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Prognostic value of HLA class I expression in patients with oral squamous cell carcinoma

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Abstract

Human leukocyte antigen (HLA) class I molecules play a central role in anticancer immunity, but their prognostic value in oral squamous cell carcinoma (OSCC) remains unclear. We examined HLA class I expression in 2 distinct tumor compartments, namely, the tumor center and invasive front, and evaluated the association between its expression pattern and histopathological status in 137 cases with OSCC. Human leukocyte antigen class I expression was graded semiquantitatively as high, low, and negative. At the invasive front of the tumor, HLA class I expression was high in 72 cases (52.6%), low in 44 cases (32.1%), and negative in 21 cases (15.3%). The HLA class I expression in the tumor center was high in 48 cases (35.0%), low in 58 cases (42.4%), and negative in 31 cases (22.6%). The 5-year overall survival and disease-specific survival rates were good in cases with high HLA class I expression at the invasive front; however, there was no significant difference in survival based on HLA class I expression in the tumor center. In addition, high HLA class I expression was correlated with high $CD8^+$ T cell density, whereas negative HLA class I expression was correlated with low $CD8^+$ T cell density at the invasive front. These results suggest that it is easier for $CD8^+$ T cells to recognize presented peptides in the case of high HLA class I expression at the tumor invasive front and could be a prognostic factor for OSCC.

KEYWORDS

HLA class I, oral squamous cell carcinoma, prognostic factor, survival, tumor-infiltrating lymphocyte

Abbreviations: DSS, disease-specific survival; HLA, human leukocyte antigen; IF, invasive front; IFN-γ, interferon gamma; NK, natural killer; OS, overall survival; OSCC, oral squamous cell carcinoma; RFS, recurrence-free survival; TCe, tumor center.

1 | INTRODUCTION

Oral cancer is the ninth most common malignancy worldwide,[1] with OSCC representing approximately 90% of oral malignancies. Despite advanced knowledge of cancer therapy, more than 145 000 deaths from oral cancer occur annually,[1] with a 5-year survival rate of approximately 50% for OSCC.[2] Unfortunately, this rate has not improved in recent decades, indicating a need for new treatments and therapeutic approaches.[2]

Cancer cells can evade immune surveillance, allowing them to grow and metastasize. One mechanism of immune escape during cancer development is the downregulation of the expression of HLA class I molecules,3-6 which are expressed on all nucleated cells and present intracellular protein fragments on the cell surface, particularly for recognition by CTLs, eg, $CD8^+$ T cells.[7] The status of the immune system is an important factor in the prognosis of cancer patients, and HLA class I antigen downregulation has been used as a prognostic biomarker in esophageal cancer,[8] malignant melanoma,[9] lung cancer,[10] ovarian cancer,[11] colon cancer,[12] renal cell carcinoma,[13] and bladder cancer,[14] thus improving the survival of patients. However, the downregulation of HLA class I expression is not observed in all types of cancer, such as gastric cancer.[15] Regardless, the loss of HLA class I molecules has been discussed in the context of tumor aggressiveness, including differentiation, invasiveness, and metastatic potential.[16,17]

Because most anti-HLA class I Abs recognize the allele-specific native structure of HLA class I molecules, these Abs are unable to react with denatured HLA class I molecules in formalin-fixed paraffin-embedded tissue sections. However, a monoclonal pan-HLA class I Ab, EMR8-5, has been developed that is suitable for immunostaining formalin-fixed tissue specimens.[18] Therefore, we are now able to retrospectively investigate HLA class I expression in surgically resected cancer specimens.

We recently reported that tumor-infiltrating $CD8^+$ T cell density was an independent prognostic marker for OSCC; specifically, $CD8^+$ T cells at the parenchyma of the invasive edge of the tumor were an indicator of recurrence and prognosis.[19] However, there are few reports on HLA class I expression in OSCC,[20,21] and no study has examined the association between HLA class I expression and $CD8^+$ T cell density in detail. Therefore, in this study, we focused on the expression of HLA class I molecules in cancer cells and its relationship with $CD8^+$ T cell density to investigate its prognostic value in patients with OSCC.

2 | MATERIALS AND METHODS

2.1 | Patients and tissue samples

This retrospective study was undertaken according to the principles of the 1964 Declaration of Helsinki and its subsequent revisions and was approved by the Institutional Review Board of our university on 12 September 2017 (No. 292-1116). Informed consent or acceptable substitutes were obtained from all patients before study inclusion. We used tissue samples collected from patients who were diagnosed with OSCC and underwent definitive surgery between January 2004 and December 2014 at Sapporo Medical University Hospital (Table 1). Tissue samples were processed, embedded in paraffin, and sectioned into 4-μm samples. None of the patients received any form of neoadjuvant or adjuvant chemo- and/or radiotherapy before or after surgery.

TABLE 1 Characteristics of patients and oral squamous cell carcinoma tumors

Characteristic	No. of patients	Percentage
Sex		
Male	76	55.5
Female	61	44.5
Age, y		
<68	63	46.0
≥68	74	54.0
Anatomical site		
Tongue/floor of the mouth	89	65.0
Other	48	35.0
Primary tumor		
T1	46	33.6
T2	77	56.2
T3/4	14	10.2
Regional lymph nodes		
N (−)	108	78.8
N (+)	29	21.2
Stage grouping		
Stage I	42	30.7
Stage II	61	44.5
Stage III/IV	34	24.8
Histopathological grading		
Grade 1	73	53.3
Grade 2	59	43.1
Grade 3	5	3.6
Lymphovascular invasion		
Absent	115	83.9
Present	22	16.1
Perineural invasion		
Absent	125	91.2
Present	12	8.8

2.2 | Immunohistochemistry

The monoclonal anti-pan-HLA class I Ab EMR8-5 was established by Torigoe et al[18] This mouse mAb is commercially available (Hokudo) and reacts with the extracellular domains of HLA-A*2402, A*0101,

A*1101, A*0201, A*0207, B*0702, B*0801, B*1501, B*3501, B*4001, B*4002, B*4006, B*4403, Cw*0102, Cw*0801, Cw*1202, and Cw*1502.[22] Immunohistochemical staining with EMR8-5 was carried out on formalin-fixed, paraffin-embedded tissue sections after steam heat-induced epitope retrieval. Subsequent incubations with a secondary biotinylated Ab, avidin-conjugated peroxidase complex, and chromogen were undertaken on a Ventana NexES system (Ventana Medical Systems).[23] The slides were counterstained with hematoxylin, rinsed, dehydrated in a graded ethanol series, and coverslipped with mounting media. Positive reactivity to EMR8-5 was confirmed by staining vascular endothelial cells and lymphocytes in sections of tumor specimens.[22] In addition, tissue sections were incubated with a primary mAb against pan-cytokeratin (1:200, clone AE1/AE3; Abcam) to detect multiple cytokeratins and identify tumor cells. $CD8^+$ T cells were stained according to our previous report.[19]

2.3 | Histopathological and immunohistopathological evaluation

The histological slides were evaluated for lymphovascular invasion, perineural invasion, and histopathological grading. Tumor extent and histopathological grading were classified according to the cancer staging manual of the American Joint Committee on Cancer.[24] Human leukocyte antigen class I expression was evaluated at the TCe and IF at ×200 magnification (Figure 1). For the assessment of HLA class I density in each compartment (ie, TCe and IF), at least 5 fields were viewed, and in heterogeneous cases, the most representative area of the entire section was selected. The HLA class I expression was assessed on a personal computer with DP2-BSW software and an Olympus microscope digital camera by three individuals (authors KK, SS, and HD) (Figure 2). Labels bearing the patient pathology number were covered. Human leukocyte antigen class I expression was classified into 3 categories as previously described[25]: high (more than 50% positive cells), low (5%-50% positive cells), and negative (less than 5% positive cells). Briefly, positivity was defined as complete and homogeneous membrane staining in more than 50% of tumor cells; low positive expression was defined as faint, incomplete, and heterogeneous membrane staining in 5%-50% of tumor cells; and negative expression was defined as membrane staining in less than 5% of tumor cells. $CD8^+$ T cell expression was also evaluated at the TCe and IF at ×200 magnification (Figure 3). The mean $CD8^+$ T cell density for each compartment was used to stratify the samples into the high- and low-density groups according to our previous report.[19]

2.4 | Statistical analysis

Disease-specific survival was calculated from the date of definitive surgery to date of death. Overall survival was defined as the time between the date of definitive surgery and date of death from any cause. Recurrence-free survival was defined as the time between the date of definitive surgery and locoregional or distant tumor recurrence, or death from any cause. Disease-specific survival, OS, and RFS were analyzed using the Kaplan-Meier method and compared using a log-rank test for each group. Variables that had prognostic potential indicated by univariate analysis were evaluated using multivariate analysis with Cox proportional hazard regression models. Associations between HLA class I expression and $CD8^+$ T cell infiltration density were evaluated using a χ^2 test. Two-tailed *P*-values less than 0.05 were considered statistically significant. SPSS version 23.0 for Windows (IBM) was used for statistical analyses.

3 | RESULTS

3.1 | Patient and tumor characteristics

From January 2004 to December 2014, a total of 221 patients with primary OSCC were treated by curative surgery. Of these, 137 patients were treated without neoadjuvant or adjuvant therapy, and sufficient tissue samples from these patients were available for further analysis. The 137 patients with OSCC comprised 76 men (55.5%) and 61 women (44.5%) with a median age of 68 (range, 33-93) years at first presentation. Anatomical sites included the tongue/floor of the mouth (89 tumors, 65.0%) and other regions of the oral cavity (48 tumors, 35.0%). Using the UICC staging classification system, the participants were classified as follows: 42 patients in

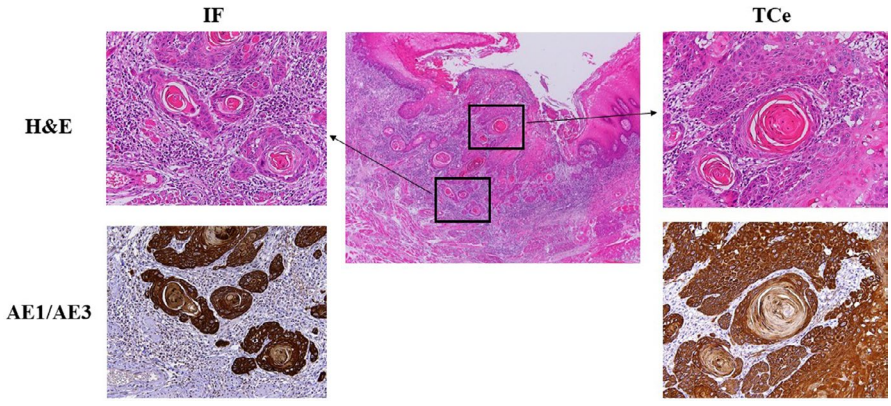

FIGURE 1 Representative H&E staining and immunohistochemical pan-cytokeratin labeling of oral squamous cell carcinoma sections for the assessment of 2 distinct tumor compartments. Tumor center (TCe) and invasive front (IF) were evaluated. Pan-cytokeratin expression was detected using a primary mAb (clone AE1/AE3; Abcam)

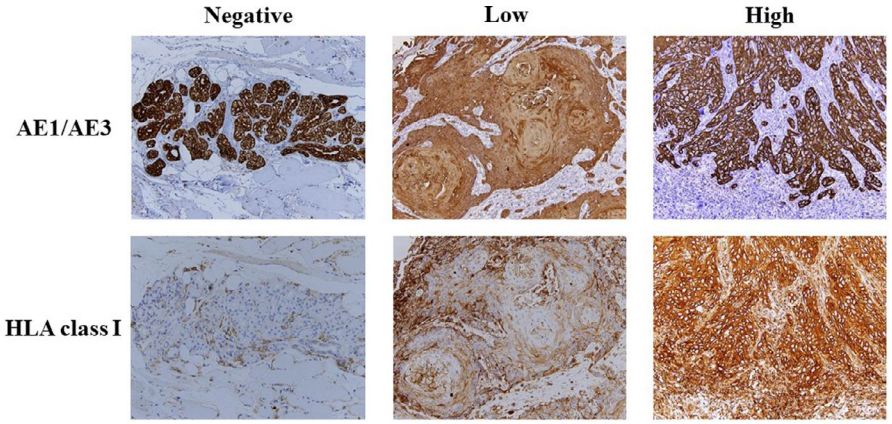

FIGURE 2 Immunohistochemical labeling of oral squamous cell carcinoma sections for the assessment of human leukocyte antigen (HLA) class I expression. HLA class I expression was classified into 3 categories: high (>50% positive cells), low (5%-50% positive cells), and negative (<5% positive cells). To detect tumor cells, immunohistochemistry was carried out using a primary mAb against pan-cytokeratin (clone AE1/AE3; Abcam)

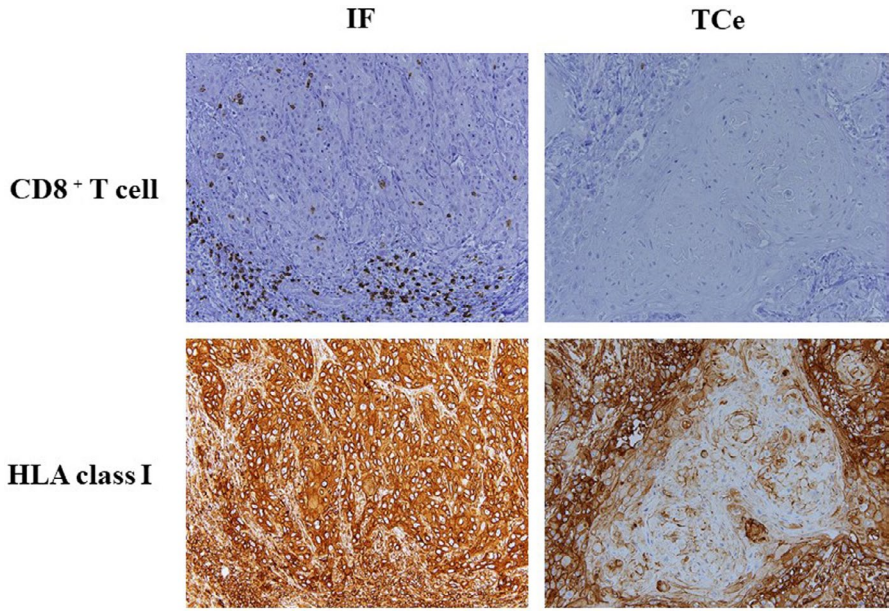

FIGURE 3 Representative immunohistochemical $CD8^+$ T cell and human leukocyte antigen (HLA) class I density of oral squamous cell carcinoma sections for the assessment of 2 distinct tumor compartments. Tumor center (TCe) and invasive front (IF) were evaluated

stage I (30.7%), 61 in stage II (44.5%), and 34 in stage III/IV (24.8%). Histopathological grading was as follows: 73 patients were assigned grade 1 (53.3%), 59 were assigned grade 2 (43.1%), and 5 were assigned grade 3 (3.6%). One hundred and fifteen patients (83.9%) did not have lymphovascular invasion, whereas it was present in 22 patients (16.1%); 125 patients (91.2%) did not have perineural invasion, but it was present in 12 patients (8.8%).

3.2 | Relationship between clinicopathological data and survival rates

The median follow-up period for all patients was 79 (range, 4-164) months. Primary tumors recurred in 16 patients (11.6%), and regional lymph node relapse was observed in 24 patients (17.5%). Five-year DSS, OS, and RFS rates for all patients were 90.5%, 83.2%, and 62.8%, respectively (Table 2). Age and perineural invasion were associated with DSS, OS, and RFS and thus considered important indicators.

4 | Expression of HLA class I in OSCC sections

Expression of HLA class I was measured in the OSCC sections to evaluate different tumor regions. At the IF, 72 cases (52.6%) had high HLA class I expression, 44 (32.1%) had low expression, and 21 (15.3%) were negative. At the TCe, 48 cases (35.0%) had high HLA class I expression, 58 (42.4%) had low expression, and 31 (22.6%) were negative. These results indicated that HLA class I molecules are expressed differentially in different compartments of OSCC.

5 | Relationship between HLA class I expression and survival rates

Patients with high HLA class I expression at the IF had significantly better OS than those with low or negative expression (90.3%, 79.5%, and 66.7%, respectively, $P = 0.03$). In contrast, there was no significant difference in survival based on HLA class I expression in the TCe (Figure 4). Disease-specific survival was better in patients with

TABLE 2 Five-year overall survival (OS), disease-specific survival (DSS), and recurrence-free survival (RFS) according to clinicopathological variables in patients with oral squamous cell carcinoma

	OS		DSS		RFS	
Observed findings	Survival rate (%)	Log-rank test (*P* value)	Survival rate (%)	Log-rank test (*P* value)	Survival rate (%)	Log-rank test (*P* value)
Sex						
Male	82.9	0.930	90.8	0.890	65.8	0.340
Female	83.6		90.2		59.0	
Age, y						
<68	95.2	**0.001**	98.4	**0.003**	74.6	**0.010**
≥68	73.0		83.8		52.7	
Anatomical site						
Tongue/floor of the mouth	88.8	**0.009**	94.4	**0.020**	66.3	0.160
Other	72.9		83.3		56.3	
Primary tumor						
T1	95.7	**0.001**	97.8	**0.030**	69.6	0.120
T2	80.5		88.3		62.3	
T3/4	57.1		78.6		42.9	
Regional lymph nodes						
N (−)	88.0	**0.001**	95.4	**<0.001**	65.7	0.170
N (+)	65.5		72.4		51.7	
Stage grouping						
Stage I	97.6	**<0.001**	100.0	**0.001**	71.4	0.140
Stage II	83.6		91.8		63.9	
Stage III/IV	64.7		76.5		50.0	
Histopathological grading						
Grade 1	87.7	0.330	94.5	0.210	71.2	**0.003**
Grade 2	78.0		86.4		55.9	
Grade 3	80.0		80.0		20.0	
Lymphovascular invasion						
Absent	86.1	0.050	92.2	0.140	67.0	**0.010**
Present	68.2		81.8		40.9	
Perineural invasion						
Absent	86.4	**<0.001**	92.8	**0.001**	66.4	**0.001**
Present	50.0		66.7		25.0	

Two-tailed *P*-values less than 0.05 were considered statistically significant.

high HLA class I expression at the IF compared with patients with low and negative expression ($P = 0.04$), whereas there was no significant difference in DSS according to HLA class I expression in the TCe (Figure 5). Recurrence-free survival was not significantly different among all patients (Figure 6). Together, these results indicated that HLA class I expression at the IF could be a prognostic factor for OS and DSS in patients with OSCC.

In multivariate analysis, HLA class I expression at the IF was an independent prognostic factor for OS (hazard ratio 0.43; 95% confidence interval, 0.20-0.91; $P = 0.02$). Age, stage grouping, and perineural invasion were independent prognostic factors for OS and DSS. Moreover, none of the parameters were independent prognostic factors for RFS. Overall, these results suggested that HLA class I expression at the IF could be a prognostic factor for OS (Table 3).

6 | Relationship between HLA class I expression and CD8$^+$ T cell density

At the IF, high HLA class I expression was correlated with high CD8$^+$ T cell density. In addition, negative HLA class I expression was correlated with low CD8$^+$ T cell density. In contrast, there was no significant difference in the TCe (Table 4). These results suggested that it is easier for CD8$^+$ T cells

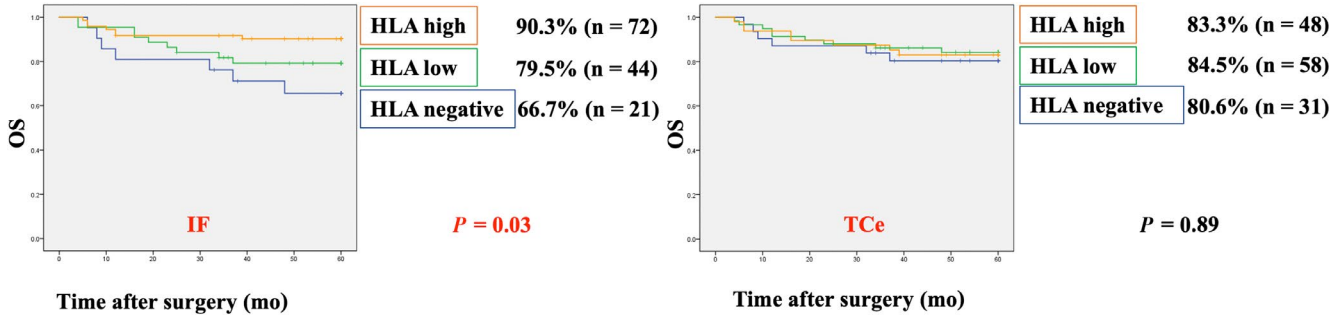

FIGURE 4 Relationship between human leukocyte antigen (HLA) class I expression and 5-year overall survival (OS) of patients with oral squamous cell carcinoma. The orange line indicates high HLA class I expression, the green line indicates low HLA class I expression, and the blue line indicates negative HLA class I expression. Patients with high HLA class I expression at the invasive front (IF) showed better OS than those with low or negative expression. However, there was no difference in OS based on HLA class I expression in the tumor center (TCe)

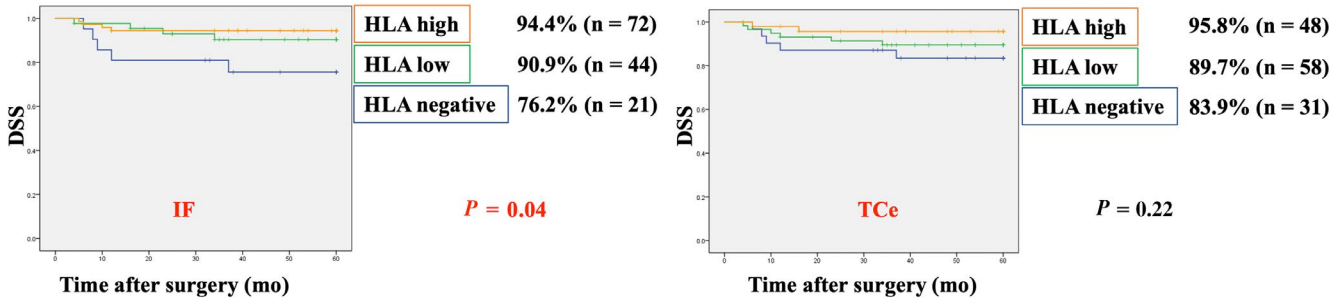

FIGURE 5 Relationship between human leukocyte antigen (HLA) class I expression and 5-year disease-specific survival (DSS) of patients with oral squamous cell carcinoma. The orange line indicates high HLA class I expression, the green line indicates low HLA class I expression, and the blue line indicates negative HLA class I expression. Patients with high HLA class I expression at the invasive front (IF) had a better DSS rate than those with low and negative expression. In contrast, there was no difference in DSS based on HLA class I expression in the tumor center (TCe)

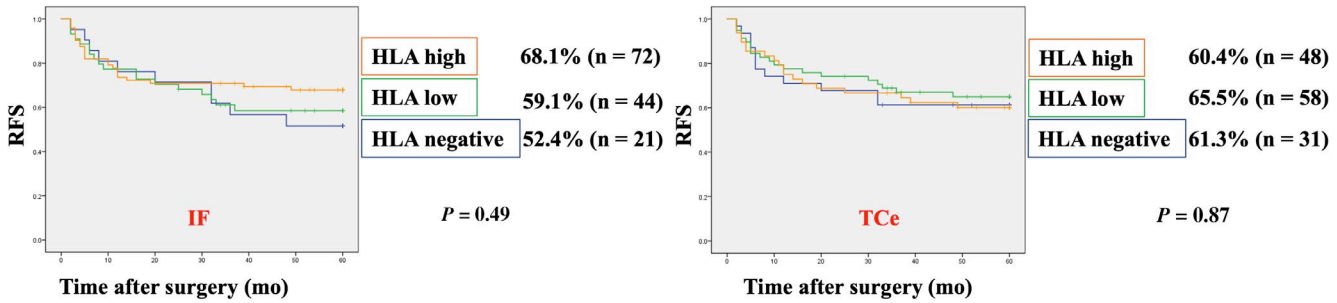

FIGURE 6 Relationship between human leukocyte antigen (HLA) class I expression and 5-year recurrence-free survival (RFS) of patients with oral squamous cell carcinoma. The orange line indicates high HLA class I expression, the green line indicates low HLA class I expression, and the blue line indicates negative HLA class I expression. There was no difference in RFS according to HLA class I expression (high, low, or negative) in each tumor region

to recognize presented peptides in the case of high HLA class I expression at the IF, whereas the ability of $CD8^+$ T cells to recognize presented peptides in the case of low HLA class I expression is impaired.

7 | DISCUSSION

There are several clinical and pathological prognostic factors for cancer, but only a few immunological prognostic methods. The immunological state of the host can influence the prognosis and features of cancer. Human leukocyte antigen class I molecules play a central role in cell-mediated immunity, especially as antigen-presenting molecules for CTLs, which recognize tumor antigen-bound peptides presented on the cell surface through HLA class I molecules and kill the target cancer cell.[26,27] Human leukocyte antigen class I expression appears to be lost or downregulated on the tumor cell surface, which could represent a mechanism for neoplastic cells to escape killing by CTLs, allowing tumor dissemination and metastasis.[28] The total loss of HLA class I expression reportedly occurs in approximately 15% and 40% of primary and metastatic head and neck SCC lesions,

TABLE 3 Multivariate analysis of overall survival (OS), disease-specific survival (DSS), and recurrence-free survival (RFS) in patients with oral squamous cell carcinoma

	OS			DSS			RFS		
Parameters	**HR**	**95% CI**	***P* value**	**HR**	**95% CI**	***P* value**	**HR**	**95% CI**	***P* value**
Immunohistochemical findings									
TCe	0.56	0.26-1.18	0.13	0.13	0.01-1.02	0.05	0.81	0.50-1.31	0.40
IF	**0.43**	**0.20-0.91**	**0.02**	0.38	0.11-1.26	0.11	0.80	0.52-1.24	0.32
Clinical findings									
Sex	0.53	0.21-1.31	0.17	0.64	0.17-2.28	0.48	1.17	0.66-2.07	0.59
Age	**5.13**	**1.35-19.37**	**0.01**	**28.00**	**1.48-529.22**	**0.02**	1.74	0.89-3.42	0.10
Anatomical site	1.92	0.64-5.74	0.24	1.49	0.25-8.89	0.65	1.18	0.58-2.38	0.63
Primary tumor	1.63	0.64-4.16	0.30	1.49	0.37-3.87	0.76	1.11	0.54-2.30	0.76
Stage grouping	**2.82**	**1.20-6.64**	**0.01**	**7.74**	**1.43-41.89**	**0.01**	1.16	0.64-2.12	0.61
Pathological findings									
Histopathological grading	1.08	0.49-2.34	0.84	1.31	0.40-4.21	0.64	1.53	0.90-2.59	0.11
Lymphovascular invasion	2.49	0.92-6.69	0.07	3.77	0.68-14.82	0.13	1.62	0.82-3.20	0.16
Perineural invasion	**3.58**	**1.15-11.13**	**0.02**	**6.85**	**1.12-41.75**	**0.03**	2.02	0.87-4.68	0.09

Two-tailed *P*-values less than 0.05 were considered statistically significant.

Abbreviations: CI, confidence interval; HR, hazard ratio; IF, invasive front; TCe, tumor center.

TABLE 4 Relationship between human leukocyte antigen (HLA) class I expression and infiltrating $CD8^+$ T cell density in patients with oral squamous cell carcinoma

	Number of cases with $CD8^+$ T cell density		
	Low, n (%)	**High, n (%)**	***P* value**
HLA class I expression			
IF			
Negative (n = 21)	17 (81.0)	4 (19.0)	**0.003**
Low (n = 44)	31 (70.5)	13 (29.5)	
High (n = 72)	33 (45.8)	39 (54.2)	
TCe			
Negative (n = 31)	23 (74.2)	8 (25.8)	0.050
Low (n = 58)	28 (48.3)	30 (51.7)	
High (n = 48)	25 (52.1)	23 (47.9)	

Two-tailed *P*-values less than 0.05 were considered statistically significant.

Abbreviations: IF, invasive front; TCe, tumor center.

respectively.[29] Similarly, in our study, the loss of HLA class I expression at the IF was observed in 15% (21/137) of cases.

Recent reports have shed light on the immunological tumor microenvironment and immune escape mechanisms of cancer cells,[30-35] but a detailed understanding is still lacking. The clinical relevance of the immunological tumor microenvironment, including the prognostic value of $CD8^+$ T cells, is controversial and remains to be elucidated.[30-31,36,37] In addition, the prognostic value of the spatial heterogeneity (IF vs TCe) of tumor-infiltrating lymphocytes and immune escape mechanisms remains unclear.

Therefore, we focused on the expression of HLA class I molecules to identify possible novel immunological prognostic factors. Our data showed that high HLA class I expression at the IF of OSCC was significantly related to better OS and DSS compared with low or negative HLA class I expression, which was consistent with the findings of other studies[7,21] and suggests that the level of HLA class I expression at the IF could be a prognostic factor. Because HLA class I expression at the IF was associated with decreased cancer cell proliferation and metastasis based on survival, it might exert a protective effect against cancer. In addition, we previously reported that $CD8^+$ T cell density at the IF was an indicator of tumor recurrence and prognosis.[19] The cases analyzed in our previous and present studies largely overlapped, but in the present study, we analyzed the relationship between $CD8^+$ T cell density and HLA class I expression. Negative HLA class I expression was correlated with low levels of $CD8^+$ T cells at the IF, but there was no significant difference in the TCe. It is possible that $CD8^+$ T cells were concentrated at the IF because tumor vasculature was enriched in the infiltrative margin, but not in the tumor center. In addition, we found that $CD8^+$ T cells were not recruited to sites where HLA class I expression was downregulated.

Tsukahara et al reported that patients with osteosarcoma who had high HLA class I expression had significantly better OS and disease-free survival than those with HLA class I-negative osteosarcoma.[22] Most reports, including the present study, suggest that the downregulation of HLA class I expression is associated with poor prognosis. In contrast, Madjd et al reported that the total loss of HLA class I expression was an independent indicator of good prognosis in breast cancer.[38] They considered that the loss of HLA class I

molecules could make tumors more susceptible to NK cells and result in a better prognostic outcome due to the presence of HLA class I allele-specific killer cell inhibitory receptors on the surface of NK cells. Thus, in the absence of HLA class I expression, specific killer cell inhibitory receptor-mediated inhibitory signaling is lost and NK cytolytic effector functions are activated. Natural killer cell-mediated cytotoxicity is regulated by a delicate balance between activating and inhibitory signals. Therefore, the prognostic influence of HLA class I expression might depend on the status of cancer immunity.

We observed a few cases in which HLA class I expression and $CD8^+$ T cell density were not correlated, but this could be explained by the presence of IFN-γ, which is a central cytokine that coordinates tumor immune responses and the associated biological consequences.[39,40] In fact, detection of IFN-γ mRNA expression at the IF has been reported in tumor cells and immune cells in OSCC.[7] In the present study, we confirmed $CD4^+$ T cell infiltration at the IF (Figure S1). Because IFN-γ is secreted mainly by activated $CD4^+$ T cells, we considered that this IFN-γ might be responsible for the upregulation of HLA class I molecules.

Many studies on HLA class I expression in cancer have produced contradictory results, perhaps due to the use of highly heterogeneous populations and because the distribution of HLA alleles and the linkage disequilibrium among alleles of diverse HLA genes differ in various ethnic populations.[41] In addition, relatively small populations were included in several studies, and the Abs, immunostaining methods, and scoring systems differed. Finally, the cut-off used to classify downregulation and HLA class I-positive groups varied substantially. Due to the differences in these criteria, a reliable comparison of these studies is difficult to carry out.

Our study showed a survival benefit for patients expressing HLA class I molecules, suggesting that the class I-restricted CTL pathway plays a major role in immune surveillance in patients with OSCC. Due to the small sample size of the present analysis, larger studies need to be undertaken to verify the significance of HLA class I expression in prognosis and the applicability of T cell-based immunotherapy for patients with OSCC.

DISCLOSURE

The authors have no conflicts of interest to disclose.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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