

Higher human lymphocyte antigen class I expression in early-stage cancer cells leads to high sensitivity for cytotoxic T lymphocytes

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Abstract

Human lymphocyte antigen (HLA) class I molecules play a central role in cytotoxic T lymphocytes (CTL)-based antitumor immunity. However, the expression rate of HLA class I in cancer cells remains a topic of discussion. We compared HLA class I expression levels between cancer cells and surrounding non-tumorous hepatocytes in 20 early-stage hepatocellular carcinoma (HCC) patients by immunohistochemistry using EMR 8-5. The expression levels of HLA class I were classified as negative, incomplete positive or complete positive. Similarly, for various types of solid cancers, HLA class I expression was examined. For the HLA class I expression in cancer cells, among 20 HCC patients, 13 were complete positive, 3 were incomplete positive, and 4 were negative. In addition, 15 (75.0%) had higher expression levels of HLA class I in cancer cells compared with that in surrounding non-tumorous hepatocytes. An interferon- γ (IFN- γ) enzyme-linked immunospot (ELISPOT) assay indicated that cancer cells with positive expression of HLA class I had strong sensitivity to antigen-specific CTL. We suggested that HLA class I expression in cancer cells could be involved in the clinical prognosis of HCC patients. Similarly, 66.7%, 100.0%, 66.7% and 62.5% of patients with early-stage pancreatic, gallbladder, esophageal and breast cancers, respectively, had higher expression levels of HLA class I in cancer cells than in surrounding normal tissue cells. We suggest that in several early-stage solid cancers, including HCC, HLA class I expression levels in cancer cells are higher than that in surrounding normal tissue cells, which could result in the anti-tumor effect of CTL-based cancer immunotherapy.

KEYWORDS

cytotoxic T lymphocytes, EMR 8-5 antibody, hepatocellular carcinoma, human lymphocyte antigen class I expression, immunohistochemistry

1 | INTRODUCTION

In the last 10 years, based on further elucidation of the human immune system and cancer microenvironment, cancer immunotherapies have been established as new cancer treatments.¹ Notably, immune checkpoint inhibitor therapies provide adequate clinical effects in certain cases with malignant diseases.²⁻⁶ Cytotoxic T lymphocytes (CTL) play an extremely important role in antitumor immunity through their ability to recognize and eliminate cancer cells. In addition, human lymphocyte antigen (HLA) class I molecules present tumor-specific antigens to CTL which have cancer cell recognition and killing functions. Therefore, it is important that HLA class I antigens are expressed on the target cell surface, to establish cancer-specific immune reactions and exert sufficient antitumor effects.

Previously, the loss or downregulation of HLA class I expression has been shown in advanced cancer cells to be one reason for their escape from immune surveillance systems.⁷ Indeed, histological evidence and molecular mechanisms for the loss or downregulation of HLA class I expression have been reported for several types of cancers, including breast, gastric, bladder and renal cancers.⁸⁻¹⁸ In addition, some studies have suggested that the HLA class I expression could be closely related to the prognosis of patients who have solid cancers.^{9,19-24} However, the rates of HLA class I expression reported in any type of cancer have been inconsistent in published studies and this remains a topic of discussion. In addition, there have been few reports comparing HLA class I expression in cancer cells and surrounding normal tissues in patients with solid cancer.

Human lymphocyte antigen class I molecules contain classical HLA-A, -B and -C and non-classical HLA-E, -F and -G antigens, which are transmembrane glycoproteins with a non-polymorphic 12-kDa β 2-microglobulin light chain and a polymorphic 45-kDa heavy chain.²⁵ Previous anti-HLA class I monoclonal antibodies, including HC-A2 and HC-10, cannot recognize all HLA types. Moreover, W6/32 recognize an allele-specific native structure of HLA class I molecules. Therefore, the previously mentioned anti-HLA class I antibodies would not be ideal for formalin-fixed paraffin-embedded tissues that have denatured HLA class I molecules. By contrast, EMR 8-5, a novel pan HLA monoclonal antibody, is suitable for HLA class I immunostaining in formalin-fixed paraffin-embedded tissue specimens that have been treated for long-term preservation. Indeed, the expression of HLA class I has been histologically examined using EMR 8-5 in colorectal,²³ breast,^{8,14} osteosarcoma,⁹ renal^{20,24} and non-small cell lung cancers.¹⁵

In this study, we compared HLA class I expression levels in cancer cells and surrounding non-tumorous hepatocytes in early-stage hepatocellular carcinoma (HCC) patients by immunohistochemistry using EMR 8-5. In addition, we evaluated the association between the HLA class I expression level and immune responses/clinical prognosis in the HCC patients. Finally, in various solid cancers we examined the differences in HLA class I expression between cancer cells and surrounding normal tissue cells.

2 | MATERIALS AND METHODS

2.1 | Patients and tissue samples

A total of 20 patients who underwent radical surgery for primary HCC at the National Cancer Center Hospital East in Japan between February 2005 and January 2011 were enrolled in this study. No patients received any systemic chemotherapy before surgery. Patient characteristics were examined from medical records, and were assessed for age, sex, performance status (PS), hepatic virus infection, tumor size, tumor stage and lymphovascular invasion. Tumor stage was evaluated using the 8th edition of the Pathological Union for International Cancer Control (UICC)-TNM classification. In addition, survival analysis, including recurrence-free survival (RFS) and overall survival (OS), of all enrolled patients during follow up was evaluated. This study was approved by the Institutional Review Board of the National Cancer Center, conforming to the ethical guidelines of the 1975 Declaration of Helsinki (approval nos. 18-41, 21-002 and 2010-145). Informed consent was obtained from all enrolled patients.

Paraffin-embedded blocks of resected specimens were obtained from all enrolled patients to perform immunohistochemical analysis of HLA class I expression using the EMR 8-5 antibody. Fresh tissue samples were obtained from 5 enrolled patients to perform flow cytometry analysis of HLA class I using the W6/32 antibody. In addition, we collected paraffin-embedded blocks of resected specimens from 6 patients with pancreatic cancer, 5 with gallbladder cancer, 6 with esophageal cancer, 7 with colon cancer and 8 with breast cancer. These patients provided informed consent and we evaluated the HLA class I expression levels of the specimens. We defined surrounding normal tissue cells in pancreatic cancer specimens as normal acinar cells or duct cells, and in gallbladder, esophageal, colon and breast cancer specimens as normal epithelial cells.

2.2 | Immunohistochemical analysis of human lymphocyte antigen class I levels in patients with hepatocellular carcinoma and other types of cancer

In all enrolled patients, comparison of the expression level of HLA class I in cancer cells and surrounding normal tissue cells was evaluated by immunohistochemical staining using EMR 8-5 (dilution 1:100, Hokudo, Sapporo, Japan), a monoclonal anti-HLA-class I antibody that recognizes all HLA-A, -B and -C heavy chains, even in formalin-fixed tissues. Immunohistochemical reactions were performed on paraffin tissue samples according to previous reports.²⁶ Briefly, HCC tissue specimens were sliced into 5- μ m-thick sections and deparaffinized. Antigen retrieval was conducted in Target Retrieval Solution, pH 9 (Dako, Carpinteria, CA, USA) at 95°C for 15 minutes. The samples were treated with .3% H₂O₂ to block endogenous tissue peroxidases and rinsed in distilled water (DW). Slides were blocked with 4% normal swine (Vector Labs, Burlingame, CA, USA) /TBS for 10 minutes. Tissue slides were incubated with EMR 8-5 as a primary anti-HLA class I antibody for 60 minutes and then with Envision+

System-HRP anti-mouse (Dako) as a secondary antibody for 30 minutes. Then, tissue slides were stained using the DAB + 2-component system (Dako) for 1 minute. Sections were rinsed in TBS with Tween 20 (TBST), dehydrated through graded alcohols into a non-aqueous solution, and covered with a coverslip with mounting media.

All specimens were evaluated independently for at least 5 regions at a magnification of $\times 400$ using light microscopy, by 2 medical staff (D.N. and M.T.), who were blinded to the data of patients' characteristics. The expression levels of HLA class I were classified into the following 3 groups according to the membrane immunoreactivity level: negative expression when staining cells comprised $< 20\%$, incomplete positive expression when staining cells comprised $20\% - 80\%$, and complete positive expression when staining cells comprised $> 80\%$ of cancer or surrounding normal tissue cells.

The expression of HLA class I in pancreatic, gallbladder, esophageal, colon and breast cancer specimens was also investigated by immunohistochemical staining using EMR 8-5.

2.3 | Flow cytometry analysis of human lymphocyte antigen class I in patients with hepatocellular carcinoma

In 5 of the enrolled HCC patients, the expression of HLA class I was compared between cancer cells and surrounding non-tumorous hepatocytes using W6/32 (AbD), which is a monoclonal anti-HLA class I antibody. Specifically, the fresh tissue samples obtained from 5 HCC patients were cut into small pieces and divided into tumorous and surrounding non-tumorous parts. They were then digested with collagenase (1.5 mg/mL, Wako Pure Chemical, Osaka, Japan) for 30 minutes at 37°C . Cancer cells and surrounding non-tumorous hepatocytes obtained from digestive tissue of tumorous and surrounding non-tumorous parts, respectively, and flow cytometry analysis was conducted using a FACSAria cell sorting instrument (BD Biosciences, San Jose, CA, USA). IgG2a isotype control antibody (MBL, Nagoya, Japan) was used as a negative control. The mean fluorescence intensity (MFI) of HLA class I staining was examined in cancer cells and surrounding non-tumorous hepatocytes in each patient sample. The MFI rate of HLA class I staining was calculated as follows: MFI rate = MFI with the anti-HLA-class I Ab/MFI with isotype control Ab.

2.4 | Interferon- γ enzyme-linked immunospot assay

In cancer cells and surrounding non-tumorous hepatocytes from Case 7 who had HLA-A*24:02, interferon- γ (IFN- γ) enzyme-linked immunospot (ELISPOT) assays were performed, and the IFN- γ spot numbers for each concentration of antigen-specific CTL were compared between cancer cells and surrounding non-tumorous hepatocytes. The HLA-A*24:02-restricted cytomegalovirus (CMV)₃₄₁₋₃₄₉ peptide (QYDPVAALF) (ProImmune, Rhinebeck, NY, USA) and the HLA-A*24:02-restricted HIV peptide (RYLKDQQLL; ProImmune, Rhinebeck, NY, USA) as a negative control were used in this study. Target CD3 and CD56 cells were sorted from isolated cancer cells

and surrounding non-tumorous hepatocytes with a FACSAria cell sorter, and then pulsed (1×10^4 cells/well) with peptides (10 $\mu\text{g}/\text{mL}$) in RPMI 1640 medium with 10% FBS added overnight. We used CMV₃₄₁₋₃₄₉ peptide-specific CTL clones as effector cells. Briefly, peripheral blood samples were collected from HLA-A*24:02-positive healthy donors, after informed consent was obtained. Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation using Ficoll-Paque gradient, and frozen until the time of immunological analysis. PBMC were cocultured (2×10^6 cells/well) with the CMV₃₄₁₋₃₄₉ peptide (10 $\mu\text{g}/\text{mL}$) in RPMI-1640 that was supplemented with 10% heat-inactivated FBS for 14 days. Administration of 10 IU/mL IL-2 (Proleukin, Novartis, Basel, Switzerland) and 10 ng/mL IL-15 (PeproTech, Rocky Hill, NJ, USA) was performed at 3-4-day intervals between stimulations. CD8⁺ cells were isolated using human CD8 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) from these PBMC. CD8⁺ CD107a⁺ cells were sorted using a FACSAria cell sorter. Sorted CTL were stimulated and the CTL clones were established as previously described.²⁷ Effector cells were incubated with target cells for 20 hours at 37°C in 5% CO₂. The BD ELISPOT set (BD Biosciences, San Jose, CA, USA) was used for an IFN- γ ELISPOT assay. The spot numbers that indicate a peptide-specific CTL response were automatically calculated using the Eliphoto system (Minerva Tech, Tokyo, Japan).

2.5 | Statistical analysis

Comparison of the HLA class I expression by EMR 8-5 between cancer cells and surrounding non-tumorous hepatocytes in enrolled HCC patients was conducted using the Mann-Whitney *U*-test. Clinical prognosis, including RFS and OS, was evaluated using the Kaplan-Meier analysis, and the differences between 2 groups were compared using the log-rank test. RFS and OS were calculated from the date of surgery to the date of recurrence and death, respectively, or the date of last follow up if there were none of these events. In addition, association between the HLA class I expression level in cancer cells and tumor volume in HCC patients was evaluated using Pearson's correlation coefficient. Differences were considered significant at $P < 0.05$. All analyses were conducted using R version 3.5.1.

3 | RESULTS

3.1 | Clinical characteristics of enrolled patients with hepatocellular carcinoma

Patient characteristics are shown in Table 1. The mean age of enrolled patients was 58.6 years (range: 25-85 years); these patients included 13 men and 7 women. In addition, 17 and 3 patients had PS 0 and 1, respectively, and there were no patients with PS ≥ 2 . In addition, 5 patients had hepatitis B and 9 had hepatitis C virus infections. There were 13 and 7 patients with stage I and II HCC, respectively. No patients had stage III or IV cancer. There were 13 patients who died from HCC during the observation period, and 16 patients had

TABLE 1 Clinical characteristics and expression of HLA class I in enrolled patients with hepatocellular carcinoma

No.	Age/Sex	PS	Hepatic virus infection	Stage ^a	PFS, mo	OS, mo	HLA class I expression					
							Immunohistochemical staining ^b		Flow cytometry analysis ^c			
							Cancer cells	Non-tumorous hepatocytes	Cancer cells	Non-tumorous hepatocytes		
1.	77/M	0	C	2	66.1	96.9	>	++	-	1051.0	>	13.5
2.	79/F	0	C	1	37.1	37.1	>	++	+			
3.	65/M	0	C	2	3.8	18.5	>	+	-	20.8	>	1.0
4.	61/M	0	NBNC	2	6.3	20.7	>	++	+	1.0	<	3.3
5.	51/M	0	B	1	93.4	93.4	=	-	-			
6.	62/M	0	B	2	17.9	56.9	>	++	-	85.6	>	1.0
7.	69/F	0	NBNC	2	1.9	9.4	>	++	-	18.60	>	4.76
8.	75/F	1	C	1	53.3	109.7	>	++	+			
9.	71/F	0	C	2	13.3	55.7	>	++	-			
10.	66/M	0	B	2	4.8	40.2	<	-	+			
11.	81/M	0	B	2	98.6	98.6	>	++	-			
12.	70/F	0	C	1	55.0	61.7	=	++	++			
13.	75/M	1	NBNC	2	16.7	50.0	>	+	-			
14.	67/M	0	C	2	3.2	12.7	>	++	-			
15.	71/M	0	NBNC	1	18.5	83.6	>	++	-			
16.	64/M	0	B	2	8.7	70.5	=	-	-			
17.	61/M	0	C	2	3.9	10.0	>	++	+			
18.	78/M	0	NBNC	2	9.1	50.6	>	+	-			
19.	63/M	0	C	1	108.8	108.8	>	++	-			
20.	64/M	1	NBNC	2	18.5	71.4	=	-	-			

B, HBs Ag was examined by radioimmunoassay; C, HCV was detected by RT-PCR; F, female; HLA class I expression, human leukocyte antigen class I expression; M, male; mo, months; NBNC, not hepatic virus B or C; OS, overall survival; PFS, progression-free survival; PS, performance status.

^aStaging was performed according to the 8th pathological TNM classification for hepatocellular carcinoma (Union for International Cancer Control: UICC).

^bStaining of HLA class I used EMR 8-5, which is a monoclonal anti-pan HLA class I antibody. Degree of staining of target cells for HLA class I: -, negative membranous reactivity (staining cells: <20%); +, incomplete membranous reactivity (staining cells: 20%-80%); ++, complete membranous reactivity (staining cells: >80%).

^cFlow cytometry analysis evaluated the mean fluorescence intensity (MFI) ratio of HLA class I staining using W6/32.

a recurrence of HCC. The median RFS and OS in enrolled patients were 17.3 months (range: 1.9-108.8 months) and 56.3 months (range: 9.4-109.7 months), respectively.

3.2 | Immunohistochemical evaluation of human lymphocyte antigen class I expression in cancer cells and surrounding non-tumorous hepatocytes in hepatocellular carcinoma patients

We performed immunochemical staining of HLA class I using EMR 8-5 antibodies in enrolled HCC patients, and compared the expression level of HLA class I with cancer cells and surrounding non-tumorous hepatocytes. Representative images of immunostaining in 3 HCC patients are shown in Figure 1, which shows that in the tumorous sites of Cases 7 and 13, the cell membranes of many cancer cells were stained (Figure 1A,C), while in the non-tumorous sites only the stromal cells, including vascular endothelial cells and immune cells, were stained, leaving most of the surrounding non-tumorous hepatocytes

unstained (Figure 1B,D). In Case 16, both cancer cells and surrounding non-tumorous hepatocytes had no staining (Figure 1E,F). The expression of HLA class I in cancer cells indicated that among 20 HCC patients, 13 patients (65.0%) were complete positive, 3 patients (15.0%) were incomplete positive, and 4 (20.0%) had negative membrane staining. In addition, 15 patients (75.0%) had a higher expression level of HLA class I in cancer cells than in surrounding non-tumorous hepatocytes, while only 1 patient (5.0%) had a higher expression of HLA class I in surrounding non-tumorous hepatocytes than in the cancer cells (Table 1). Therefore, in our study, the HLA class I expression level by immunochemistry using EMR 8-5 was significantly higher in cancer cells than in surrounding non-tumorous hepatocytes in HCC tissue from stage I-II patients (Mann-Whitney test, $P < 0.05$).

3.3 | Human lymphocyte antigen class I expression measured by flow cytometry in cancer cells and surrounding non-tumorous hepatocytes in hepatocellular carcinoma patients

In 5 of the enrolled HCC patients, we compared the expression levels of HLA class I with cancer cells and surrounding non-tumorous hepatocytes by flow cytometry using W6/32. Representative data of the flow cytometry analysis of Case 7 is shown in Figure 2. In Case 7, the MFI of HLA class I in cancer cells was 18.60, while that in surrounding non-tumorous hepatocytes was 4.76. Therefore, in Case 7, the HLA class I expression level by flow cytometry using W6/32 was higher in cancer cells than in surrounding non-tumorous hepatocytes, which was consistent with the EMR 8-5 immunochemical staining results. In addition, in Case 7, the FACS data of HLA class I expression of non-tumorous hepatocytes was heterogenous. We investigated whether the cause would be the partial inclusion of immune cells and vasculature cells in the FACS analysis of non-tumorous hepatocytes by using digestive tissue obtained from non-tumorous parts. Overall, among the 5 HCC patients, 4 had a higher MFI of HLA class I in cancer cells than in surrounding non-tumorous hepatocytes (Table 1). In Case 4, the MFI of HLA class I was lower in cancer cells than in surrounding non-tumorous hepatocytes, which was not consistent with the EMR 8-5 immunohistochemistry analysis. This might have been because the flow cytometry data, which were obtained using tissue sections, reflect not only HLA class I expression of cancer cells or non-tumorous hepatocytes, but also that of some cells in the tumor microenvironment, including fibroblast and immune cells.

3.4 | Measurements of cytotoxic T lymphocyte responses in cancer cells and non-tumorous hepatocytes by interferon- γ enzyme-linked immunospot in hepatocellular carcinoma patients

In Case 7, which had complete positive expression of HLA class I in cancer cells and had negative expression of HLA class I in surrounding non-tumorous hepatocytes, we compared the immune response to CMV-specific CTL between CMV peptide-pulsed cancer cells and CMV peptide-pulsed surrounding non-tumorous

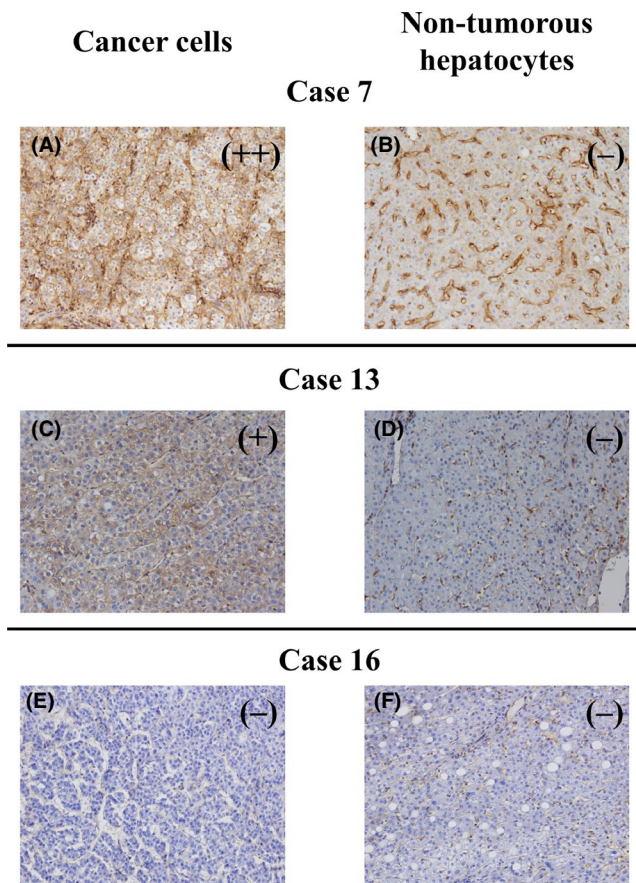


FIGURE 1 Representative staining patterns of human lymphocyte antigen (HLA) class I expression in paired cancer cells (left) and surrounding non-tumorous hepatocytes (right) from 3 hepatocellular carcinoma patients (A-B; C-D; E-F). All panels show cells stained with the monoclonal anti-HLA class I antibody, EMR 8-5. A, B, Immunohistochemical staining patterns in Case 7; C, D, Case 13; E, F, Case 16. (++) , complete membrane staining (>80%); (+), incomplete membrane staining (20%-80%); (-), negative membrane staining (<20%). Magnification, $\times 400$

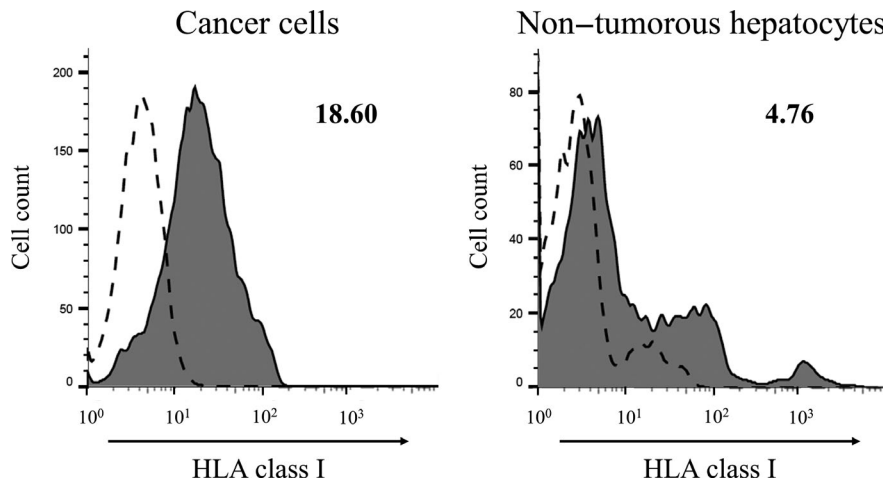


FIGURE 2 Flow cytometry analysis using W6/32 in Case 7. Numbers into the histograms show the mean fluorescence intensity of human lymphocyte antigen (HLA) class I in cancer cells and surrounding non-tumorous hepatocytes, respectively (black framed box). Horizontal axis: expression of HLA class I. Vertical axis: cell count. Solid line: cancer cells or surrounding non-tumorous hepatocytes. Dashed line: negative control

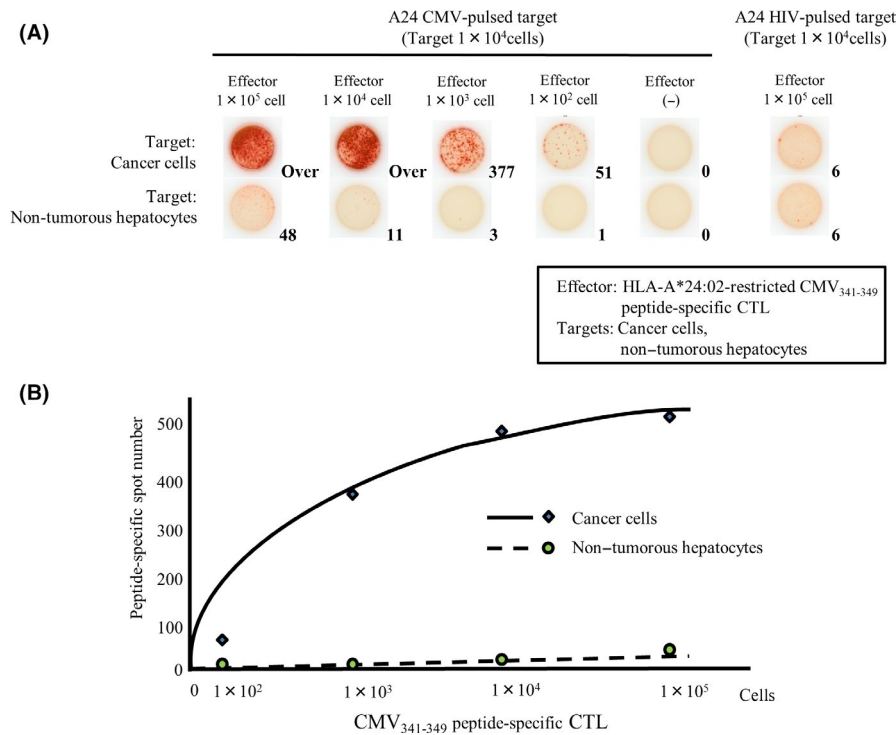


FIGURE 3 Comparison of ex-vivo IFN- γ enzyme-linked immunospot (ELISPOT) assay for the HLA-A*24:02-restricted CMV₃₄₁₋₃₄₉ peptide for each concentration of antigen-specific CTL between the cancer cells and surrounding non-tumorous hepatocytes in Case 7. A, A representative image and spot number from the IFN- γ ELISPOT assay. The numerical value in the lower right indicates the IFN- γ spot (red spot) number. B, Correlation curve between median spot number of IFN- γ ELISPOT and concentration of antigen-specific CTL

hepatocytes using an IFN- γ ELISPOT assay (Figure 3). The results suggested that peptide-pulsed cancer cells had strong reactivity with peptide-specific CTL, with an increase in the IFN- γ spot numbers (Figure 3A). In contrast, peptide-pulsed surrounding non-tumorous hepatocytes were poorly reactive with specific CTL, and showed little increase in the IFN- γ spot numbers. In addition, we found that in cancer cells, the immunological reactivity with peptide-specific CTL became stronger as the concentration

of peptide-specific CTL increased (Figure 3B). These differences in immune response of peptide-specific CTL between cancer cells and non-tumorous hepatocytes might be related to differences in the HLA class I expression level. In addition, based on these results, peptide-specific CTL might not attack surrounding normal tissue, even if injected peptides spread to the surrounding non-tumorous lesions beyond the tumor area in intratumoral peptide injection therapy, which has been previously reported.²⁸

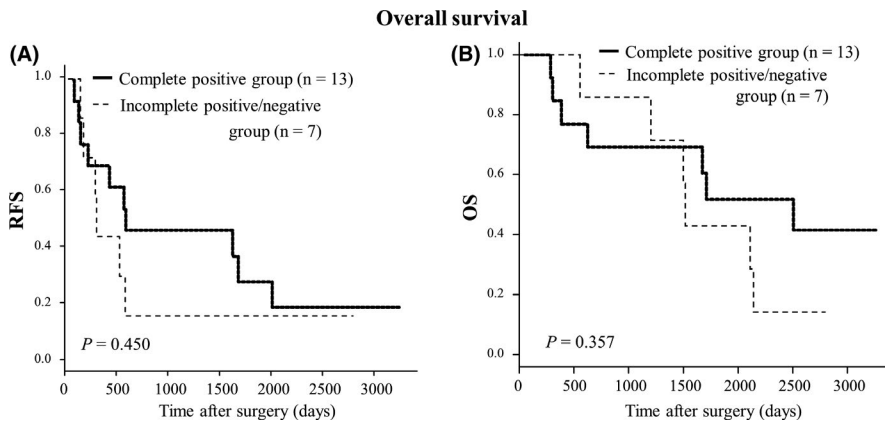


FIGURE 4 Association of human lymphocyte antigen (HLA) class I expression level in cancer cells with clinical prognosis, including (A) recurrence-free survival (RFS) and (B) overall survival (OS), in hepatocellular carcinoma patients. Kaplan-Meier graphs indicate the probabilities of RFS and OS in the patients who were distributed into 2 groups: complete positive group and incomplete positive/negative groups. Log-rank tests were used to analyze the significance of differences

3.5 | Association of human lymphocyte antigen class I expression in cancer cells and clinical prognosis in hepatocellular carcinoma patients

In enrolled HCC patients, we evaluated the association between clinical prognosis and the expression level of HLA class I in cancer cells using EMR 8-5 (Figure 4). The enrolled patients were divided into 2 groups: a “complete positive” group ($n = 13$) and an “incompletely positive and negative” group ($n = 7$). The median RFS of the “complete positive” group and the “incomplete positive and negative” group were 18.5 and 9.1 months, respectively. In the Kaplan-Meier analysis, there was no significant difference in RFS between the 2 groups (log-rank test, $P = 0.450$). The median OS of the “complete positive” group and the “incomplete positive and negative” group were 56.9 and 50.6 months, respectively. Kaplan-Meier analysis showed that there was no significant difference in OS between the 2 groups (log-rank test, $P = 0.357$).

In addition, we examined the association between the expression of HLA class I in cancer cells and HCC tumor volume in enrolled HCC patients (Figure S1). Patients with larger HCC tumor volume

tended to have lower HLA class I expression level in cancer cells (Pearson's correlation coefficient, $P = 0.34$, $r = 0.224$).

3.6 | Expression level of human lymphocyte antigen class I in cancer and surrounding normal tissue cells in various types of solid cancers

We also compared the HLA class I expression level by EMR 8-5 in cancer cells and surrounding normal tissue cells with several other solid cancers, including pancreatic, gallbladder, esophageal, colon and breast cancers (Table S1). The results are summarized in Table 2 and representative images are shown in Figure 5. In these tumorous sites, the cell membranes of many cancer cells were stained (Figure 5A,C,E,G,I), while in non-tumorous sites, only the stromal cells were stained, including vascular endothelial cells and immune cells. Almost none of the surrounding normal tissue cells were stained with the EMR 8-5 antibody (Figure 5B,D,F,H,J). We found that 66.7% (4/6), 100.0% (5/5), 83.3% (5/6), 42.9% (3/7) and 62.5% (5/8) of pancreatic, gallbladder, esophageal, colon and breast cancer cells had complete or incomplete positive expression of HLA class I,

Solid cancer types	HLA class I expression ^a	
	Positive rate in the cancer cells ^b (%)	Rate of cancer cells > normal tissue cells ^c (%)
Hepatocellular carcinoma	80.0	75.0
Pancreatic cancer	66.7	66.7
Gallbladder cancer	100.0	100.0
Esophageal cancer	83.3	66.7
Colon cancer	42.9	28.6
Breast cancer	62.5	62.5

TABLE 2 HLA class I expression by immunohistochemistry in various types of solid cancer

HLA class I expression, human leukocyte antigen class I expression.

^aHLA class I expression was examined by immunohistochemistry using EMR 8-5.

^bPositive rate in tumorous sites. HLA class I expression was defined as “positive” when degree of HLA class I staining was incomplete (staining cells: 20%-80%) and complete (staining cells: >80%) membranous reactivity.

^cHLA class I expression was compared between the cancer cells and surrounding non-tumorous hepatocytes by classifying the degree of HLA staining as negative membranous reactivity (staining cells: <20%), incomplete membranous reactivity (staining cells: 20%-80%) and complete membranous reactivity (staining cells: >80%).

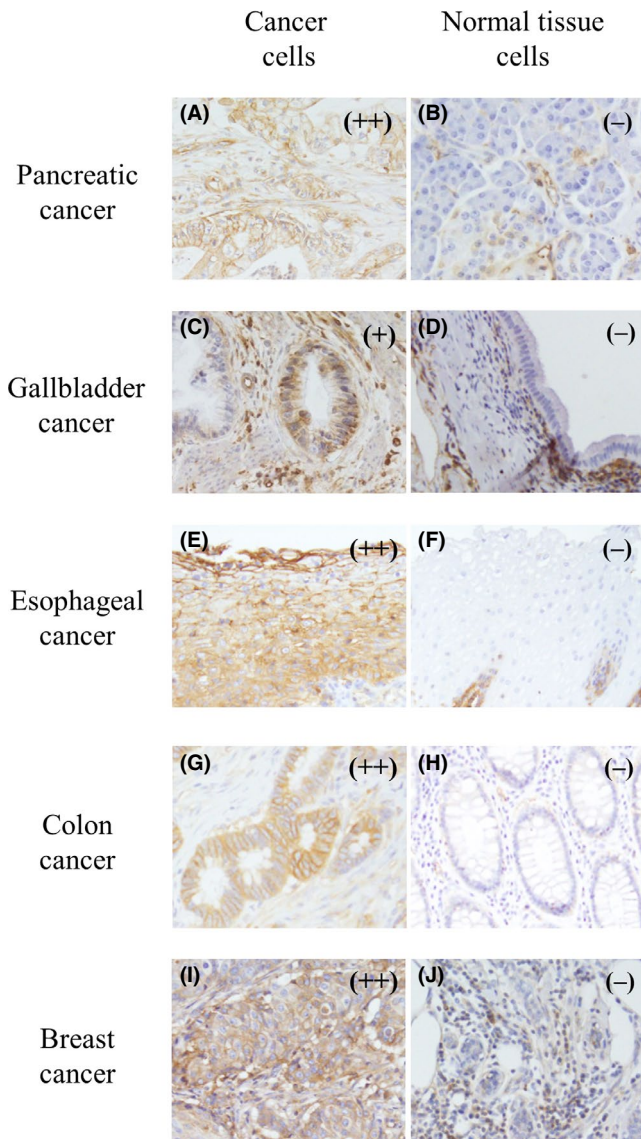


FIGURE 5 Representative images of human lymphocyte antigen (HLA) class I expression by immunohistochemical staining using EMR 8-5 between cancer and surrounding normal tissue cells in various solid cancers. (++) , complete membrane staining (>80%); (+), incomplete membrane staining (20%–80%); (–), negative membrane staining (<20%). Magnification, $\times 400$

respectively. In addition, compared to the expression of HLA class I in cancer and surrounding normal tissue cells, the expression level of HLA class I was higher in cancer cells than in surrounding normal tissue cells in 66.7% (4/6), 100.0% (5/5), 66.7% (4/6), 28.6% (2/7) and 62.5% (5/8) of the pancreatic, gallbladder, esophageal, colon and breast cancer samples, respectively.

4 | DISCUSSION

In this study, we evaluated the HLA class I expression level in cancer cells and surrounding non-tumorous hepatocytes in stage I–II HCC patients who underwent radical surgery, by immunohistochemistry

using EMR 8-5. We showed that in 15 of 20 HCC patients (75%), the expression levels of HLA class I were higher in cancer cells than in surrounding non-tumorous hepatocytes, and the IFN- γ ELISPOT assay of Case 7 indicated that cancer cells with positive expression of HLA class I had high reactivity with antigen-specific CTL. In addition, in several early-stage solid tumors, including pancreatic, gallbladder, esophageal and breast cancers, cancer cells had higher expression levels of HLA class I compared with surrounding normal tissue cells.

The W6/32 and EMR8-5 antibodies can recognize all HLA-A, -B and -C, the heavy chains. The W6/32 can evaluate HLA class I expression only in fresh frozen samples, because it would not recognize a denatured structure of HLA class I molecules. The EMR8-5 is the first anti-HLA class I monoclonal antibody that can evaluate HLA class I expression even in formalin-fixed paraffin-embedded tissue specimens. Several other HLA class I antibodies, including HC-A2 and HC-10, have also been used; HC-A2 and HC-10 could not recognize all HLA types. Remarkably, there are often differences in the expression level of HLA class I, depending on the HLA class I antibodies used. Kaneko et al⁸ showed that the positive expression rate of HLA class I in patients with breast cancer using EMR 8-5 was approximately 65%, whereas Madjd et al²⁹ suggested that the positive expression rate of HLA class I in the cases with breast cancer was approximately 50% when using HC-10. In addition, W6/32 is effective only for frozen specimens of cancer tissues and is unsuitable for evaluating the HLA class I expression level of formalin-fixed paraffin-embedded tissue sections that have been preserved for a long time. In addition, allele and haplotype frequencies of HLA loci are different among various races; thus, because EMR 8-5 reacts with extracellular domains of A *0101, A *0201, A *0207, A *1101, HLA-A *2402, B *0702, B *3501, B *4001, B *4002, B *4403, B *0801, B *1501, B *4006, Cw *0102, Cw *0801, Cw *1202 and Cw *1502,³⁰ this antibody is better suited for examining the expression of HLA class I in people around the world, especially in Japanese people who possess these HLA molecules.⁹ We believe that EMR 8-5 is an ideal antibody for evaluating the expression of HLA class I antigen in HCC patients.

Previously, it has been reported that HLA class I expression is downregulated in various cancer cells, including sarcoma (70%),⁹ and gastric (45%),¹⁶ non-small cell lung (30%)¹⁵ and esophageal (60%)¹⁷ cancer, by immunohistochemistry using EMR 8-5.^{8–18} Thus, the degree of loss of HLA class I may differ by organ specificity, suggesting the possibility that the mechanism of mutation and disappearance of HLA class I molecules is organ-dependent. Ishigami et al¹⁶ suggested that the expression of HLA class I is elevated in gastric cancer by exogenous stimulation by *Helicobacter pylori* bacterial infection or gastritis. In breast cancer, the expression of HLA class I might be related to the degree of proteolysis and inflammation that can occur at breast cancer origin sites.⁸ In this research, we evaluated the HLA class I expression in several early-stage cancer types, most of which had a higher HLA class I expression level in cancer cells than in surrounding normal tissue cells. However, colon cancer had a relatively low HLA class I expression

level in cancer cells (42.9%), and the proportion of patients with higher expression of HLA class I in cancer cells than in surrounding normal tissue cells was low (28.6%). This result might indicate one of the factors that leads to insufficient immune checkpoint inhibitor efficacy in colon cancer. In the future, we would like to further characterize the HLA class I expression in colon cancer on a larger scale.

In HCC, it has been reported using different antibodies that the expression of HLA class I is downregulated in 40%-50% of cancer cells.^{14,31-33} In our study, 16 (80.0%) of 20 HCC patients had complete or incomplete positive HLA class I expression, whereas only 4 (20.0%) had negative expression of HLA class I. The contradiction of these results could be from the different antibodies used. In addition, it might be also influenced by differences in patient backgrounds, including nationality or the organ in which the cancer originates as well as differences in the immunostaining method, including the preservation status of samples. Because patients enrolled in this study were at a relatively early stage in HCC prognosis, loss or mutations of HLA class I antigens may have not yet occurred in cancer cells, resulting in higher expression of HLA class I in our results compared to those from past literature. In addition, it has been reported that in normal human organs, HLA class I molecules exist on the surface of almost all nucleated cells, but the degree of their expression differs depending on the organ.³⁴ In particular, some reports have suggested that the HLA class I expression of normal hepatocytes was relatively low compared to that of other organs; these reports examined the HLA class I expression using W6/32.^{35,36} In addition, in this study using EMR8-5, we suggested that the expression of HLA class I in surrounding non-tumorous hepatocytes was negative in 75% of enrolled patients. Previous reports of several cancers, including breast, gastrointestinal and sarcoma, indicated that the expression of HLA class I was negative in normal tissues and positive in stage I-II tumors, and then decreased in stage III-IV tumors.^{8,9,16,17} In our study, the HLA class I expression level in cancer cells decreased as the HCC tumor volume increased. In addition, previously, we evaluated HLA class I expression using EMR 8-5 in patients with advanced HCC.²⁶ As a result, in 10 stage III HCC patients, 1 and 9 had complete positive and incomplete positive membrane staining of HLA class I, respectively. In addition, in 13 stage IV HCC patients, 2, 8 and 3 had complete positive, incomplete positive and negative membrane staining of HLA class I, respectively. Compared to the enrolled HCC patients with early stage disease in this study, the rate of complete positive staining of HLA class I was lower in HCC patients with stage III and IV disease. Thus, natural cancer progression would lead to cancer cells gradually losing the expression of HLA class I, which results in escaping antigen-specific CTL and could allow for tumor spread and metastasis. This transition to the tumor with negative expression of HLA class I is characterized by important changes in the tumor microenvironment and tissue re-organization.^{18,37,38} We also found that in patients with hepatic B virus infection, the expression rate of HLA class I in cancer cells compared to surrounding non-tumorous hepatocytes was relatively low (40.0%, $n = 2/5$). Thus, the patient's

clinical characteristics may also be involved in HLA class I expression level; however, this observation needs further investigation in the future.

Human lymphocyte antigen class I molecules play an important role in anti-cancer immune systems, especially as molecules with the ability to present antigen-specific CTL.¹⁴ In this study, we demonstrated that antigen-specific CTL had high reactivity toward HCC cells with strongly positive HLA class I expression (Figure 3). Previously, we identified glypican-3 (GPC3), which is a tumor-associated antigen, and conducted clinical trials of GPC3-peptide vaccination against patients with several cancer types, including advanced HCC.^{26,39-43} As a result of these trials, we found an adequate anti-tumor effect in advanced HCC with this peptide vaccination, which led to a better OS in HCC patients.^{26,39} We also developed a method of immunotherapy using intratumoral peptide injection that could induce additional peptide loading onto tumor cells. We demonstrated that this therapy enhanced the invasion of peptide-specific CTL into the tumor site in a mouse model.²⁸ The HLA class I expression level in cancer cells is an important indicator of the clinical effect of these peptide therapies. Similarly, in the novel immunotherapies such as T Cell Receptor-T cell therapy, the expression of HLA class I in tumor cells is indispensable.^{44,45} In the patients with stage I-II HCC, the expression of HLA class I in cancer cells is high and, therefore, these immunotherapies are more likely to be effective. In addition, the HLA class I expression level was low in surrounding non-tumorous hepatocytes, indicating that normal cells are not targeted by CTL in HCC patients. This suggests that even if injected peptides spread into surrounding non-tumorous lesions beyond the target area when using intratumoral peptide injection techniques, normal cells are less likely to load the peptide onto their HLA class I molecules and be targeted by CTL cytotoxicity.

Some studies have reported that HLA class I expression could be a prognosis factor in a variety of carcinomas.^{9,19-23} Tsukahara et al⁹ showed that patients with osteosarcoma, who had positive expression of HLA class I, had better PFS or OS than those who had negative expression of HLA class I. Iwayama et al²³ also suggested that positive expression of HLA class I was associated with better PFS in patients with stage II colorectal cancer. Thus, patients with high expression levels of HLA class I in cancer cells have a more favorable clinical outcome than patients with low HLA class I expression level in cancer cells. These results showed that cancer cells with high HLA expression are recognized and attacked by antigen-specific CTL, which leads to good clinical prognosis including OS and PFS. In contrast, Madjd et al²⁹ showed that HLA class I loss was an independent factor of good prognosis in breast cancer. Based on this result, they speculated that HLA class I loss could make tumors more susceptible to natural killer (NK) cell targeting, resulting in a better prognosis. In addition, Lee et al³⁰ suggested that in gastric cancer, the loss of HLA class I expression was not associated with clinical outcome. In this study, the RFS and OS of the "complete positive" group tended to be better than those of "incomplete positive and negative" groups, during 1.5-5 and >5 years after surgery, respectively.

Therefore, our findings showed that the expression of HLA class I in cancer cells might be involved in prolonging RFS and OS in the relatively long-term period after surgery in patients with stage I-II HCC. Therefore, the prognostic influence of the expression level of HLA class I might be complicated and dependent on various cancer immune circumstances.

In conclusion, we showed using EMR 8-5 immunohistochemistry that in patients with several early-stage cancers, including HCC, the HLA class I expression was higher in cancer cells than in surrounding normal tissue cells, which could result in the anti-tumor effect of CTL-based cancer immunotherapies. We hope that prospective large-scale studies of HLA class I expression with appropriate patient inclusion criteria will be performed in the future.

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DISCLOSURE

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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