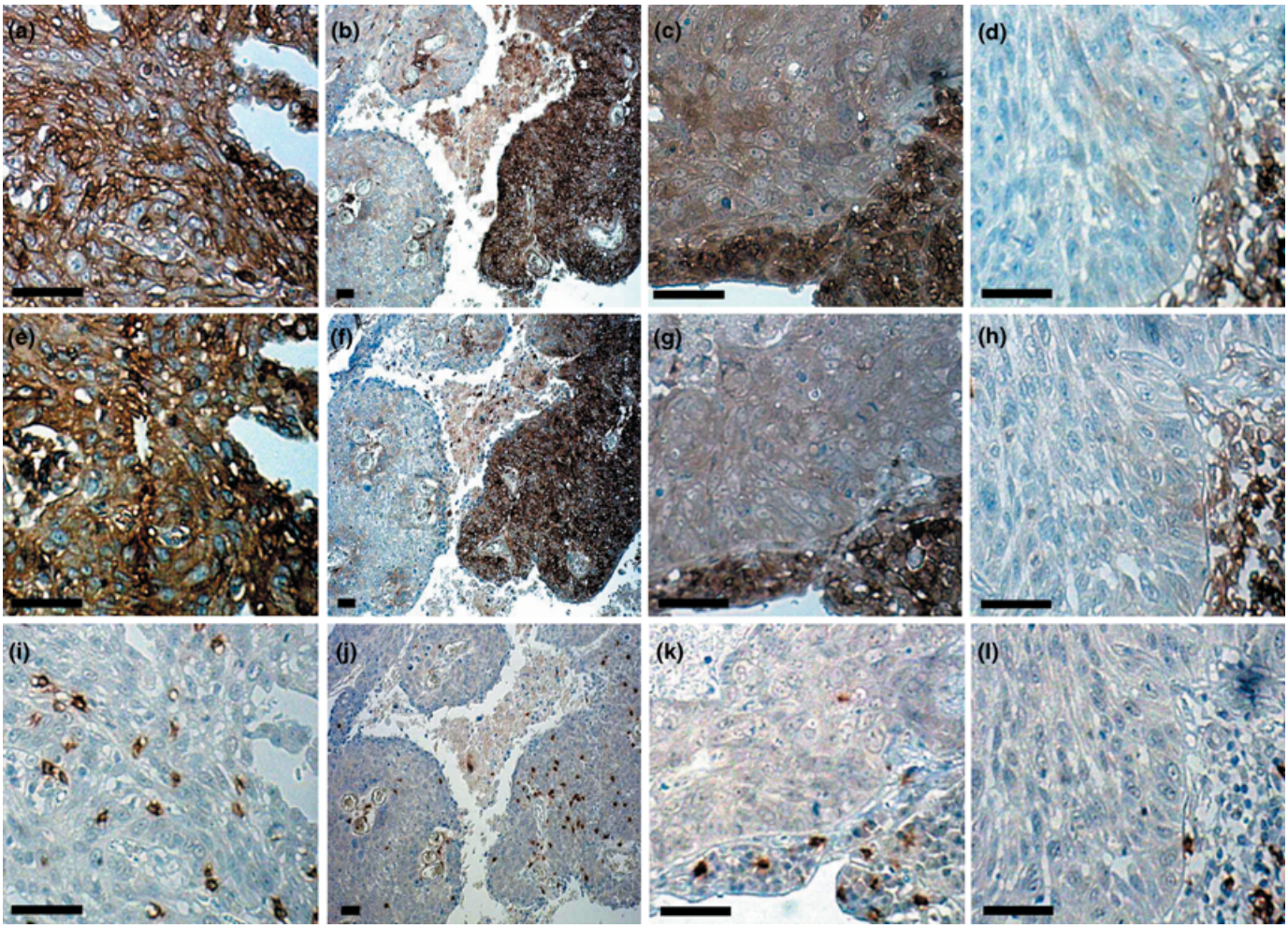

Fig. 1. Representative images of immunostaining with (a–d) EMR8-5 anti-human leukocyte antigen (HLA) class I heavy chain antibody, (e–g) EMR-b6 anti-β_2-microglobulin antibody, and (i–l) anti-CD8 antibody (scale bar = 50 μm). (a,e,i) Sequential sections from a case with strongly positive expression of HLA class I. (b,f,j) Sequential sections from a case with weakly positive (heterogeneous) expression of HLA class I. This specimen contained areas with both strongly positive and negative expression of HLA class I. (c,g,k) Sequential sections from a case with weakly positive (dim) expression of HLA class I. Tumor cells and cytoplasmic membrane showed weaker staining than lymphocytes in the stroma. (d,h,l) Sequential sections from a case with negative expression of HLA class I.

Expression of HLA class I and β_2-microglobulin. Expressions of HLA class I and β_2-microglobulin were assessed by two investigators (E.K. and K.Y.) who were blinded to the status of other immunohistologic and clinical data. The expression level of HLA class I on tumor cells was defined as previously described.[17] Briefly, expression was defined as strongly positive when tumor cells for which the membrane was strongly and homogenously stained at the same level as stromal lymphocytes or endothelial cells comprised >80% of tumor cells (Fig. 1a), weakly positive for 20–80% (heterogeneous pattern; Fig. 1b), or tumor cells with the membrane stained more weakly than stromal lymphocytes or endothelial cells comprising >20% (dim pattern; Fig. 1c), and negative when stained cells comprised <20% (Fig. 1d). Cases with negative or weakly positive staining were judged as representing down-regulation.

Measurement of tumor-infiltrating lymphocytes. Based on previous reports,[18,21] tumor-infiltrating immunoreactive cells were classified into two groups according to location: cells within cancer stroma adjacent to cancer cell nests; and cells within cancer cell nests themselves. For the measurement of tumor-infiltrating cells, microscopic fields were chosen under high magnification (×200) using an IX71 light microscope (Olympus, Tokyo, Japan), and digitized using a Camedia C-7070 digital camera (Olympus) at 0.30 mm^2/field, then histological images were saved on a computer. Analysis was performed using ImageJ software (available in the public domain from http://rsb.info.nih.gov/ij/). Each of 10 areas containing the highest abundance of positively stained immune cells within cancer cell nests or stroma were selected in every sample for closer examination. Next, the numbers of positively stained immune cells within cancer cell nests or stroma in these 10 areas were counted manually, and areas of interest (AOI) in cancer nests or stroma were drawn as appropriate to determine immune cell density in cancer nests or stroma, respectively. Mean cell density in the highest six fields was determined as the number of immune cells infiltrating into cancer nests or stroma.[22] Tumors showing replacing growth of pulmonary alveolar structure and not forming tumor cell nests were excluded from evaluation of tumor-infiltrating lymphocytes.

Statistical analysis. Concordance between the expression of HLA class I and β_2-microglobulin was assessed using Cohen's kappa. The χ^2 test (or extended Fisher's exact test when appropriate)

Table 1. Human leukocyte antigen (HLA) class I expression, clinicopathologic factors, and β_2-microglobulin expression

		HLA class I			*P*-value
		Strongly positive	Weakly positive	Negative	
All patients (%)		50 (31.1)	57 (35.4)	54 (33.5)	
Age (years)	<65	29	34	21	0.056†
	≥65	21	23	33	
Sex	Male	27	36	47	0.00083†
	Female	23	21	7	
Smoking	Never smoking	16	17	5	0.0082‡,§
	Ever smoking	30	34	44	
	Unknown	4	6	5	
Stage	I	32	35	28	0.41†
	II/III/IV	18	22	26	
T factor#	T1	16	19	9	0.097†
	T2/T3/T4	34	38	45	
N factor#	N0	35	38	34	0.75†
	N1/N2/N3	15	19	20	
Histology	Adenocarcinoma	36	28	19	0.00079†,¶
	Squamous cell carcinoma	11	26	31	
	Others	3	3	4	
Grade	Well	20	17	8	0.016†,††
	Moderate/poor	27	37	40	
	Unclassified	3	3	6	
β_2-microglobulin	Strongly positive	35	0	0	
	Weakly positive	12	45	4	
	Negative	3	12	50	

†*P*-value is calculated using χ^2 test. ‡*P*-value is calculated using extended Fisher's exact test. §*P*-value is for the comparison between never smoking patients and ever smoking patients. ¶*P*-value is for the comparison between adenocarcinoma and the others. ††*P*-value is for the comparison between well-differentiated carcinoma and the others. #T factor (the extent of the primary tumor) and N factor (lymph node metastasis) are determined according to the American Joint Committee on Cancer guidelines for post-operative tumor–node–metastasis (TNM).

was used to assess the significance of correlations between the expression of HLA class I and clinicopathologic factors. Densities of tumor-infiltrating immune cells were compared using the non-parametric Mann-Whitney *U*-test. Holm's correction was taken into consideration to avoid multiple testing errors. Overall patient survival was calculated from the date of surgery to the date of last follow up (censored) or date of patient death by any cause (event). Survival probabilities were calculated using the Kaplan–Meier method. Differences in survival time between patient subgroups were analyzed using the log-rank test. Uni- and multivariate analysis using Cox proportional hazards modeling was used to measure correlations between clinicopathologic variables and overall survival. A backward elimination method was used to determine independent variables with values of $P < 0.05$ for elimination. Values of $P < 0.05$ were considered statistically significant in all tests.

Results

Expression of HLA class I and β_2-microglobulin. Staining revealed 50 tumors (31.1%) with strongly positive expression of HLA class I antigen, 57 tumors (35.4%) with weakly positive expression, and 54 tumors (33.5%) with negative expression. Strongly positive, weakly positive, and negative expressions of β_2-microglobulin were found in 35 tumors (21.7%), 61 tumors (37.9%), and 65 tumors (40.3%), respectively. According to χ^2 testing, down-regulation of HLA class I expression significantly correlated with male sex, a history of smoking, non-adenocarcinoma histology, and moderate-/low-grade differentiation (Table 1). A significant correlation was identified between expressions of HLA class I and β_2-microglobulin, with Cohen's kappa coefficient of 0.71 (95% confidence interval [CI], 0.62–0.80; Table 1).

Cancer nest- and stroma-infiltrating CD8$^+$ cells. Tumor-infiltrating immune cells were assessed in 96 cases, except for tumors showing replacing growth of the pulmonary alveolar structure and not forming cancer cell nests. The median count of cancer nest-infiltrating CD8$^+$ cells was 137.2/mm^2 (range, 21.1–496.9/mm^2) in HLA class I-negative tumors, 277.3/mm^2 (range, 28.2–1415.0/mm^2) in HLA class I-weakly positive tumors, and 503.2/mm^2 (range, 105.4–1273.0/mm^2) in HLA class I-strongly positive tumors. The density of cancer nest-infiltrating CD8$^+$ cells in tumors with negative expression of HLA class I was significantly decreased compared to that in tumors with weakly positive expression of HLA class I ($P = 0.0019$), and compared to that in tumors with strongly positive expression of HLA class I ($P = 0.0000012$; Fig. 2a). A significant difference was also found between densities of CD8$^+$ cells infiltrating tumors with weakly positive and strongly positive expression of HLA class I ($P = 0.010$; Fig. 2a). Conversely, the median count of stroma-infiltrating CD8$^+$ cells was 805.3/mm^2 (range, 304.0–2061.0/mm^2) in HLA class I-negative tumors, 1013.0/mm^2 (312.3–2620.0/mm^2) in HLA class I-weakly positive tumors, and 899.0/mm^2 (421.0–2310.0/mm^2) in HLA class I-strongly positive tumors, with no significant correlations between HLA class I expression status and density of stroma-infiltrating CD8$^+$ cells (Fig. 2b).

The density of CD8$^+$ cells between HLA class I-negative areas and strongly positive areas in sections that displayed both negative and strongly positive areas ($n = 7$) was also compared. A significant scarcity of cancer nest-infiltrating CD8$^+$ cells was seen in HLA class I-negative areas ($P = 0.0023$; Fig. 3).

In contrast, very few CD56$^+$ cells were observed both in cancer nests and in stroma. Median counts of cancer nest-infiltrating

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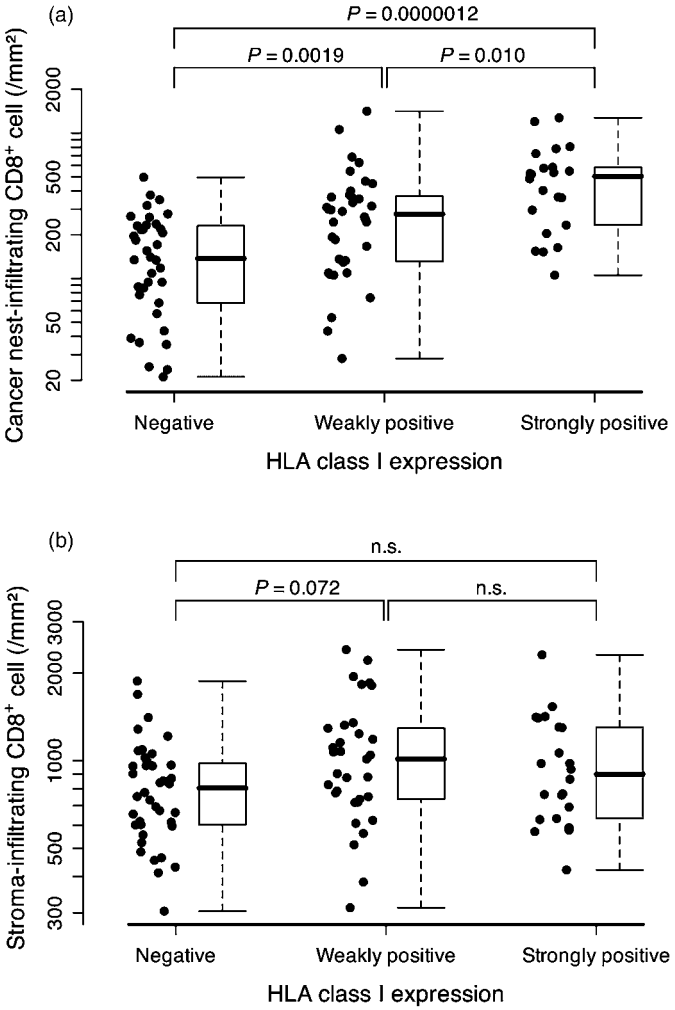

Fig. 2. Density of CD8⁺ cells infiltrating into (a) cancer nests and (b) stroma with negative, weakly positive, and strongly positive expression of human leukocyte antigen (HLA) class I. Box-and-whisker plots display median, interquartile range and extremes. *P*-values were calculated using the Mann–Whitney *U*-test with Holm's correction.

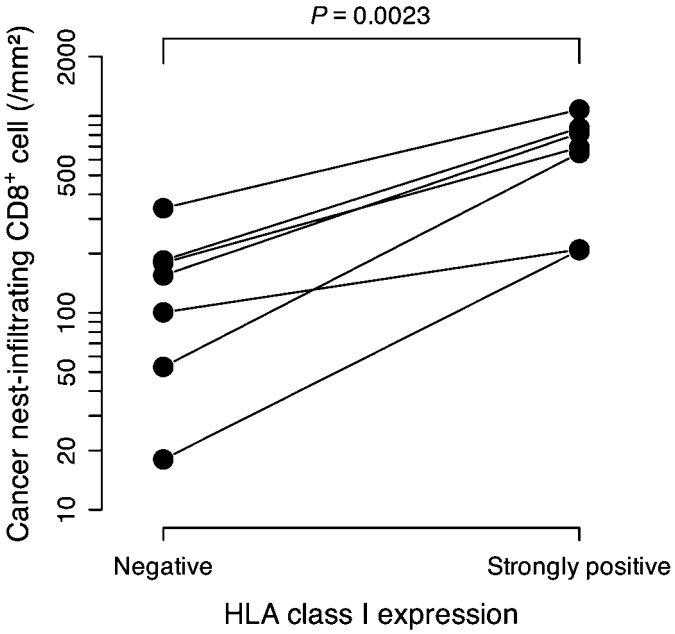

Fig. 3. Comparison of CD8⁺ cells infiltrating into cancer nests of human leukocyte antigen (HLA) class I-negative and -strongly positive areas within the same sections. *P*-values were calculated using the Mann–Whitney *U*-test.

and stroma-infiltrating CD56⁺ cells were 0.0/mm^2 regardless of expression status of HLA class I, with no significant correlation.

HLA class I expression and survival. In 150 patients for whom survival data was available, Kaplan–Meier analysis using the log-rank test revealed a significant favorable influence on overall survival for patients who had tumors with strongly positive expression of HLA class I compared to the down-regulation group ($P = 0.0051$; Fig. 4a). When stratified by pathological stage, stage I patients who had tumors with strongly positive expression of HLA class I showed significantly prolonged survival compared to the down-regulation group ($P = 0.0044$; Fig. 4b). Conversely, no correlation was found between HLA class I expression and survival of patients with pathologic stage II–IV NSCLC ($P = 0.85$ Fig. 4c). Significant poor prognostic factors in pathological stage I patients determined using univariate analysis comprised down-regulation of HLA class I expression and patient age (Table 2). Moreover, multivariate analysis using Cox's proportional hazards model revealed down-regulation of HLA class I expression as an independent factor associated with poor prognosis (adjusted hazard ratio, 2.59; 95% CI, 1.13–5.93) in patients with pathological stage I NSCLC (Table 2). Although HLA class I expression was correlated with sex, smoking history and histology in pathologic stage I patients, these clinicopathologic factors were not significant poor prognostic factors by univariate analysis, Moreover, the density of cancer nest-infiltrating CD8⁺ cells or stroma-infiltrating CD8⁺ cells was not a significant poor prognosis factor.

Discussion

Evasion of anti-tumor immunity has been thought to be critical to progress for cancers. Several studies have shown that down-regulation of HLA class I expression is observed in various tumor cells and it would cause a malignant phenotype. This is the first report demonstrating the significance of immune evasion by down-regulation of HLA molecules through assessing the correlation between expression of HLA class I and β_2-microglobulin, clinicopathologic factors, and tumor-infiltrating CD8⁺ T cells in NSCLC.

Although the present study showed the rate of HLA down-regulation was approximately 69% in NSCLC, the ratio has been reported to vary widely, from 25–90%.[11–13] The unsettled ratio of HLA down-regulation must be caused by several factors, such as different antibodies,[11–13] or preparation of the section.[11,12] The authors thought that the method should be standardized and so used EMR8-5, which can recognize HLA-A, -B and -C.[14] Subsequently, the staining of stromal lymphocytes was introduced as an internal positive control and the membrane staining of tumor cells was assessed as reported by Kitamura *et al*.[17] Furthermore, immunohistochemical analysis for β_2-microglobulin was introduced and it confirmed the validity in HLA class I immunohistochemistry.

The HLA class I heavy chain forms a stable complex with β_2-microglobulin and endogenous peptide antigen within the endoplasmic reticulum (ER), and the HLA class I complex is then transported to the plasma membrane where it plays a major role in interactions with CD8⁺ T cells. The mechanisms involved in down-regulation of HLA class I have been reported as follows:[23]

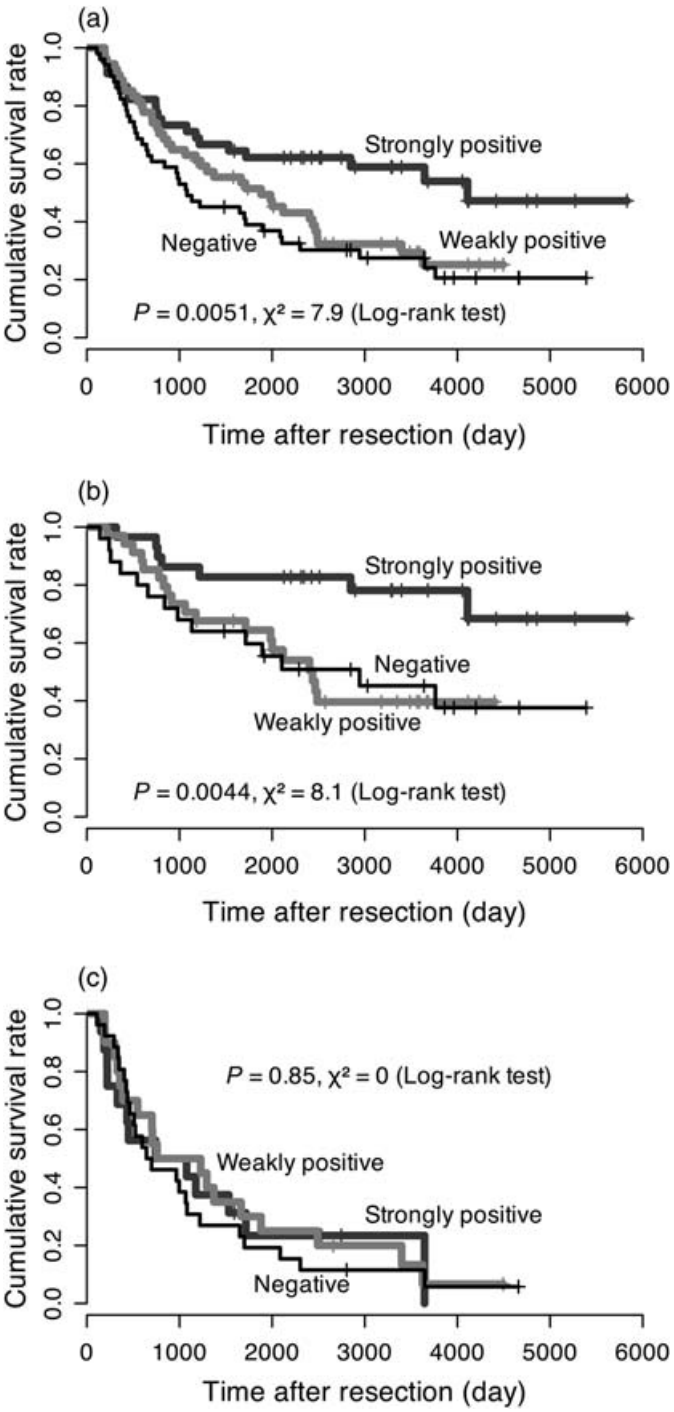

Fig. 4. Kaplan–Meier curves showing overall survival after resection according to HLA class I expressions in (a) all patients with non-small cell lung cancer (NSCLC; $n = 150$) in (b) pathologic stage I patients ($n = 88$) and (c) pathologic stage II–IV patients ($n = 62$). *P*-values were calculated using the log-rank test between the strongly positive group and the down-regulation group.

(i) genetic mutation or suppressed transcriptional activity of HLA class I heavy chain; (ii) genetic mutation or suppressed transcriptional activity of β_2-microglobulin; (iii) mutation in the transporter associated with antigen processing (*TAP*) gene; (iv) inhibition of glycosylation or transport of HLA class I molecules; or (v) *cis-trans* oncogene and viral down-regulation of HLA class I expression. The assembly of HLA class I heavy chain, β_2-microglobulin, and peptide antigen, is indispensable for the expression of HLA class I molecules. The correlation between HLA class I and β_2-microglobulin expression may be due to the assembly of HLA class I molecules, supporting the validity of immunostaining in the present study.

HLA class I expression was significantly correlated with a history of smoking, sex, and histology. Strongly positive expression of HLA class I was observed in adenocarcinoma in non-smoking female patients, while negative expression of HLA class I was observed at a high rate in squamous cell carcinoma in smoking male patients. Genetic mutation of epidermal growth factor receptor (EGFR) has recently been identified in adenocarcinoma in non-smoking female patients at a high frequency,[24] and is mutually exclusive with k-ras oncogene mutation, which occurs frequently in lung cancers found in smoking patients. Lung cancers with these two mutations could conceivably show different status of epigenetic alteration,[25] and these findings suggests that tobacco-mediated and non-smoker NSCLC display different tumorigenesis, different pathways for the development of cancer, and different genetic/epigenetic changes.[25] These differences may affect the correlation of HLA class I expression with smoking status, sex and histology.

In the present study, down-regulation of HLA class I expression represented an independent factor associated with poor prognosis in pathologic stage I NSCLC, but it did not correlate with survival in the late stage. Watson *et al*. reported that patients who had colon cancer with low-level expression of HLA class I displayed significantly worse prognosis compared with those with HLA class I-positive cancer, particularly in the early stages (TNM 0–II),[10] consistent with the results of the current study. The present result that the expression of HLA class I correlates with survival after resection in stage I, but exhibits no correlation with tumor stage, may suggest that immune response against cancer cells is effective when tumor burden is minimal, but is ineffective against overt cancer. Conversely, Watson *et al*. reported that the loss of HLA class I expression showed a favorable prognosis in colon cancer,[10] and Coca *et al*. reported that extensive intratumoral infiltration of $CD57^+$ natural killer (NK) cells in colorectal cancer was associated with favorable tumor outcomes.[26] This point contradicts the findings of the present study. Tumor-infiltrating $CD56^+$ NK cells showed no significant correlation with neither HLA class I expression by tumor cells nor the overall survival. Immune functions of NK cells might not be important in NSCLC.

Correlations between expression of HLA class I and the density of tumor-infiltrating $CD8^+$ lymphocytes have been reported in esophageal cancer,[9] pancreatic cancer,[22] colon cancer,[27] and ovarian cancer.[28] However, no previous reports have investigated correlations between HLA class I expression and two classes of tumor-infiltrating immune cells, that is, cancer nest-infiltrating cells and stroma-infiltrating cells. The authors revealed that the density of $CD8^+$ cells infiltrating into cancer nests was decreased in tumors with negative expression of HLA class I, but density of $CD8^+$ cells infiltrating in stroma was unaffected by expression of HLA class I. A significant scarcity of cancer nest-infiltrating $CD8^+$ cells in areas with negative expression of HLA class I compared to those in areas with strongly positive expression of HLA class I in the same sections was also shown. When referring to prognosis, the abundance of tumor-infiltrating $CD8^+$ cells has been reported to correlate with better prognosis in colon cancer,[21] esophageal cancer,[29,30] and kidney cancer.[31] However, several previous reports and the present study revealed no association between tumor-infiltrating $CD8^+$ cells and prognosis in NSCLC.[18,32] Other factors such as $CD4^+$ cells might be involved, as Hiraoka *et al*. reported that prognosis of NSCLC patients correlated with the number of both $CD4^+$ and $CD8^+$ cells in stroma.[32]

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Table 2. Uni- and multivariate analysis using Cox proportional hazards model for clinicopathologic factors affecting overall survival after resection in pathologic stage I non-small cell lung cancer

Variable		Hazard ratio (95% CI)	*P*-value of univariate analysis	Adjusted hazard ratio (95% CI)	*P*-value of multivariate analysis
Human leukocyte antigen class I	Down-regulation versus strongly positive	3.10 (1.37–7.03)	0.0068	2.59 (1.13–5.93)	0.024
Age	1-year increment	1.05 (1.01–1.09)	0.0099	1.04 (1.00–1.09)	0.043
Histology	Non-adenocarcinoma versus adenocarcinoma	1.82 (0.967–3.41)	0.064	–	–
Smoking	Ever smoking versus never smoking	2.04 (0.989–4.64)	0.089	–	–
Sex	Male versus female	1.83 (0.896–3.76)	0.097	–	–
Tumor grade	Non-well versus well	1.71 (0.853–3.42)	0.13	–	–
Cancer nest-infiltrating $CD8^+$ cell	1-cell increment	0.999 (0.997–1.00)	0.65	–	–
Stroma-infiltrating $CD8^+$ cell	1-cell increment	0.999 (0.998–1.00)	0.059	–	–
Cancer nest-infiltrating $CD56^+$ cell	1-cell increment	0.892 (0.732–1.09)	0.26	–	–
Stroma-infiltrating $CD56^+$ cell	1-cell increment	0.985 (0.885–1.09)	0.77	–	–

–, eliminated by backward elimination procedure; CI, confidential interval.

In conclusion, down-regulation of HLA class I expression in NSCLC represents a marker of poor prognosis, and may play a critical role in immune surveillance of patients with NSCLC. The present results provide critical information for successful immunotherapy against NSCLC.

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