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Clinicopathological and Prognostic Analysis of PD-L1 and PD-L2 Expression in Surgically Resected Primary Tongue Squamous Cell Carcinoma

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Abstract. *Background/Aim: The expression of tumor-associated programmed death-ligand 1 (PD-L1) predicts clinical responses to PD-1-directed immunotherapy. The expression levels of PD-L2, another PD-1 ligand, and its relationship with responses to PD-1-targeting therapy in oral squamous cell carcinoma (OSCC) remain unclear. Furthermore, the clinicopathological characteristics and prognostic effects of the expression of PD-L1 and PD-L2 in OSCC have not yet been elucidated. Materials and Methods: The expression of PD-L1 and PD-L2 was immunohistochemically examined in 98 tongue carcinomas. Furthermore, the expression levels of PD-L1 and PD-L2 in OSCC cell lines and their relationships with those of MMP2 and MMP9 were assessed. Results: The expression levels of PD-L1 and PD-L2 correlated with those of MMP2 and MMP9. The expression of PD-L1 and/or PD-L2 was detected in OSCC cells, and their levels correlated with those of MMP9. The prognosis of patients with PD-L1- and PD-L2-positive tumors was significantly worse. Conclusion: PD-L1 and PD-L2 status is potentially a novel predictor of the prognosis of OSCC and provides a rationale for the development of novel immunotherapies.*

Malignant tumors evade immune surveillance (1), and the underlying mechanisms in human cancers have been suggested to involve the manipulation of costimulatory signaling (2), which is crucial for the initiation and termination of immune responses *via* the activation of T cells (1, 3).

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Signaling by programmed death 1 (PD-1), a costimulatory molecule, suppresses the activation of T cells (4). PD-1 is expressed by T cells, B cells, and myeloid cells. PD-1 ligands (PD-Ls) belong to the B7 family of molecules and include PD-1 ligand 1 (PD-L1) and PD-1 ligand 2 (PD-L2) (5, 6). Interactions between PD-1 and PD-Ls have been shown to suppress the functions of T cells (1, 7).

Immunocytes, such as T cells, B cells, regulatory T cells, natural killer T cells, dendritic cells (DCs), and tumor cells, express PD-L1 (8). PD-L2 is expressed by antigen-presenting cells, including DCs, macrophages, B cells, and tumor cells (5, 9). The abnormal expression of PD-L1 by cancer cells has recently been demonstrated in human malignancies (10-12). PD-L1 on tumor cells inhibits T-cell antitumor responses and facilitates cancer development (13). The findings of clinical trials on the systemic administration of therapeutic antibodies to block PD-1 or PD-L1 demonstrated the potential of this approach in the treatment of various tumors (14-16).

PD-L2, the other ligand of PD-1, was found to be moderately or strongly expressed in some tumor cells, suggesting a functional role in the tumor microenvironment (13). Furthermore, PD-L2 has been shown to play an inhibitory role by interacting with the PD-1 receptor (17, 18). However, the expression of PD-L2 in tumor tissue and its contribution to responses to PD-1-axis-targeted therapy have been examined in less detail than the expression and significance of PD-L1 (19). Similar to PD-L1, the interaction between PD-1 and PD-L2 inhibits T-cell proliferation, cytokine production, and T-cell cytolysis. A few studies have detected the expression of PD-L2 as well as the absence of PD-L1 in human tumors (19, 20); however, its relationship with clinical responses remains controversial (19).

Clinical responses to PD-1-targeting therapies vary among different tumor types, and extensive efforts have been made to identify predictive biomarkers that will be used to select patients who will benefit the most from these therapies (19).

Squamous cell carcinomas account for more than 95% of malignant tumors in the head and neck region (5). Oral squamous cell carcinoma (OSCC), including squamous cell carcinoma of the tongue, is the most common malignancy of the head and neck region, accounting for nearly 3% of all cancer cases worldwide (5). OSCC severely impairs the quality of life of patients because it adversely affects speech, swallowing, and mastication. The survival of patients with metastatic OSCC has been improved by the use of platinum-based salt drugs and, more recently, by immune checkpoint-targeting therapies. Clinical studies have reported durable tumor regression by PD-1/PD-L1 checkpoint blockade, which prompted the recent registration of anti-PD-1 antibodies for the treatment of head and neck cancer, including OSCC (5, 21). However, PD-1-targeting therapies only appear to be beneficial for some patients (22), and only a few clinical studies have been conducted on the PD-1/PD-L pathway in OSCC. Many aspects of the fundamental mechanisms involved in resistance to immune-checkpoint inhibitor therapies remain unclear (23).

The present study investigated the expression of PD-L1 and PD-L2 in OSCC, and assessed the relationships between the expression levels of these proteins and clinical histopathological parameters. We also used in vitro assays to examine the roles of PD-L1 and PD-L2 in tumor proliferation and invasion. Collectively, the present results demonstrate that PD-L1 and PD-L2 play important roles in cancer invasion and suggest that they suppress cancer immune surveillance.

Materials and Methods

Patients and data collection. The medical records of 98 patients with pathologically proven tongue cancer, who were diagnosed between April 2001 and December 2015, were retrospectively analyzed. All patients underwent surgical treatment for tongue cancer and did not previously receive any treatment. Information on white blood cell counts from routine laboratory examinations conducted before treatment onset, such as lymphocyte and monocyte counts, was retrospectively collected from medical records. Tumor stages were categorized using the TNM classification of the International Union Against Cancer (24). Data on age at diagnosis, sex, and the pretreatment tumor stage were also collected. The status of patients (alive/dead) 5 years after the date of diagnosis was obtained from medical records. In the survival analysis, overall survival (OS) was defined as the time from diagnosis until death, and the follow-up of patients who were still alive was censored at the date of their latest follow-up examination. The present study was approved by the Institutional Review Board of Nagasaki University (IRB No. 19090909). Informed consent was obtained from each subject in accordance with the Declaration of Helsinki.

Immunohistochemistry. Paraffin-embedded sections were produced from the biopsy specimens of 98 patients. The degree of histological differentiation of tumors was assessed according to the WHO classification (25), and the Yamamoto-Kohama (YK) mode of invasion was employed to evaluate the grade of invasion (26). Sections deparaffinized in xylene were soaked in 10 mmol/l citrate buffer (pH 6.0) and then autoclaved at 121˚C for 5 min for antigen retrieval. The Envision system (Dako, Glostrup, Denmark) was used to conduct immunohistochemical staining. Primary antibodies were used against PD-L1 (1:100), PD-L2 (1:100), PD-1 (1: 250) (Abcam, Cambridge, UK), Ki-67 (MIB-1, 1:100) (DAKO, Japan), matrix metalloproteinase 2 (MMP2) (1:100), and MMP9 (1:100) (Abcam, Cambridge, UK). The results obtained were evaluated by calculating the number of immunohistochemically positive cells around cancer cell nests at five randomly selected fields. Proportional scores were based on the estimated percentage of positively stained tumor cells (1, <10%; 2, 10–50%; 3, >50%). Intensity scores, which represented the estimated staining intensity, were as follows: 0, no staining; 1, weak; 2, moderate; 3, strong. These two items were then multiplied to identify positive cases. A total score of ≥3 indicated immunohistochemical overexpression. PD-L1(–) and PD-L2(–) were defined as a total immunostaining score of between 0 and 3, and PD-L1 (+) and PD-L2 (+) were defined as total immunostaining scores of ≥4. Patients were also grouped based on the proportion of PD-1-positive inflammatory cells around cancer cell nests at the invasive front: those with PD-1-positive rates of ≥10% were assigned to the PD-1-positive group and those with PD-1-positive rates of <10% to the PD-1-negative group. The cut-off point (10%) for PD-1 positivity was selected based on the mean PD-1 positivity rate.

Cell culturing and chemicals. The human OSCC cell lines HSC-3, OSC-19, and SCC-25 were obtained from the Human Science Research Resource Bank (Osaka, Japan). All cells were cultured in a 1:1 mixture of Ham's F-12/Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Trace Scientific, Melbourne, Australia). All cells were maintained under a humidified atmosphere of 5% CO_2 at 37˚C.

Flow cytometry. The standard method for flow cytometry (FCM) was employed in the present study. Data acquisition was performed using a FACSLyric cytometer (Becton-Dickinson, Franklin, NJ, USA) and assessed using FlowJo Pro software (Becton-Dickinson). The expression levels of PD-L1 and PD-L2 in OSCC cell lines were measured using the following monoclonal antibodies (mAbs): allophycocyanin-conjugated mouse anti-human CD274 and phycoerythrin-conjugated mouse anti-human CD273 antibodies (Becton-Dickinson). IgG isotype controls were used for FCM.

Cell proliferation assay. Proliferation was assessed using Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Cells were seeded in 96-well plates at a concentration of 1.5×10^3 per well and incubated for 24 h. Cells were exposed to various concentrations of anti-PD-L1 and anti-PD-L2 mAbs. At the end of the 48-h treatment, 10 μl of CCK-8 reagent were added to each well, and the plates were incubated at 37˚C for 2 h. The absorbance of cells was then analyzed at 450 nm using a plate reader according to the manufacturer's protocol. Culture solution containing CCK-8 reagent, but without antibodies, was used as a blank control.

Measurement of MMP2 and MMP9 levels in the culture medium. Cells were cultured and exposed to various concentrations of anti-PD-L1 and PD-L2 mAbs. After treatment for 0, 24, and 48 h, the culture medium was collected, and total MMP2 and MMP9 levels were measured in ELISA using commercially available kits (MMP2: ProteinTech Group,

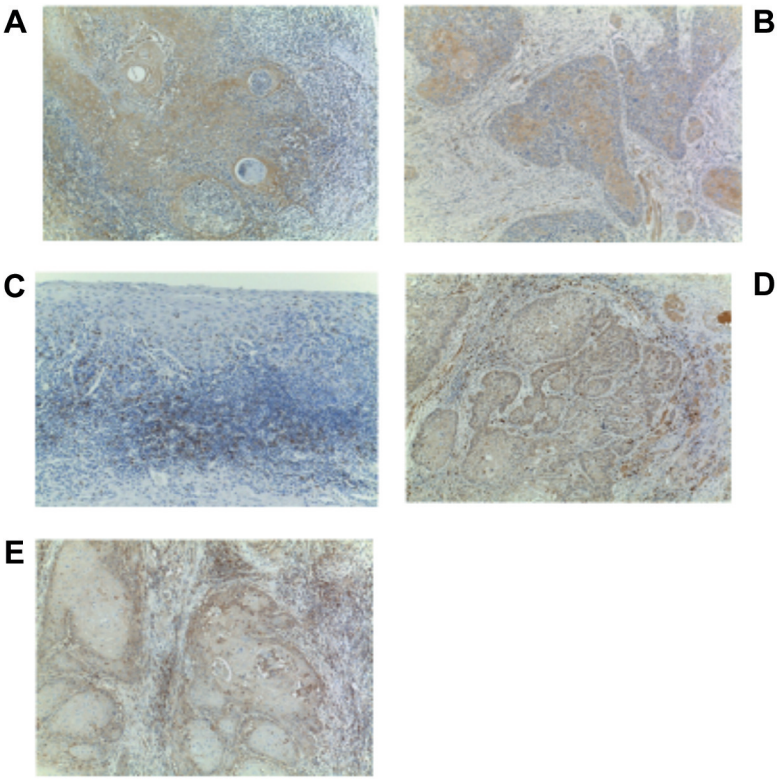

Figure 1. *Immunohistochemical staining of human oral squamous cell carcinoma (OSCC) tissues. (A) PD-L1-positive, (B) PD-L2-positive, (C) PD-1-positive, (D) MMP2-positive, (E) MMP9-positive. Original magnification: ×100.*

Rosemont, IL, USA; MMP9: R&D Systems, Minneapolis, MN, USA). Absorbance was read at 450 nm. Results were expressed as the absolute concentrations of total MMP2 and MMP9.

Statistical analyses. Survival curves were generated by the Kaplan-Meier method and compared using the Log-rank test. The relationships between the expression levels of PD-Ls and clinicopathological factors were assessed using Fisher's exact test. Cox's proportional hazards model was used in univariate and multivariate analyses to identify prognostic factors. All *p*-values <0.05 were considered to indicate statistically significant differences. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan) (27), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, it is a modified version of R commander designed to add statistical functions that are used in biostatistics.

Results

Patient characteristics. The median age of patients was 62 years (range=26-92 years), and the percentages of males and females were 54.1 and 45.9%, respectively. There were 51, 31, 5, and 11 patients with clinical stage I, II, III, and IV disease, respectively. The 5-year OS rate of the cohort was 83.5%.

PD-L1 and PD-L2 expression levels in OSCC and their relationship with clinicopathological parameters. PD-L1 and PD-L2 were detected in tumor cells (Figure 1). In total, 79.6 and 71.4% of OSCC tissue samples were positive for PD-L1 and PD-L2, respectively. However, immunoreactivity was not detected in the surrounding normal oral mucosal tissues. No significant differences were observed between the expression levels of PD-L1 and PD-L2. Although the relationships between PD-L1 or PD-L2 expression levels and various clinical parameters were investigated, no correlations were found with sex, age, the TNM classification, or the clinical stage (Table I). However, among the pathological parameters examined, a correlation was noted between PD-L1 expression and the Y-K classification (Table II).

Peripheral blood lymphocyte count and PD-1 expression. The mean peripheral blood lymphocyte count was 1.83×10^9 cells/l (range: $0.63\text{-}4.79\times10^9$), and the mean peripheral blood monocyte count was 0.35×10^9 cells/l (range=$0.04\text{-}0.88\times10^9$). The expression levels of PD-Ls did not correlate with the lymphocyte or monocyte count (Table I). PD-1 was expressed by lymphocytes around cancer cell nests (Figure 1). The PD-1 positivity rate was 34.2%. PD-1 expression was inversely

Table I. *Correlation between PD-L1 or PD-L2 and clinical factors.*

	PD-L1			PD-L2		
	Positive	Negative	*p*-Value	Positive	Negative	*p*-Value
Age			0.802			0.611
≤63	42	12		43	11	
>63	36	8		37	7	
Gender			0.803			0.605
Male	43	10		42	11	
Female	35	10		38	7	
cT classification			0.786			0.105
T1 T2	72	18		39	13	
T3 T4	6	2		5	2	
cN classification			0.294			0.677
N0	66	19		70	15	
N1 N2	12	1		2	1	
Stage			0.964			0.498
1,2	64	18		39	12	
3,4	14	2		4	1	
Clinical inspection			0.02			0.296
External, surface	32	14		35	11	
invasive	44	5		42	7	
Smoking status			1			1
No	56	14		57	13	
Yes	22	6		23	5	
Peripheral blood (cells/ml)						
Lymphocyte	1.78±0.52	2.04±0.80	0.0887	1.85±0.60	1.77±0.55	0.601
Monocyte	0.35±0.16	0.35±0.14	0.963	0.35±0.16	0.33±0.15	0.485
Neutrophil	3.49±1.38	3.68±1.62	0.592	3.41±1.32	4.02±1.77	0.106

associated with PD-L1 expression, and no correlation was observed between PD-L2 or PD-1 expression (Table II).

Relationship between Ki-67 and PD-L expression. The immunohistochemical expression of Ki-67 was detected in cancer cells. The mean Ki-67 labeling index (LI) was 11.4 (range=0-48.6). Ki-67 expression levels were higher in PD-L-positive cases than in PD-L-negative cases (Table II).

MMP2 and MMP9 expression in OSCC and their relationship with PD-L expression. Immunohistochemical staining showed that MMP2 and MMP9 were both present in the cytoplasm of tumor cells (Figure 1). We divided cases into the following three groups according to MMP-positive rates: group 1 (<25%), group 2 (≥25%, <50%), and group 3 (≥50%). PD-L1 and PD-L2 positive groups had significantly higher positive rates of MMP2 and MMP9 than PD-L1 and PD-L2 negative groups (Table II).

Relationship between PD-L expression levels and prognosis. OS did not significantly differ between patients with PD-L-positive tumors and those with PD-L-negative tumors. Disease-specific survival was worse in patients with PD-L1-positive tumors than in those with PD-L1-negative tumors. It was also slightly worse in patients with PD-L2-positive tumors than in those with PD-L2-negative tumors. Furthermore, disease-specific survival was significantly worse in patients with PD-L1- and PD-L2-positive tumors than in those with tumors that were negative for these molecules (Figure 2). Univariate and multivariate analyses were performed to investigate the relationships between disease-specific survival and clinicopathological factors (Table III). The univariate analysis revealed that sex, clinical T and N classifications, the clinical stage, pathological N classification, perineural invasion, late lymph node metastasis, and the expression of PD-L1 and/or PD-L2 were associated with disease-specific survival. Moreover, the multivariate analysis identified sex, the clinical stage, late lymph node metastasis, and the expression of PD-L1 and/or PD-L2 as independent predictors of poor disease-specific survival in patients with tongue cancer (Table III).

Expression of PD-L1 and PD-L2 in oral cancer cell lines. PD-L1 and PD-L2 were both expressed in HSC-3 cells. PD-L1 was expressed in OSC-19 cells, whereas PD-L2 was not. SCC-25 cells did not express PD-L1 or PD-L2 (Figure 3).

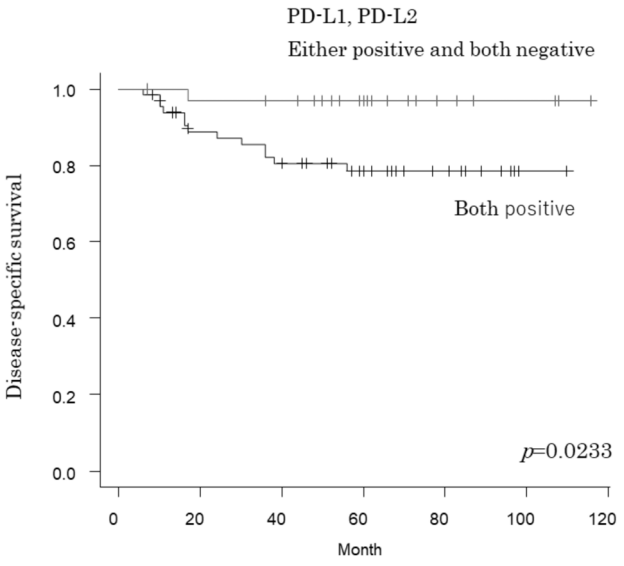

Figure 2. *Kaplan-Meier analysis of PD-L1 and PD-L2 expression in oral squamous cell carcinoma. Disease-specific survival was significantly worse in patients with PD-L1- and PD-L2-positive tumors than in those with PD-L1- and PD-L2-negative tumors.*

Proliferation of cancer cell lines. The sensitivity of PD-L mAbs in OSCC cell lines was assessed using the Cell Counting Kit-8. PD-L1 and PD-L2 mAbs dose-dependently inhibited the proliferation of HSC-3 cells but did not suppress the proliferation of SCC-25 at any dose (Figure 4).

Expression of MMP2 and MMP9 in OSCC cell lines. MMP2 and MMP9 were detected in the supernatants of all three cancer cell lines. MMP2 and MMP9 levels were measured in cell culture supernatants after PD-L blockade. In the HSC-3 and OSC-19 cell lines, the expression of MMP9 was suppressed by the blockade of PD-L1 and/or PD-L2, whereas that of MMP2 was not. In the SCC-25 cell line, neither MMP2 nor MMP9 expression was suppressed by blocking the expression of PD-L1 or PD-L2 (Figure 4).

Discussion

Recent studies suggested a novel mechanism involving the expression of PD-L1 and PD-L2 by which tumors evade host immune responses (1). The binding of PD-L1 and PD-L2 to PD-1 receptors on activated T and B cells is considered to negatively regulate cellular and humoral immune responses (1).

However, the expression of PD-L1, PD-L2, and PD-1 in OSCC has not yet been simultaneously examined. The present results revealed the abnormal overexpression of PD-L1 and PD-L2 in OSCC cells, and PD-1 was also expressed by lymphocytes around cancer cell nests. PD-1 has two ligands, PD-L1 and PD-L2, and the ligand-receptor interactions induce

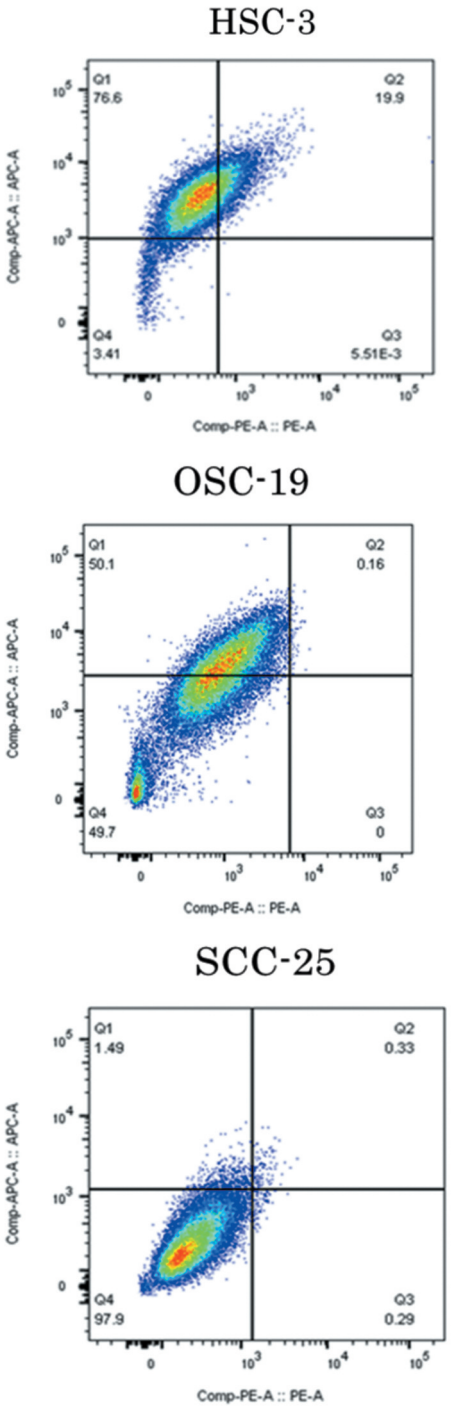

Figure 3. *Expression of PD-L1 and PD-L2 in human oral squamous cell carcinoma (OSCC) cell lines (HSC-3, OSC-19, and SCC-25). HSC-3 cells expressed PD-L1 and PD-L2. OSC-19 cells expressed PD-L1, but not PD-L2. SCC-25 cells did not express PD-L1 or PD-L2.*

immune tolerance through the inhibition of activated immune cells, which allows tumor cells to evade T cells (28). Although the mechanisms underlying PD-L1 and PD-L2 expression

Table II. *Correlation between PD-L1 or PD-L2 and pathological or immunohistochemical factors.*

	PDL1			PDL2		
	Positive	Negative	*p*-Value	Positive	Negative	*p*-Value
pN			0.888			0.719
pN0	58	17		59	16	
pN1, pN2	20	3		21	2	
Histological grade			0.184			1
Well	76	18		76	18	
Moderate, poor	2	2		4	0	
Perineural invasion			0.261			0.733
No	55	17		58	14	
Yes	23	3		22	4	
Local relapse			1			1
No	68	18		70	16	
Yes	10	2		10	2	
Late lymph node metastasis			0.776			0.277
No	59	16		59	16	
Yes	19	4		21	2	
Y-K classification			<0.05			0.183
1,2	18	14		23	9	
3,4	60	6		57	9	
MMP-2			<0.05			<0.05
Score 1	2	8		7	3	
Score 2	19	10		20	9	
Score 3	57	2		53	6	
MMP-9			<0.05			<0.05
Score 1	0	11		7	4	
Score 2	28	9		28	9	
Score 3	50	0		45	5	
PD-1			1			0.589
Positive	27	7		29	5	
Negative	50	13		50	13	
Ki-67 LI	12.26±9.35	8.10±12.57	0.102	11.34±10.00	11.76±11.10	0.876

overlap, some differences have been reported (5). Inflammatory cytokines have been shown to up-regulate the expression of PD-L1 and PD-L2; however, the mechanisms slightly differ (28). PD-1 exhibits greater affinity for PD-L2 than for PD-L1 due to its lower dissociation rate for PD-L2 binding (28). Furthermore, differences in PD-L1 and PD-L2 expression levels may be attributed to the different regulatory cytokine profiles involved in their expression (28). The up-regulation of PD-L1 depends on interferon regulatory factor 1 (IRF1) and signal transducer and activator of transcription 1 (STAT1) binding to the PD-L1 promoter, while that of PD-L2 appears to depend on IRF1 and STAT1/3 binding to the PD-L2 promoter (2). A previous study has reported that PD-L1 and PD-L2 play different prognostic roles in various tumors, suggesting the existence of different tumor subsets, including among cancer stem cells (2, 28).

Although PD-L1 and PD-L2 were both strongly expressed in OSCC in the present study, a correlation was not observed between their expression levels. This result indicates the independent expression of PD-L1 and PD-L2.

The expression of PD-L1 has been detected in most human cancers (11, 15). Furthermore, correlations have been reported between the expression of PD-L1 in desmoplastic melanoma and rapid cancer progression, greater invasion depth and perineural invasion (12). In head and neck cancer, previous studies reported PD-L1-positive rates of between 46.4 and 100% (29). The variability in PD-L1-positive rates among these studies may be due to differences in the antibodies and tissue samples used. However, Lenouvel *et al*. (21) reviewed the expression of PD-L1 in OSCC series and found that previous studies all identified its overexpression in OSCC as a negative prognostic marker, similar to other types of cancer. In the present study, the overexpression of PD-L1 correlated with the prognosis of patients, which is consistent with previous findings.

Limited information is currently available on the role of PD-L2 in malignant tumors, and the findings obtained to date remain controversial (30). In previous studies on ovarian cancer and hepatocellular carcinoma (31, 32), the

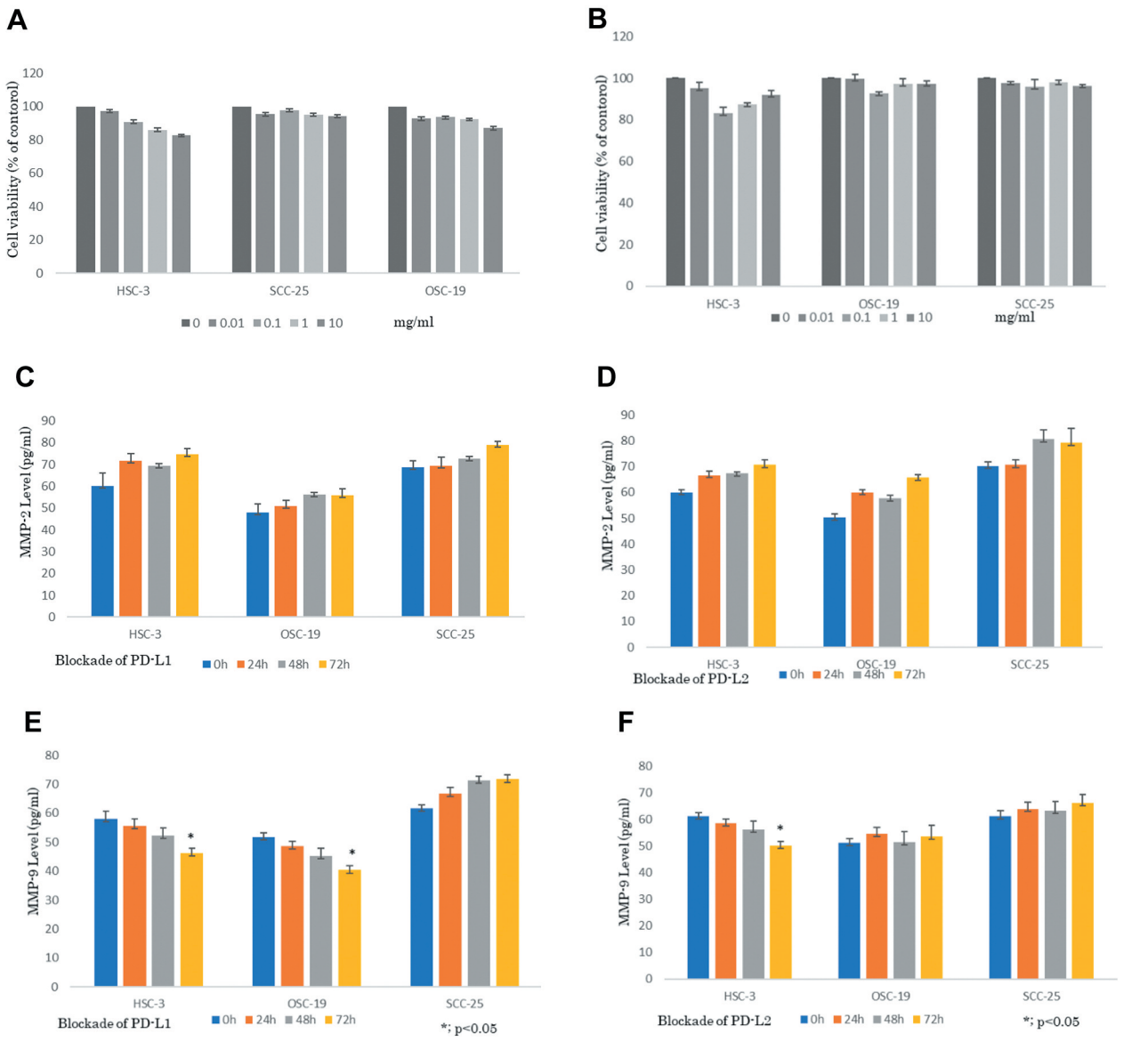

Figure 4. *Proliferation of oral squamous cell carcinoma (OSCC) cells and expression of MMP2 and MMP9 on OSCC cells. The proliferation of OSCC cell lines was not significantly affected by the blockade of PD-L1 or PD-L2, whereas that of HSC-3 cells was slightly decreased by the blockade of PD-L1 (A) or PD-L2 (B). All OSCC cell lines expressed MMP2 and MMP9. MMP2 expression was not affected by the blockade of PD-L1 (C) and/or PD-L2 (D). MMP9 expression on HSC-3 cells was decreased by the blockade of PD-L1 (E) and/or PD-L2 (F), and its expression on OSC-19 cells was decreased by the blockade of PD-L1.*

expression of PD-L2 was associated with impaired survival. However, in another study, a correlation was observed between the expression of PD-L2 and the prognosis of esophageal squamous cell carcinoma (1).

Discrepancies have also been reported in the prognostic effects of the expression of PD-L1 and PD-L2. Takada *et al*. (33) reported a shorter postoperative OS in PD-L1-positive patients than in PD-L1-negative patients. In contrast, OS was significantly worse in PD-L2-negative patients, and PD-L1 positivity and PD-L2 negativity were identified as independent predictors of worse OS (28). On the other hand, the expression of PD-L2 in renal cell carcinoma was identified as an independent poor prognostic indicator of cancer-specific survival and progression-free survival. Furthermore, prognosis was worse in patients with positivity for both PD-Ls than in those who were positive for PD-L1 or PD-L2 (30). In the present study, the prognosis of patients with PD-L1- and PD-L2-positive tumors was poor.

Table III. *Univariate and multivariate analysis of clinicopathological factors associated disease-specific survival.*

	Univariate analysis		Multivariate analysis	
	HR	*p*-Value	HR	*p*-Value
Age				
<63/≥63	1.26 (0.47-3.22)	0.66	1.74 (0.45-6.81)	0.43
Gender				
Male/Female	3.17 (1.03-9.74)	0.04	3.63 (1.12-11.72)	0.03
cT classification				
T1, T2/ T3, T4	0.23 (0.07-0.71)	0.01	0.56 (.06-4.87)	0.6
cN classification				
N0/N1, N2	3.26 (1.14-9.27)	0.03	0.42 (0.04-4.35)	0.47
Stage				
1,2/3,4	0.22 (0.08-0.59)	0.002	0.12 (0.04-0.40)	0.0005
Clinical inspection				
External, surface / invasive	17.53 (2.32-233.3)	0.006	6.35 (0.57-70.2)	0.13
Smoking status				
No/Yes	1.01 (0.34-2.87)	0.98	0.79 (0.23-2.68)	0.7
pN				
pN0/pN1, pN2	4.91 (1.89-12.8)	0.001	1.92 (0.41-8.95)	0.4
Perineural invasion				
No/Yes	2.78 (1.07-7.22)	0.04	0.82 (0.17-3.91)	0.81
Local relapse				
No/Yes	1.08 (0.25-4.71)	0.92	1.09 (0.10-12.0)	0.94
Late lymph node metastasis				
No/Yes	3.77 (1.45-9.79)	0.007	6.51 (2.12-19.97)	0.001
Y-K classification				
1, 2/3, 4	8.77 (1.16-66.22)	0.04	0.60 (0.05-7.60)	0.69
PD-1				
Positive/Negative	0.70 (0.25-2.00)	0.51	1.81 (0.48-6.80)	0.38
PD-L1 and/or PD-L2				
Both positive/both negative	0.13 (0.02-0.56)	0.04	0.10 (0.01-0.78)	0.03

Therefore, we suggest that PD-L2 is associated with a poor prognosis in OSCC, as has been demonstrated for PD-L1.

Differences have been reported in the molecular mechanisms underlying the PD-L1/PD-1 and PD-L2/PD-1 interactions (30). Furthermore, unknown receptors of PD-L2 have been suggested to exist. Cytokine production has been previously shown to be inhibited by PD-L2, whereas the double blockade of PD-Ls synergistically increased it over that with the single blockage of a PD-L in PD-L-positive cases (30). Therefore, the expression of PD-L2 in OSCC may play an important role in immune evasion by tumors and may be an additional molecular target for the treatment of OSCC.

OSCC is characterized by a high degree of local invasion and a high rate of metastasis to the cervical lymph nodes. Cancer-related death is caused by local recurrence or regional and/or systemic metastasis (34). MMPs belong to a group of extracellular matrix (ECM)-degrading enzymes. The characterization of more than 20 members of the MMP family has so far been achieved (35). Among them, MMP2 and MMP9 play important roles in ECM degradation because of their ability to destroy collagen in the ECM. Many studies have demonstrated the significance of both MMP2 and MMP9 in the aggressive growth of OSCC. In particular, increased MMP expression levels correlated with tumor cell proliferation and invasion (35). Although a previous study suggested that MMP enzymes play a role in the regulation of surface PD-L1 expression, the immunomodulatory effects of MMP9 remain unclear (23). The ability of MMP9 to negatively regulate anti-PD-1 responses by reducing the tumor surface expression of PD-L1 is consistent with the immunological impact of other mechanisms that have been found to regulate the surface expression of this ligand (23). The inhibition of the TGF-β pathway was found to promote the proliferation of stromal fibroblasts, thereby facilitating the MMP9-dependent cleavage of PD-L1 surface expression, leading to resistance to PD-1-targeting therapies. Zhao *et al*. (23) have suggested that MMP9 expressed by stromal fibroblasts desensitized tumors to PD-1-targeting therapies and reported that the

inhibition of TGF-β may be more immunologically effective when administered after the development of resistance to these therapies. On the other hand, Hira-Miyazawa *et al*. (22) have proposed MMP13-induced PD-L1 shedding/cleavage as a mechanism responsible for the protection afforded by MMP13 against invasion and metastasis, indicating its potential as a marker of the efficacy of PD-1-targeting therapy. Hira-Miyazawa *et al*. (22) have also hypothesized that the MMP9-induced shedding/cleavage of PD-L1 is not the primary mechanism underlying protection against invasion and metastasis by MMP9. Therefore, further studies are needed to elucidate the other mechanisms responsible for the protective effects of MMP9.

The status of PD-L1 and PD-L2 in esophageal cancer are significant independent prognostic factors. Ohigashi *et al*. (14) have suggested that the expression of PD-L1 and PD-L2 in tumors plays critical roles in cancer metastasis and progression in human esophageal cancer. Nomi *et al*. (36) have reported that anti-PD-1 and anti-PD-L1 mAbs exerted significant inhibitory effects on tumor growth, suggesting that the PD-L1/PD-1 pathway is critical for the growth of pancreatic cancer. The results of the present study showed a correlation between PD-L and MMP levels. Collectively, these findings and the present results suggest that PD-L1 and PD-L2 play critical roles in the progression and invasion in OSCC; however, further studies on the roles of cytokines, such as the TGF-β pathway, are needed.

Tumor-infiltrating lymphocytes (TILs) are a manifestation of antitumor immunity in a host (1). PD-1 is mainly expressed on TILs. Leng *et al*. (1) reported a negative correlation between the PD-1-positive TIL count and the expression of both PD-L1 and PD-L2 in esophageal squamous cell carcinoma. They also indicated that PD-L1 and PD-L2 expression suppressed PD-1-positive TIL activity or enhanced PD-1-positive TIL apoptosis in esophageal squamous cell carcinoma, and, thus, promoted immune evasion *via* the PD-1/PD-L pathway. On the other hand, a correlation was found between the overexpression of both PD-L1 and PD-1 in OSCC (29). Although PD-1 was expressed in TILs around cancer cell nests in the present study, a correlation was not observed between PD-1 expression on TILs and PD-L1 or PD-L2 expression on cancer cells. As discussed above, the expression of PD-1 is mainly detected on TILs; therefore, any discrepancies may be derived from the functions of TILs.

We have previously reported that preoperative CD8-positive TIL and peripheral blood lymphocyte counts may be used as independent prognostic biomarkers in OSCC (37). CD8-positive T cells are considered to play a central role in antitumor responses, and the presence of CD8-positive T cells has been identified as a prognostic factor (14). The number of tumor-infiltrating CD8-positive T cells has been shown to positively correlate with the expression of PD-L1 on tumor cells (38). In addition, Nomi *et al*. (36) have reported that PD-L1 expression inversely correlated with the TIL count in pancreatic cancer, particularly the CD8-positive T cell count. Ohigashi *et al*. (14) did not detect a correlation between the PD-L1 status and the TIL count, suggesting that the expression of PD-L1 contributes to tumor growth and metastasis independently of reductions in the TIL count. However, an inverse correlation was observed between the PD-L2 status and CD8-positive TIL count in the same case series, suggesting that PD-L2 is a better target than PD-L1 for immunotherapy for esophageal cancer.

PD-Ls have been suggested to dampen T-cell responsiveness and promote immune tolerance (38). As described above, contradictory findings have been obtained on the relationships between the PD-1-positive TIL count and PD-L expression on cancer cells. Activated effector cytotoxic T cells may produce interferon (INF)-γ, whereas exhausted CD8-positive T cells do not (38). Furthermore, a stimulation with INF-γ enhanced PD-L1 and PD-L2 expression (39). Therefore, we suggest that some unknown factors, such as INF-γ, regulate the expression of PD-Ls and PD-1, and the balance between the expression of these molecules in OSCC may modulate the effects of PD-1-targeting therapy. Further studies are warranted to elucidate the underlying mechanisms in more detail.

Conclusion

PD-Ls may play critical and independent roles in the invasion and progression of OSCC. Further studies are important for predicting the outcomes of patients and planning clinical treatments. Clinical studies are also needed to allow for the effective clinical use of PD-L1 and PD-L2-targeting therapies.

Conflicts of Interest

The Authors declare that they have no conflicts of interest related to this study.

Authors' Contributions

GK designed the experiments. KF, GK and TY performed the experiments and acquired the data. GK performed the literature review and took the lead in writing the manuscript. GK, KF and MU revised the manuscript. KF, GK, TY and MU interpreted the data and contributed advice for the manuscript. All Authors have read and agreed to the final manuscript.

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