学　位　論　文

Down-regulated expression of human leukocyte antigen class I heavy chains is linked to poor prognosis in non-small cell lung cancer.

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Down-regulated expression of human leukocyte antigen class I heavy chains is linked to poor prognosis in non-small cell lung cancer

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Key words

HLA class I antigen, β_2-microglobulin antigen, non-small-cell lung cancer, immunohistochemistry

Running title

ICHINOKAWA et al. DOWN-REGULATED EXPRESSION OF HLA CLASS I HEAVY CHAINS AND POOR PROGNOSIS FOR NSCLC

ABSTRACT

The aim of this study was to clarify the associations between expression of human leukocyte antigen (HLA) class I on 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 undertaken using Cox's proportional hazard model. The cases were divided into five classes based on expression of HLA class I heavy chain and β_2-microglobulin. Overall survival for patients with tumors lacking HLA class I heavy chain (30 cases; 27.0%) was significantly compromised. Multivariate analysis revealed the absence of HLA class I heavy chain to be an independent predictor of poor prognosis. There was a trend towards unfavorable prognosis for those patients whose tumors did not express β_2-microglobulin (57 cases; 51.4%). Down-regulation of HLA class I heavy chain expression significantly correlated with down-regulation of β_2-microglobulin expression (χ^2; $p<0.01$). Cases lacking both HLA class I heavy chain and β_2-microglobulin expression (23 cases; 20.7%) had a statistically significant unfavorable prognosis compared with other cases ($p<0.05$). Our data demonstrate that lack of HLA class I heavy chain expression in tumor cells is an independent poor prognostic factor for NSCLC survival, and is likely to have 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, over 60% of NSCLC patients relapse after surgery (1-3).

The immune system can suppress tumor growth by not only destroying cancer cells or inhibiting their outgrowth, but also by promoting tumor progression either by selecting for tumor cells that can survive in an immunocompetent host, or by establishing conditions within the tumor microenvironment that facilitate tumor outgrowth (4, 5). The immune system's anti-tumor arsenal includes CD4 T cells that kill tumor cells in a MHC class II expression-dependent manner, and NK cells/macrophages that mediate cell killing by antibody dependent cellular cytotoxicity (ADCC). In addition, CD8 T cells are thought to play an important role in the elimination of tumor cells by recognition of HLA class I expression on tumor cells. The association between HLA class I expression on tumor cells and $CD8^+$ T cell infiltration has been found in laryngeal squamous carcinoma (6), pancreatic carcinoma (7), and ovarian carcinoma (8).

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. There are three HLA class I heavy chains, HLA-A, -B, and –C, encoded by three separate genes on chromosome 6p21. Loss or down-regulation of HLA class I has been reported in various cancers (7-13), and many studies have described HLA class I down-regulation in NSCLC (14-19). However, in these studies there were discrepant correlations between HLA class I down-regulation 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 can recognize HLA-A, -B, and -C (20), and the establishment of a novel control method for staining using SCID mice xenografts to examine expression of CD40 and CD154 in NSCLC (21). However, a classification system based on immunostaining for loss or down-regulation of HLA class I

has not been established.

In this study, we assessed the influence of down-regulation of HLA class I expression in tumors on the survival of patients with NSCLC using EMR8-5 immunostaining, SCID mice xenografts as quantitative controls, and new classification schemes based on tumor heterogeneity, and reviewed the correlation between loss or down-regulation of HLA class I expression and NSCLC prognosis.

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-LCMS cell lines were grown in RPMI-1640 (Sigma-Aldrich Co., Ltd., Irvine, CA, USA) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. ABC-1, VMRC-LCD and RERF-LCOK cells were maintained in Minimum Essential Medium Eagle (M-EME, Sigma-Aldrich Co., Ltd., Irvine, CA, USA) with 10% FBS and 1% penicillin/streptomycin. All cell lines were maintained at 37°C in a humidified incubator with 5% CO_2.

Mice and xenograft models

CB17/SCID mice were obtained from Charles River Japan (Yokohama, Japan). All mice were 4- to 6-week-old females maintained under specific pathogen-free conditions. All animal procedures were conducted in accordance with the guidelines of the Hokkaido University Institutional Animal Care and Use Committee.

Xenografts were generated from all the lung cancer cell lines by injecting $5x10^6$ cells in 200 μl phosphate-buffered saline (PBS) subcutaneously into the flanks of CB17/SCID mice. When the tumor diameter exceeded 10 mm, the mice were sacrificed and tumors were separated into two portions. One portion was snap frozen using liquid nitrogen to extract

proteins for Western blot analysis, and the other was immersed in formalin for immunohistologic analysis.

Reagents and antibodies

The pan anti-human HLA-A, -B, and –C antigen mouse monoclonal antibody (EMR8-5: FA01), and anti-β_2-microglobulin mouse monoclonal antibody (EMRB6-12: GB-01B) were purchased from Hokudo (Sapporo, Japan). Negative control mouse IgG1 (X0931) was purchased from DAKO Japan (Kyoto, Japan). Peroxidase conjugated goat anti-mouse IgG was purchased from Jackson ImmunoResearch (West Grove, PA, USA).

Western blot analysis

Western blots were used to analyze expression of HLA class I heavy chain and β_2-microglobulin in lung cancer cells. Briefly, lysates from cell lines and SCID mice xenografts were resolved using 15% SDS-PAGE, transferred to nitrocellulose membranes (Amersham, Aylesbury, UK), and screened with EMR8-5: FA01 (1:1000) and EMR-B6: GB-01B (1:400) antibodies. The peroxidase-conjugated goat anti-mouse IgG secondary antibody was used at a dilution of 1:10000. Bound antibodies were detected using the ETL system (Amersham). Lysate from normal human peripheral blood mononuclear cells (PBMCs) was used as positive control for HLA class I heavy chain and β_2-microglobulin expression.

Patients and tissue specimens

Whole surgical specimens of NSCLC obtained between December 1992 and January 2001 were utilized in this study. All patients lacked any evidence of metastases to secondary sites and had not received prior anticancer treatment. Cases of in-hospital and non-cancer-related death were excluded. Patients gave informed consent to the processes involved, which were conducted in accordance with the guidelines of the Hokkaido University Institutional

Review Board. We examined 111 NSCLC surgical specimens from patients meeting these criteria who had undergone curative resection of the primary tumor, including systematic lymph node dissection, at a Hokkaido University hospital.

Surgical specimens were obtained from 72 men and 39 women with a mean age of 62.7 years (range 36-80 years). Median duration of follow-up was 60.9 months (range 4.0-100.7 months), and 43 patients (38.7%) died during the follow-up period.

Our results were correlated with the patients' clinical records. A single section from deep within the tumor specimen was selected for analysis, and resected specimens were examined histopathologically following staining with hematoxylin and eosin. According to the World Health Organization classification system the specimens consisted of 56 adenocarcinomas, 39 squamous carcinomas, 3 adenosquamous carcinomas, 12 large-cell carcinomas, and 1 carcinosarcoma. Site and size of the lesion were assessed on the gross specimen.

Clinicopathological stage of the tumor was determined according to the standard tumor node and metastasis (TNM) classification system. Fifty patients were T1, and 61 were T2-4. Lymph nodes were negative in 79 patients, and positive in 32 patients. Stage was defined on the surgical specimen according to the seventh International Union Against Cancer Classification (22). Diagnosis was made independently by at least two pathologists.

Immunohistochemistry

Immunohistochemical reactions were carried out using the universal immuno-enzyme polymer method. Surgical specimens were fixed in 10% formalin solution and embedded in paraffin for sectioning at 4 μm. 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.

Following three washes in PBS containing 0.1% Tween 20, sections were incubated in 10% normal goat serum (Histofine SAB-PO kit; Nichirei, Tokyo, Japan) for 30 min. 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-β_2-microglobulin mouse monoclonal antibody (1:400 dilution). After three additional washes, sections were incubated with Histofine Simple Stain MAX-PO (Multi) (Nichirei, Tokyo, Japan) for 30 min at room temperature and reaction products were visualized by incubation for approximately 10 min with 3,3′ -diaminobenzidine tetrahydrochloride (Nichirei, Tokyo, Japan), followed by washing in distilled water. Sections were counterstained in hematoxylin for 1.5 min, and then mounted in Permount (MICRO SLIDES; MUTO-GLASS, Tokyo, Japan).

Statistical analyses

The statistical significance of correlations between HLA class I heavy chain and β_2-microglobulin expression in cancer cells was evaluated by the Mann-Whitney U test. The χ^2–test (or extended Fisher's exact test where appropriate) was used to analyze correlations between HLA class I heavy chain or β_2-microglobulin expression in cancer cells and patient parameters, including histopathological findings.

The cumulative survival rate was calculated using the Kaplan-Meier method. Statistical significance was analyzed by the log-rank test. Cancer-specific survival rates were also calculated from date of surgery to date of cancer-related death. Univariate and multivariate analyses utilized the Cox proportional hazard regression model. P values of less than 0.05 were considered to be statistically significant. All analyses were performed using Statview-J ver. 5.0 software (SAS Institute, Inc., Cary, NC, USA).

Results

Expression of HLA class I heavy chain and β_2-microglobulin in non-small-cell lung

cancer cell lines

On Western blotting, HLA class I heavy chains were detected most strongly at 45 kDa in two cell lines, RERF-LCOK and H226 (Fig. 1A**,** top panel)*,* and expression was lowest in the VMRC-LCD and LK2 xenografts (Fig. 1A**,** lower panel). β_2-microglobulin was expressed strongly at 12 kDa in five cell lines: PC3, RERF-LCMS, RERF-LCOK, VMRC-LCD and H226 (Fig. 1B, top) and down-regulated in four xenografts: ABC-1, VMRC-LCD, LC-1, and LK2 (Fig. 1B, bottom). HLA class I heavy chain and β_2-microglobulin were expressed in normal human lung tissue (NHLT), at similar levels to PBMC (Fig. 1A top and 1B top).

Of the 10 cell lines, HLA class I heavy chain and β_2-microglobulin were down-regulated in A549 and PC10**,** although xenografts of these cell lines showed higher expression of these molecules.

PC10 xenografts were used as positive tissue controls for HLA class I heavy chain and β_2-microglobulin expression because the respective proteins were highly expressed and clearly detected by xenografts (Fig. 1A, 2A, and 2**C**). LK2 xenografts, which exhibited down-regulated HLA class I heavy chain and β_2-microglobulin expression by Western blotting, were used as negative tissue controls (Fig. 1B, 2B**,** and 2D). Isotype matched negative control antibodies were used to control for non-specific staining.

Establishment of quantitative evaluation methodology for tumor HLA class I expression

Immunostaining results were grouped into three categories: strongly positive (equivalent to staining of alveolar epithelium) (Fig. 2E, 2H); weakly positive relative to alveolar epithelium; (Fig. 2F, 2I); and absent (no detectable staining) (Fig. 2G, 2J). The cases were further divided into five categories: homogeneous strong staining, heterogeneous strong-weak staining, homogeneous weak staining, heterogeneous strong-weak-absent staining, and heterogeneous weak-absent staining. Strong staining was defined by the positive control and stages were graded in 10% steps (Fig. 3A, 4A).

Expression of HLA class I heavy chain and β_2-microglobulin.

We used the PC10 and LK2 xenografts as positive and negative controls, respectively, for HLA class I expression.

Immunohistochemical staining for HLA class I was categorized into three grades relative to alveolar epithelium: strong, weak, and absent. Moreover, the cases were divided into five groups with reference to expression patterns.

Staining revealed 31 tumors (27.9%) with homogeneous strong expression of HLA class I heavy chain, 38 (34.2%) with heterogeneous strong-weak expression, 12 (10.8%) with homogeneous weak expression, 21 (18.9%) with heterogeneous strong-weak-absent expression, and 9 (8.1%) with heterogeneous weak–absent expression (Fig. 3A).

With regard to β_2-microglobulin, 12 tumors (10.8%) showed homogeneous strong expression, 37 (33.3%) had heterogeneous strong-weak expression, 5 (4.5%) had homogeneous weak expression, 48 (43.2%) had heterogeneous strong-weak-absent expression, and 9 (8.1%) had heterogeneous weak–absent expression (Fig. 4A).

With reference to HLA class I heavy chain and β_2-microglobulin, we found 46 tumors (41.4%) to be double positive, 35 (31.5%) to be positive only for HLA class I heavy chain, 8 (7.2%) positive only for β_2-microglobulin antigen, and 22 (19.8%) to be double negative (Table I). There was a significant correlation between the expression of HLA class I heavy chain and β_2-microglobulin (p=0.005) (Table I).

Survival analysis and HLA class I heavy chain and β_2-microglobulin expression.

The 5-year disease-specific survival was 62.2% for all patients. The survival curves for each HLA class I heavy chain expression group are shown in Fig. 3B**.** Survival curves constructed according to the Kaplan-Meier method are shown in Fig. 3D and 3E.

The overall prognosis for cases lacking HLA class I heavy chain expression was

unfavorable compared with positive groups (p=0.039; Fig. 3D). When divided by cancer stage, stage I-II cases lacking HLA class I heavy chain expression had an unfavorable prognosis compared with positive groups (p=0.012; Fig. 3E), but correlation with survival was not significant in stages II-III (p=0.104; data not shown).

The survival curves of each β_2-microglobulin expression group are shown in Fig. 4B. There were no significant correlations between overall prognosis and lack of β_2-microglobulin expression (p=0.092; Fig. 4D), or stage I-II cancer (p=0.996; Fig. 4E).

Correlation between HLA class I heavy chain expression and clinicopathological features.

No significant correlations were detected between expression of HLA class I heavy chain and gender, 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, gender, T classification, N classification, TNM stage, histopathological grade and absence of HLA class I heavy chain. Multivariate analysis of the same set of patients was performed for T classification, N classification, and absence of HLA class I heavy chain for survival time using the Cox regression model.

The results indicated that absence of HLA class I heavy chain is an independent poor prognostic factor (p=0.032). T and N classification also 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 this study was to use to develop a quantitative immunohistochemistry

protocol to investigate whether expression of HLA class I in NSCLC correlates with patient survival. Previously reported correlations were controversial and difficult to compare because of differences in antibodies used, positive controls and classification systems (**Table III**). We developed a reproducible and carefully controlled quantitative immunohistochemistry methodology that provided a robust approach to accurately classify NSCLC specimens into categories based on HLA class I expression levels and characteristics. One of the most important issues in past studies was a lack of consideration of tumor heterogeneity in IHC evaluation. Therefore, in this study, we classified HLA Class I down regulation in consideration of tumor heterogeneity assessed using EMR8-5 and SCID mice xenografts. Our 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.

While 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, we divided the NSCLC cases into five groups with reference to the expression pattern of HLA class I, and then divided the cases into a positive group (A-C), and a negative group (D-E), excluding those cases that did not express HLA class I. Also, in terms of the intra-tumor heterogeneity for the mutations in localized lung cancer, Zhang J et al reported that single-region sequencing might be adequate to identify the majority of known cancer gene mutations (23) Our present data may reflect the result of emerging immune-escaping variants reflecting intra-tumor heterogeneity for HLA class I and β2-microglobulin.

There were 80 tumors with weak HLA class I expression, included in groups B-E. The rate of down-regulation shown by immunohistochemistry corresponded to that shown by Western blotting (~80%). In group A vs. group B-E, and group A-B vs. group C-E, there was no correlation between expression of HLA class I and prognosis, which is consistent with

previous studies (14-19).

We found that absence of HLA class I expression on tumors was an independent factor predicting poor prognosis, although expression of HLA class I heavy chain did not correlate with pathological variables. These results suggest that NSCLC would progress regardless of the expression of HLA class I heavy chain, and host immunity plays a limited role in influencing tumor growth prior to surgery. However, after surgery, more cases lacking HLA class I relapsed than cases with HLA class I expression. Moreover, the prognosis was good for early stage HLA class I heavy chain-positive cases, although in later stage cases there was no correlation between prognosis and expression of HLA class I heavy chain consistent with previous studies(14-19).

It is well-accepted that inflammation or an inflammatory gene expression signature in tumors correlates with anti-tumor immune responses (24, 25). MHC class I expression is predominantly up-regulated by interferon gamma in the tumor microenvironment. In addition, interferon gamma is essential to elicit immune elimination of tumor cells (26). Therefore, our data, which link HLA class I expression with prognosis, might be a reflection of interferon gamma signature. Some active immunotherapies, such as novel agents capable of generating anti-tumor immunity, have been found to have clinical efficacy against lung cancer (27), and may act by boosting the interferon gamma signature in the tumor microenvironment. Interestingly, immunologic responses were independent of stage and prior therapy in a study of NSCLC dendritic cell vaccines (28), consistent with the possibility that the effectiveness of immunotherapy may be related to expression of HLA class I heavy chains on tumor cells.

Currently, recurrent or surgically non-resectable cases are often treated with immunotherapy,

even though the patients' immune systems have a limited capacity to deal with the heavy tumor burden**.** Our data suggest that immunotherapy would be beneficial as postoperative adjuvant therapy in NSCLC.

Acknowledgements

We would like to acknowledge the excellent technical assistance of Mr. Hiraku Shida and Ms. Naomi Saito. We would also like to thank the many physicians at the affiliated Departments of Surgical Oncology who cared for the patients included in this study.

This study was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

Figure Legends

Figure 1: Western blotting for HLA class I heavy chain (A) and β2-microglobulin (B).

(A, top) Western blot analysis of HLA class I heavy chain expression in human lung cancer cell lines and homogenized normal human lung tissue (NHLT).

(A, bottom) Western blot analysis of HLA class I heavy chain expression in lysates from CB17/SCID mice xenograft models. Each cell line was implanted subcutaneously into the flanks of CB17/SCID mice.

(B, top) Western blot analysis of β2-microglobulin expression in lysates from human lung cancer cell lines and NHLT were subjected to Western blotting.

(B, bottom) Western blot analysis of β2-microglobulin expression in CB17/SCID mouse xenograft models. Lysates from human peripheral mononuclear cells (PBMCs) were used as a positive control for HLA class I heavy chain and β2-microglobulin.

LCMS: RERF-LCMS; LCOK: RERF-LCOK; LCD: VMRC-LCD; Heavy chain: HLA class I heavy chain; β2m: β2-microglobulin; AC: Adenocarcinoma; SCC: Squamous-Cell Carcinoma; P.C.: Positive Control

Figure 2: Immunohistochemical staining for HLA class I heavy chain and β2-microglobulin.

(A-D): HLA class I heavy chain and β2-microglobulin SCID mouse xenograft samples.

(A): Expression of HLA heavy chain in PC10.

(B): Absence of HLA heavy chain expression in LK2.

(C): Expression of β2-microglobulin in PC10.

(D): Absence of β2-microglobulin expression in LK2.

(E-G): Cell membranes stained for HLA class I heavy chain in NSCLC samples.

(E): Strongly stained.

(F): Weakly stained

(G): No staining

(H-J): Cell membranes stained for β_2-microglobulin in NSCLC samples.

(H): Strongly stained

(I): Weakly stained

(J): No staining

(A-D, E-J lower right): Magnification: x400

(E-J): Magnification: x100

Figure 3

A. HLA class I heavy chain expression in NSCLC

Group A: Homogeneous strong staining (n=31)

Group B: Heterogeneous strong-weak staining (n=38)

Group C: Homogeneous weak staining (n=12)

Group D: Heterogeneous strong-weak-absent staining (n=21)

Group E: Heterogeneous weak-absent staining (n=9)

B. Survival curves of patients with NSCLC according to their HLA class I heavy chain expression of each group.

There was no significant differences in prognosis between groups.

C. Survival analyses

Patients were divided into two groups according to expression of HLA class I heavy chain.

D. Survival curves of patients with NSCLC according to their HLA class I heavy chain

expression

Patients were divided into two groups: positive (n=81) vs. negative (n=30)

E. Survival curves of patients with early stage NSCLC according to their HLA class I heavy chain expression (stage I-II)

Patients were divided into two groups: positive (n=67) vs. negative (n=24)

Figure 4

A. β_2-microglobulin expression in NSCLC

Group A: Homogeneous strong staining (n=12)

Group B: Heterogeneous strong-weak staining (n=37)

Group C: Homogeneous weak staining (n=5)

Group D: Heterogeneous strong-weak-absent staining (n=48)

Group E: Heterogeneous weak-absent staining (n=9)

B. Survival curves of patients with NSCLC according to β_2-microglobulin expression

There were no significant differences in prognosis between the groups.

C. Survival analyses

Patients were divided into two groups according to expression of β_2-microglobulin.

D. Survival curves of patients with NSCLC according to β_2-microglobulin expression

Patients were divided into two groups: positive (n=64) vs. negative (n=57)

E. Survival curves of patients with early stage NSCLC according to β_2-microglobulin expression (stage I-II)

Patients were divided into two groups: positive (n=84) vs. negative (n=7)

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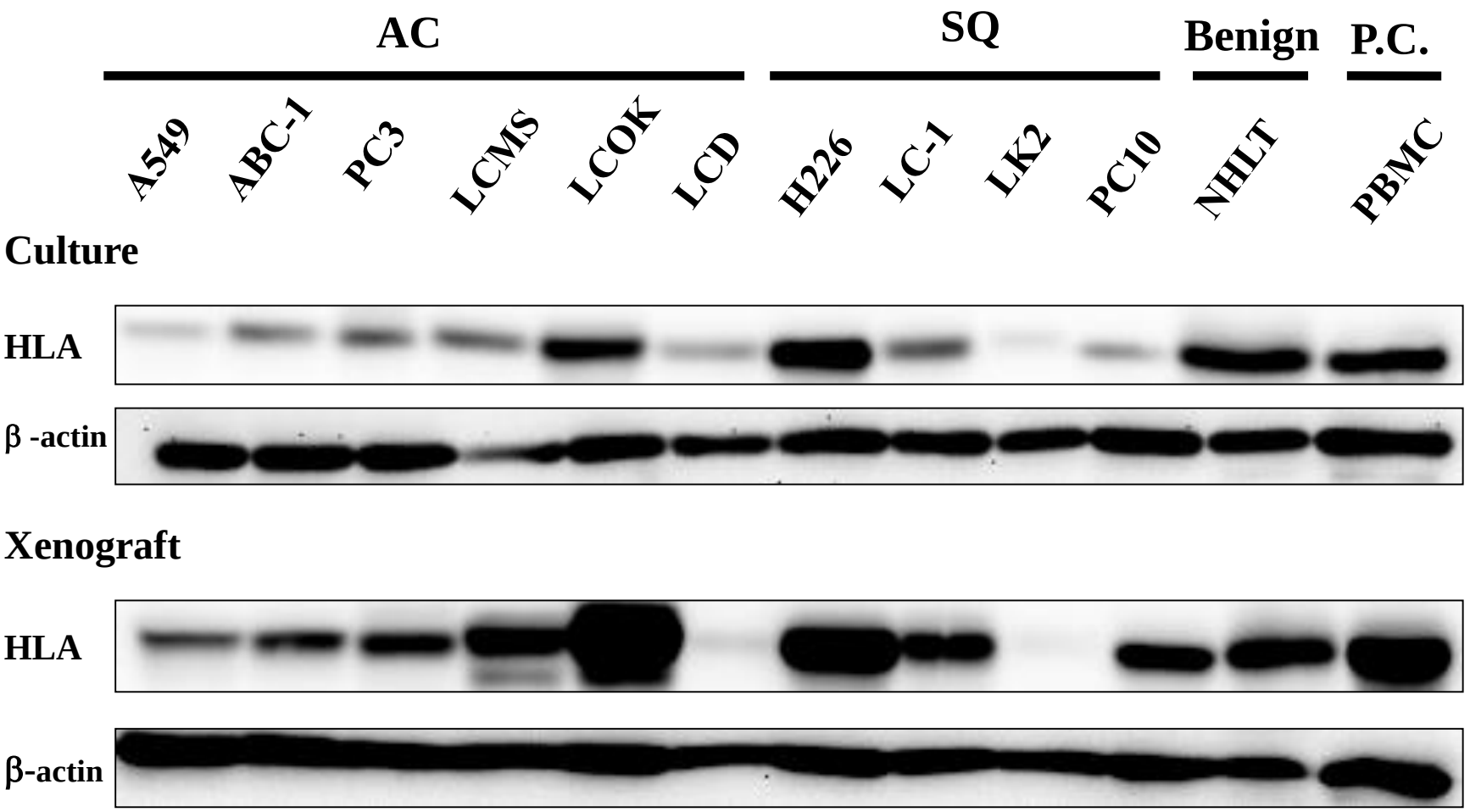
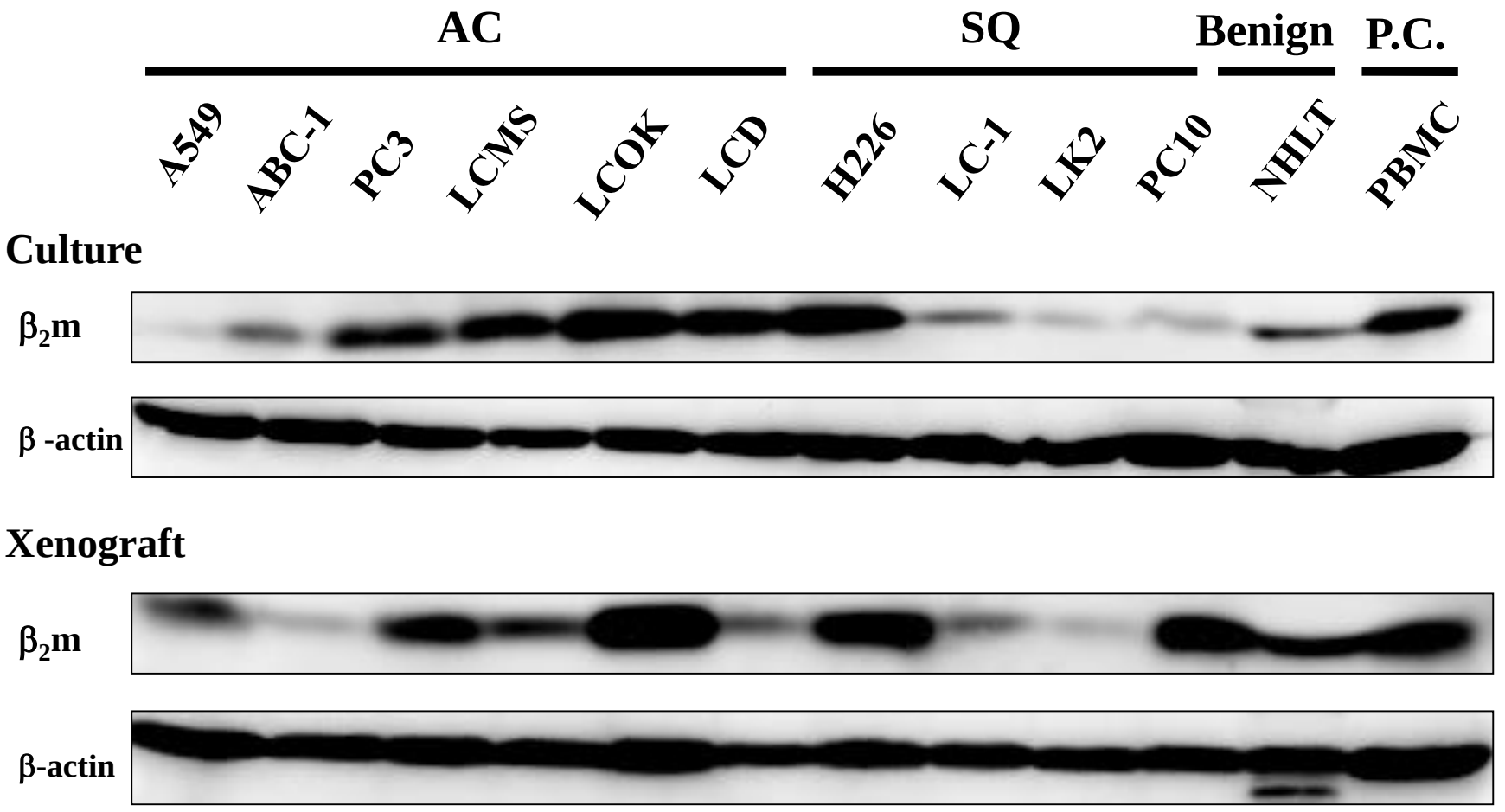
Figure 1A



HLA: HLA class I Heavy chain

Figure 1B



β_2m: β_2-microglobulin

Figure 2

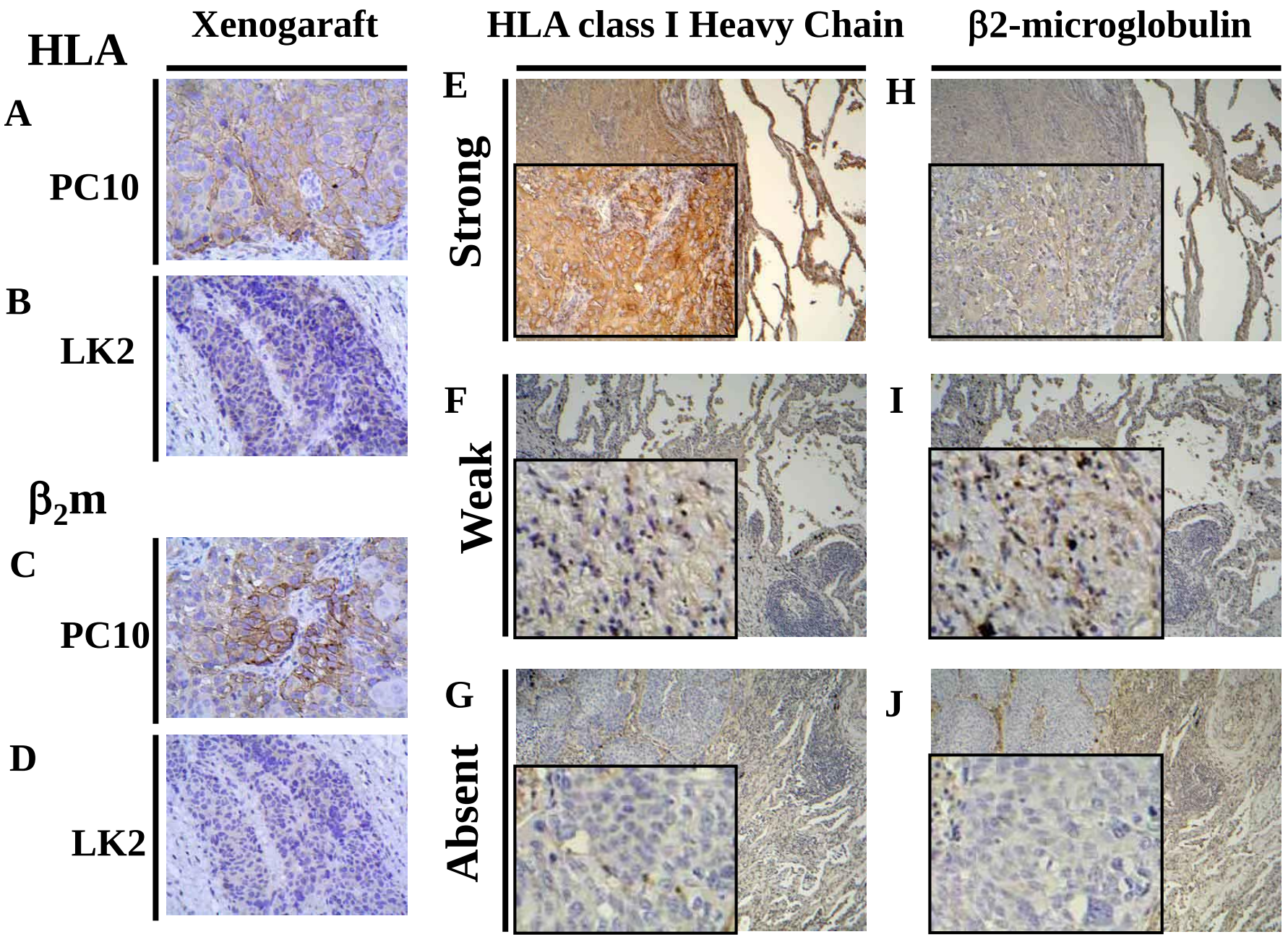
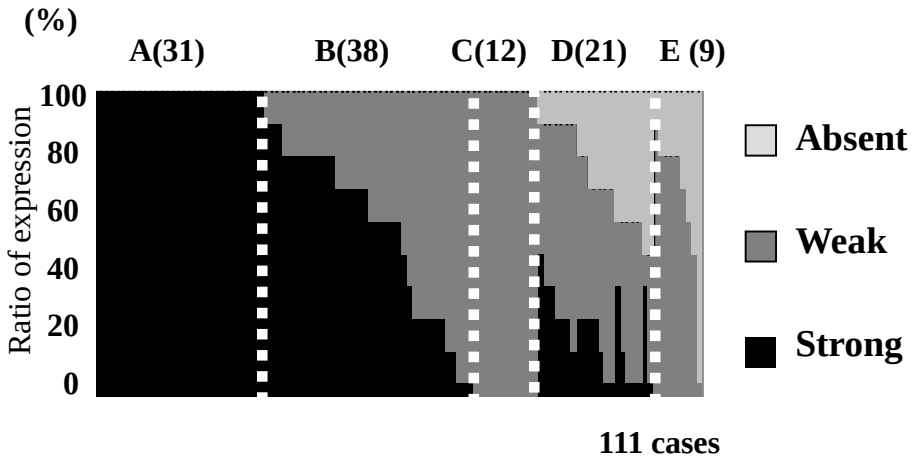

Xenogaraft
HLA class I Heavy Chain
β2-microglobulin
HLA
A
PC10
B
LK2
β2m
C
PC10
D
LK2
E
Strong
H
F
Weak
I
G
Absent
J

Figure 3-1

A


(%)
A(31)
B(38)
C(12)
D(21)
E (9)
Ratio of expression
100
80
60
40
20
0
Absent
Weak
Strong
111 cases

B

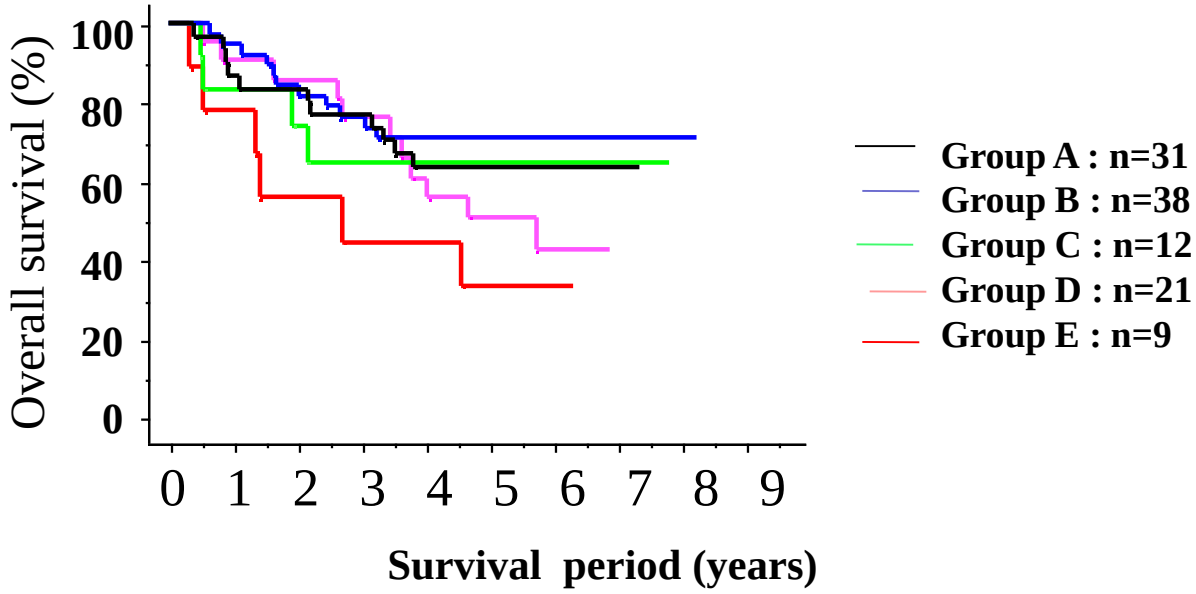

Overall survival (%)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9
Survival period (years)
Group A : n=31
Group B : n=38
Group C : n=12
Group D : n=21
Group E : n=9

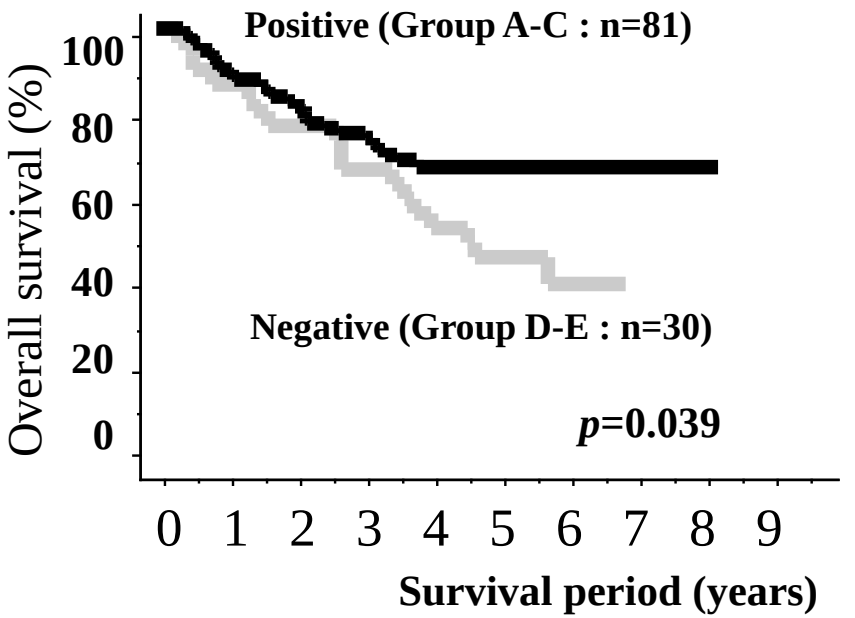
Figure 3-2

C

Each group of survival analyses

Group	5 years survival rate (%)	p-value
A v.s. B-E	63.4% v.s. 60.4%	*p*=0.735
A-B v.s. C-E	64.7% v.s. 50.7%	*p*=0.073
A-C v.s. D-E	67.1% v.s. 45.1%	*p*=0.039
A-D v.s. E	63.7% v.s. 33.3%	*p*=0.031

D



E

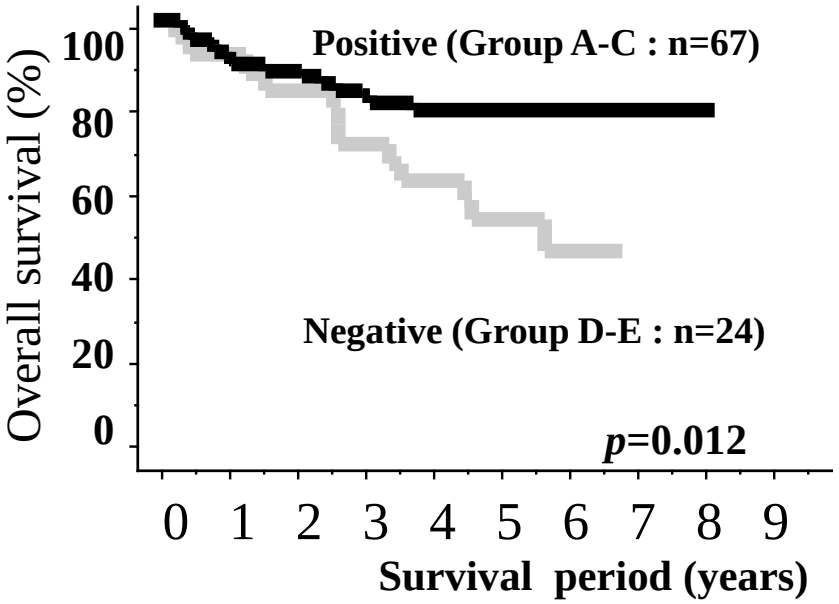
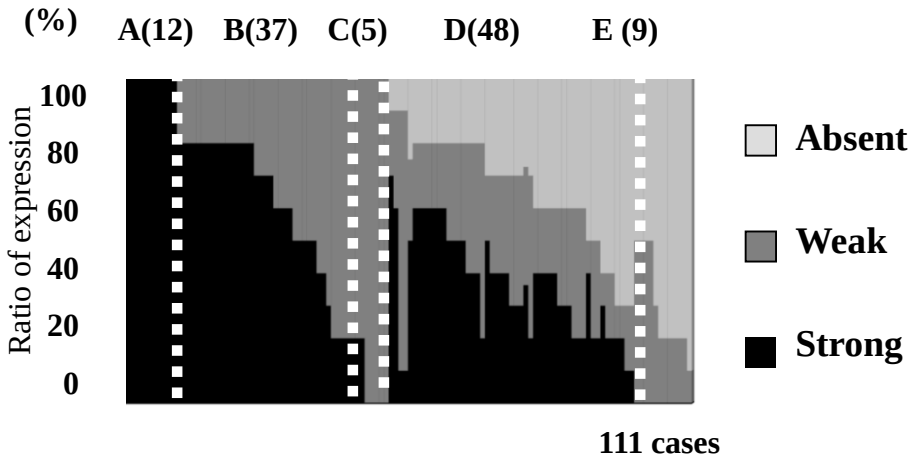


Figure 4-1

A


(%)
A(12)
B(37)
C(5)
D(48)
E (9)
Ratio of expression
100
80
60
40
20
0
Absent
Weak
Strong
111 cases

B

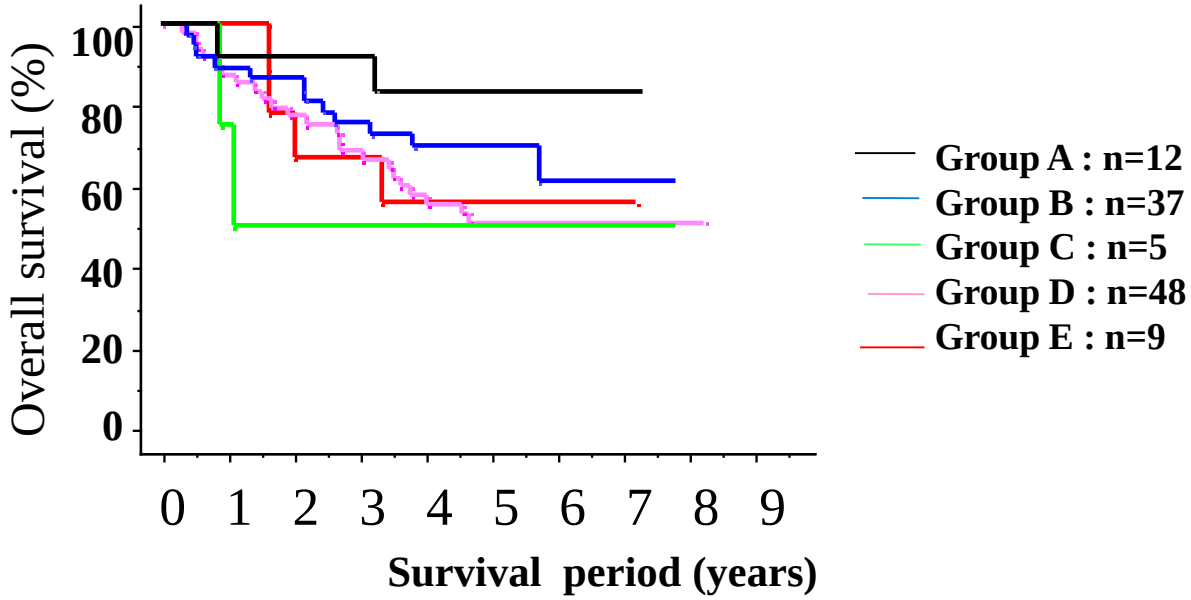
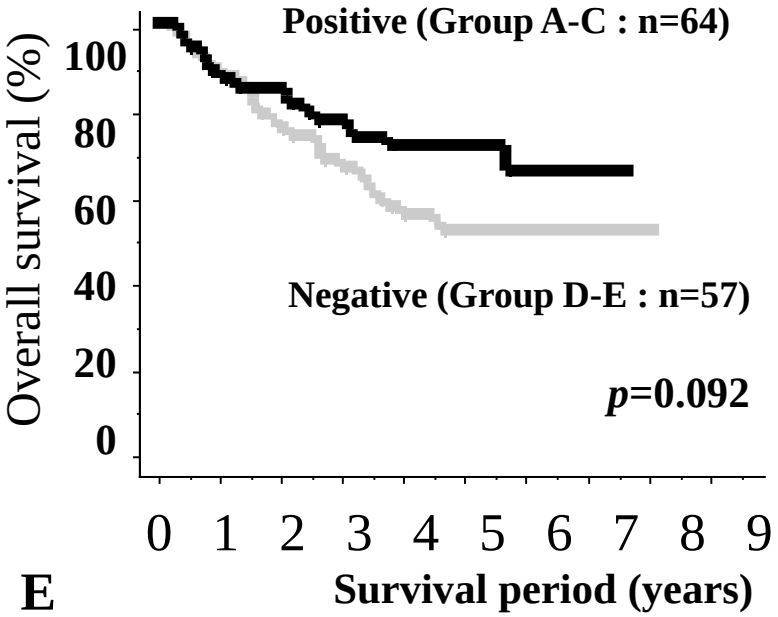

Overall survival (%)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9
Survival period (years)
Group A : n=12
Group B : n=37
Group C : n=5
Group D : n=48
Group E : n=9

Figure 4-2

C

Each group of survival analyses					
Group	5 years survival rate (%)			p-value	
A v.s. B-E	83.3%	v.s.	58.4%	*p*=0.114	
A-B v.s. C-E	73.1%	v.s.	51.5%	*p*=0.057	
A-C v.s. D-E	71.5%	v.s.	51.5%	*p*=0.092	
A-D v.s. E	61.7%	v.s.	55.6%	*p*=0.777	

D



E

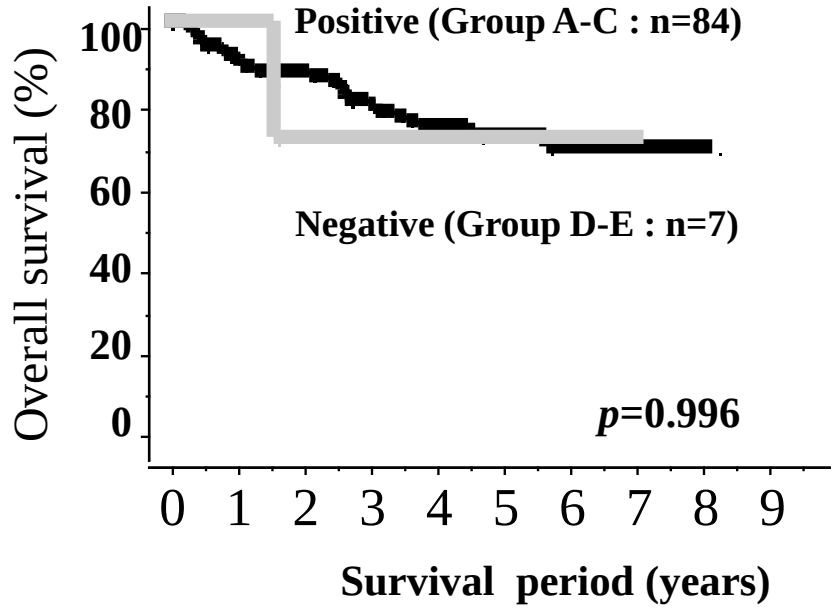


Table I: Relationships between HLA class I heavy chain expression in NSCLC and clinicopathological variables.

	HLA class I Heavy chain staining in cancer cells		
	(-)	(+)	
	N = 30	N = 81	p-value
Variable			
Age (years)			
≤62	11	47	0.045
>62	19	34	
Gender			
Male	22	50	0.255
Female	8	31	
Histology			
AC	13	43	0.312*
SCC	13	25	
LCC	2	11	
Other	2	2	
T-classification			
pT1	13	37	0.825
pT2-4	17	44	
N-classification			
pN (-)	22	57	0.76
pN (+)	8	24	
Histopathological grading			
G1	9	22	0.835
G2-3	17	46	
TNM stage			
Stage I	20	51	0.718
Stage II-III	10	30	
b2-microglobulin			
(+)	8	46	0.005
(-)	22	35	

* Fisher's exact test. AC: Adenocarcinoma; SCC: Squamous-Cell Carcinoma;

LCC: Large-Cell Carcinoma; Other: Adenosquamous Carcinoma (3 cases) and Carcinosarcoma (1 case)

Table II: Univariate and multivariate analysis of clinicopathological factors affecting overall survival after resection.

	Univariate			Multivariate		
	Hazard ratio	95%CI*	p-value	Hazard ratio	95%CI*	p-value
Variable						
Age (years)						
>62 /≤62	1.77	0.97-3.25	0.065			
Gender						
Male / Female	1.98	0.99-3.94	0.051			
T-classification						
pT2-4 / T1	4.08	2.00-8.33	<0.001	3.48	1.70-7.14	0.001
N-classification						
pN (+) / N(-)	4.17	2.25-7.69	<0.001	3.72	1.99-6.94	<0.001
TNM stage						
Stage II-III / Stage I	4.61	2.45-8.62	<0.001			
Histopathological grading						
G1 / G2-3	1.31	0.67-2.54	0.433			
HLA class I Heavy Chain						
Negative / Positive	1.88	1.02-3.47	0.043	1.96	1.06-3.62	0.032

95% CI*: 95% confidence interval

Table III: Literature describing HLA class I heavy chain expression in NSCLC.

First author / Year	No. reference	Histology	No. of patients	Loss or down regulation, No (%)	Sample	Antibody	Definition of loss or down-regulation	Positive control	The association between prognosis and loss or down-regulation
Redondo / 1991	(14)		59 (100%)	16 (27%)	frozen	W6 / 32, HC-10	Less 5% of the staining the tumor	Not described	Not examined
		AC	15 (25%)						
		SCC	40 (68%)						
Korkoloppoulou / 1996	(15)		93 (100%)	37 (40%)	frozen	W6 / 32, HCA2, MA2.1	Less or equal 75% of the Staining the tumor	Normal respiratory epithelium and lymphocyte	No association
		AC	29 (31%)	10 (35%)					
		SCC	57 (61%)	27 (47%)					
Redondo / 1997	(18)		123 (100%)	36 (29%)	frozen	W6 / 32, HC-10	Less 20% of the staining the tumor	Stromal cell	Not examined
		AC	38 (31%)						
		SCC	30 (24%)						
Ramnath / 2006	(16)		190 (100%)	178 (94%)	paraffin	HC-10	Less or equal 75% of the staining tumor	Non-neoplastic lung tissue	Down-regulation is associated with improved survival
		AC	109 (57%)	104 (95%)					
		SCC	64 (34%)	58 (91%)					
Kikuchi / 2007	(17)		161 (100%)	111 (69%)	paraffin	EMR8-5	Less 80% of the tumor	Endothelial cells	Expression is interrelated with favorable prognosis in early stage
		AC	83 (52%)	47 (57%)				Stromal lymphocytes	
		SCC	68 (42%)	57 (84%)					
Torigoe / 2012	(20)	Not described	35 (100%)	7 (20%)	paraffin	EMR8-5	Less 75% of the staining tumor	Endothelial cells Stromal lymphocytes	Not examined
Hanagiri / 2013	(19)		136 (100%)	87 (64%)	paraffin	EMR8-5	Less 80% of the tumor	Endothelial cells	Expression is interrelated with favorable prognosis in early stage
		AC	96 (71%)	56 (58%)				Stromal lymphocytes	
		SCC	33 (24%)	25 (76%)					
This study / 2016			111	30 (27%)	paraffin	EMR8-5	Absence of the staining the tumor	SCID mice xenograft	Expression is interrelated with favorable prognosis
		AC	56	13 (23%)				Endothelial cells	
		SCC	38	13 (34%)					

AC: adenocarcinoma. SCC: squamous cell carcinoma