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Prognostic value of programed death ligand-1 and ligand-2 co-expression in salivary gland carcinomas

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ABSTRACT

Objectives: The aim of the present study was to investigate the molecular basis for the use of immune checkpoint inhibitors to treat salivary gland carcinomas (SGC).

Materials and methods: We examined the clinical and prognostic significance of programed death ligands 1 and 2 (PD-L1 and -L2) expression using immunohistochemistry and *in situ* hybridization, as well as microsatellite instability (MSI) status using polymerase chain reaction, along with tumor-infiltrating lymphocytes (TILs) in 30 cases of SGC.

Results: The SGC cases studied included adenoid cystic carcinoma (AdCC, 36.7%), salivary duct carcinoma (SDC, 26.7%), mucoepidermoid carcinoma (MEC, 23.3%), and carcinoma ex pleomorphic adenoma (CxPA, 13.3%). Either PD-L1 or PD-L2 overexpression was observed in 36.7% patients. PD-L2 expression was associated with reduced disease-specific survival (DSS) and disease-free survival (DFS) ($P = 0.0266$ and $P = 0.0209$, respectively). Simultaneous PD-L1 and PD-L2 overexpression was detected in 13.3% of cases, and was correlated with reduced DSS ($P = 0.0113$). Among non-AdCCs, all cases that developed distant metastasis were positive for PD-L2 ($P = 0.001$). Cases showing low-TILs that were positive for either PD-L1 or L2 were associated with poor DFS. No MSI was detected in the SGC cases studied.

Conclusion: To our knowledge, this is the first comprehensive study examining PD-L1 and PD-L2 status, MSI status, and TILs in SGC. Our results indicate that the PD-1/PD-L1 or PD-L2 pathway, which is associated with poor clinical outcomes, may provide promising therapeutic targets against SGC in selected patients. Further experimental and clinical studies are encouraged.

Introduction

Salivary gland carcinomas (SGC) are relatively rare malignancies, accounting for only 3%–6% of head and neck carcinomas [1]. Surgery is the main primary therapy for this malignancy, and patient prognosis is predicted and graded (low, intermediate, and high) based on the postoperative pathological findings [2]. For patients with positive surgical margins, multiple lymph nodes metastases, and/or high-grade tumors, adjuvant radiotherapy is recommended [3–6]; however, the clinical benefits of these therapies remain controversial [7–12]. In addition, there is no clinically effective treatment for locally advanced inoperable tumors, invasive local recurrences, and/or distant metastases [1,12,13]. Therefore, it is an urgent task to establish a novel treatment modality reflecting the biology of SGC.

In view of the promising results obtained in recent clinical trials examining immune checkpoint control inhibitors (ICI), the programed cell death-1 (PD-1) antibody has been approved worldwide for the treatment of head and neck cancers. It is noteworthy that, in Japan, the application of nivolumab (PD-1 antibody) is not restricted to squamous cell carcinoma, and SCG is a potential target of this drug. However, owing to the paucity in studies examining the SGC tumor immune microenvironment, the scientific basis for using ICI to treat SGC remains unclear. To address this issue, we evaluated the expression levels of programed cell death ligand-1 and -2 (PD-L1 and PD-L2), microsatellite instability (MSI), and tumor-infiltrating lymphocytes (TIL) in SGC. The pathway of PD-1 and its ligands, PD-L1 and PD-L2, is associated with the negative feedback of cell-mediated immune responses. Several studies have reported that PD-1, PD-L1, or PD-L2 overexpression is correlated with poor prognosis in various cancers [14–17], as well as with the effect of ICI [18–21]. However, little is known about

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the frequency and prognostic significance of PD-L1 and PD-L2 expression in SGC [22–26]. Microsatellites are short DNA sequences consisting of tandem repeats of two to four nucleotides, which are scatted throughout the human genome. Microsatellite instability (MSI) is caused by a deficiency in the mismatch repair (MMR) gene. MSI and its prognostic significance have been reported in several cancers, including head and neck squamous cell carcinoma [27,28]. In addition, it was reported that mismatch repair deficiency is significantly correlated with the effectiveness of ICI [29]. However, the precise frequency of MSI and its prognostic value for SGC is currently unknown [30–33]. In the present study, we investigated the expression levels of PD-L1 and PD-L2, the MSI status, and the degree of TILs to analyze their prognostic significance in 30 cases of SGC.

Materials and Methods

Case selection

We retrospectively reviewed the medical records of patients who were diagnosed with malignant SGC, dating from 1987 to 2016 at the National Kyushu Cancer Center. Then, we selected 30 representative cases whose surgical specimen contained sufficient tumor tissue and normal salivary gland tissues for further analyses. The tumors were staged according to the American Joint Committee on Cancer (AJCC 7th edition) [34], and were pathologically graded into three categories: low-, intermediate-, and high-risk. An observer-blind assessment of the slides was conducted by two pathologists (K.T. and T.N.) with no knowledge of the clinical outcomes. The institutional review board of National Kyushu Cancer Center approved this study (2015–5).

Immunohistochemistry for PD-L1: Procedure and evaluation

Immunohistochemical staining (IHC) was performed using 4-μm-thick formalin-fixed, paraffin-embedded tissue sections and the primary antibody for VENTANA PD-L1 (SP263; Roche) using a Ventana Benchmark Ultra slide processor, according to the manufacturer's instructions. PD-L1 protein expression was judged as positive when the tumor cells showed complete membranous staining in > 1% of the tumor cells [23].

In situ hybridization for PD-L2: Procedure and evaluation

For the detection of PD-L2 expression, we employed a PD-L2 *in situ* hybridization (ISH) system, the RNAscope platform (RNAscope 2.0 High Definition Kit, Advanced Cell Diagnostics). This is because the reliable antibody for PD-L2 IHC (clone 3G2), which showed good concordance with the RNAscope ISH, was not commercially available at the time of the experiment [35]. The hybridization was conducted using anti-sense and sense probes for human PD-L2 (test probe: 316291 and negative control: 55189, respectively), and an anti-sense probe for PPIB (positive control: 313901) according to the manufacturer's instructions. PD-L2 ISH was scored as follows: 0: no staining or < 1 dot/10 cells; 1: 1–3 dots/cells; 2 : 4–9 dots/cell and none or very few dot clusters; 3: 10–15 dots/cell and < 10% dots are in clusters; 4: > 15 dots/cell and > 10% dots are in clusters. We defined PD-L2 ISH-positive as a score greater than 2 in > 1% of the tumor cells.

Microsatellite instability analysis

Genomic DNA was extracted from formalin-fixed paraffin-embedded tissues using the QIAamp DNA FFPE Tissue Kit (Qiagen) according to the manufacturer's instructions. The microsatellite markers used in the present study were BAT25, BAT26, NR-21, NR24, and NR-27. Fluorescence-based polymerase chain reaction (PCR) (Promega, MSI Analysis System, Version 1.2) was used to conduct MSI analysis. The PCR products were analyzed via capillary electrophoresis using a 3130xl Genetic Analyzer (Thermo Fisher Scientific, San Jose, CA, United States). The tumors were classified as microsatellite instability-high (MSI-H) if more than two markers showed MSI, as microsatellite instability-low (MSI-L) if only one maker showed MSI, and as microsatellite-stable if none of the markers showed MSI.

Table 1
Clinicopathological findings of salivary gland carcinoma (N = 30).

Variables		N	(%)
Age (years), median (range)		64	(13–81)
Sex	Male	17	(56.7)
	Female	13	(43.3)
Location	Parotid	22	(73.3)
	Submandibular	3	(10.0)
	Sublingual	5	(16.7)
T stage	Low (T1/T2)	7	(23.3)
	High (T3/T4)	23	(76.7)
N stage	N0	19	(63.3)
	N1-3	11	(36.7)
Stage	Low (I/II)	5	(16.7)
	High (III/IV)	25	(83.3)
Histopathological subtype	Adenoid cystic carcinoma	11	(36.7)
	Salivary duct carcinoma	8	(26.7)
	Mucoepidermoid carcinoma	7	(23.3)
	Carcinoma ex pleomorphic adenoma	4	(13.3)
Histological grade	Low	2	(6.7)
	Intermediate	11	(36.7)
	High	17	(56.7)
Surgical margin	Positive	8	(30.8)
	Negative	18	(69.2)
	not given	4	
Tumor-infiltrating lymphocyte	High (> 50%)	10	(33.3)
	Low (< 49%)	20	(66.6)
Recurrence	Local	5	(16.7)
	Regional lymph node	3	(10.0)
	Distant	12	(40.0)
Last contact	No evidence of disease	15	(50.0)
	Alive with tumor	4	(13.3)
	Died of tumor	11	(36.7)

Histopathological assessment

We evaluated the percentage of TILs on hematoxylin and eosin-stained specimens using previously reported criteria [36–38]. An observer-blinded histopathological evaluation of TILs was independently performed by two pathologists (K.T. and T.N.) with no knowledge of the clinical outcomes, and the mean value was used for the analyses. In this study, high-TILs was defined as > 50% infiltration of stromal lymphocytic infiltration [37].

Statistical analysis

For the comparison of specific groups chi-square test was employed. The Kaplan–Meier method and the log-rank test were used to calculate the disease-specific survival (DSS) and disease-free survival (DFS). The Cox proportional hazard models were used to analyze the effects of individual factors on survival. The results were considered statistically significant if the *P*-value was less than 0.05. All statistical analyses were conducted on the JMP Statistical Discovery Software (Version 14.0; SAS Inc., Cary, NC, USA).

Table 2
Association between clinicopathological findings and PL-L1 and/or PD-L2 status.

			PD-L1		*P* value	PD-L2		*P* value	PD-L1 and L2		*P* value	Tumor-infiltrating lymphocyte		*P* value
			Positive	Negative		Positive	Negative		Positive	Negative		High (> 50%)	Low (< 49%)	
Variables		N = 30	11	19		11	19		4	26		10	20	
Age (years)	≧64	16	7	9	N.S.	6	10	N.S.	2	13	N.S.	5	11	N.S.
	< 63	14	4	10		5	9		1	13		5	9	
Sex	Male	17	7	10	N.S.	7	10	N.S.	4	13	0.0252	5	12	N.S.
	Female	13	4	9		4	9		0	13		5	8	
T stage	Low (T1/T2)	7	1	6	N.S.	2	5	N.S.	0	7	N.S.	2	5	N.S.
	High (T3/T4)	23	10	13		9	14		4	19		8	15	
N stage	N0	19	3	16	0.0045	5	14	N.S.	0	19	0.0025	4	15	N.S.
	N1-3	11	8	3		6	5		4	7		6	5	
Pathological stage	Low (I/II)	5	0	5	N.S.	1	4	N.S.	0	5	N.S.	1	4	N.S.
	High (III/IV)	25	11	14		10	15		4	21		9	16	
Histopathological subtype	AdCC	11	0	11	0.0015	5	6	N.S.	0	11	0.0356	0	11	0.0032
	non-AdCC	19	11	8		6	11		4	13		10	9	
	SDC	8	4	4		4	4		3	5		5	3	
	MEC	7	4	3		1	6		0	7		4	3	
	CxPA	4	3	1		1	3		1	3		1	3	
Histological grade	Low/ Intermediate	13	1	12	0.0067	5	8	N.S.	0	13	0.0252	3	10	N.S.
	High	17	10	7		6	11		4	13		7	10	
Surgical margin	Positive	8	3	5	N.S.	3	5	N.S.	1	7	N.S.	4	4	N.S.
	Negative	18	8	10		6	12		3	15		6	12	
	not given	4	0	4		2	2		0	4		0	4	
Tumor-infiltrating lymphocyte	High (> 50%)	10	6	4	N.S.	4	6	N.S.	2	8	N.S.			
	Low (< 49%)	20	5	15		7	13		2	18				
Recurrence (Lacal/ regional LN)	Positive	6	4	2	N.S.	2	4	N.S.	1	5	N.S.	1	5	N.S.
	Negative	24	7	17		9	15		3	21		9	15	
Recurrence (Distant)	Positive	12	4	8	N.S.	8	4	0.0049	3	9	N.S.	3	9	N.S.
	Negative	18	7	11		3	15		1	17		7	11	

Abbreviation: PD-L1, programmed cell death-ligand 1; PD-L2, programmed cell death-ligand 2; AdCC, adenoid cystic carcinoma; SDC, salivary duct carcinoma; MEC, mucoepidermoid carcinoma; CxPA, carcinoma ex pleomorphic adenoma; LN, lymph node; N.S., not significant.

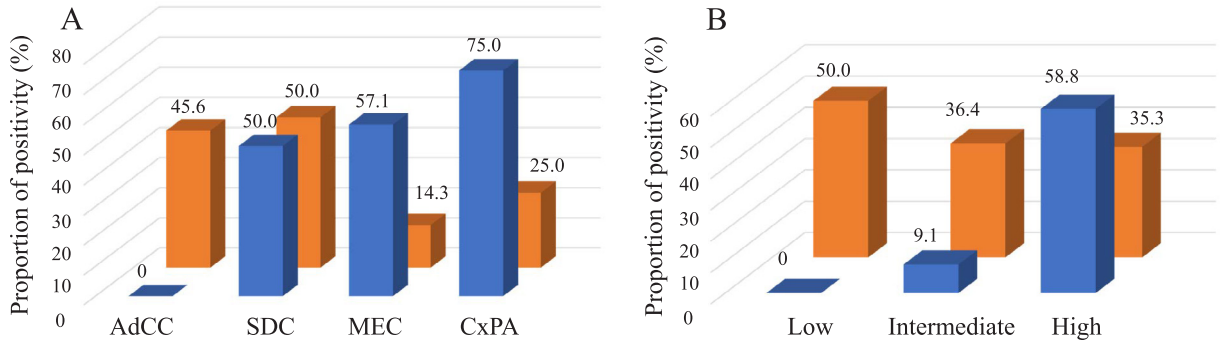

Fig. 1. Proportion of PD-L1 positivity (blue bars) and PD-L2 positivity (orange bars) according to histopathological subtypes (A) and histological grades (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Results

Clinicopathological findings

Table 1 shows the clinicopathological findings from the SGC. The cases consisted of 17 males (56.7%) and 13 females (43.3%), with ages ranging from 13 to 81 years (median age of 64 years). Primary SGC were located in 22 parotid glands (73.3%), 3 submandibular glands (10.0%), and 5 sublingual glands (16.7%). Of the 30 cases studied, 7 cases (23.3%) were low T-stage (T1 or T2), while the other 23 cases (76.7%) were high T-stage (T3 or T4). Eleven cases (36.7%) were lymph node metastasis-positive; 5 cases (16.7%) were pathological stage I/II tumors and the other 25 cases (83.3%) were pathological stage III/IV. Histopathologial examination revealed 11 adenoid cystic carcinomas (AdCC, 36.7%), 8 salivary gland carcinomas (SDC, 26.7%), 7 mucoepidermoid carcinomas (MEC, 23.3%), and 4 carcinoma ex

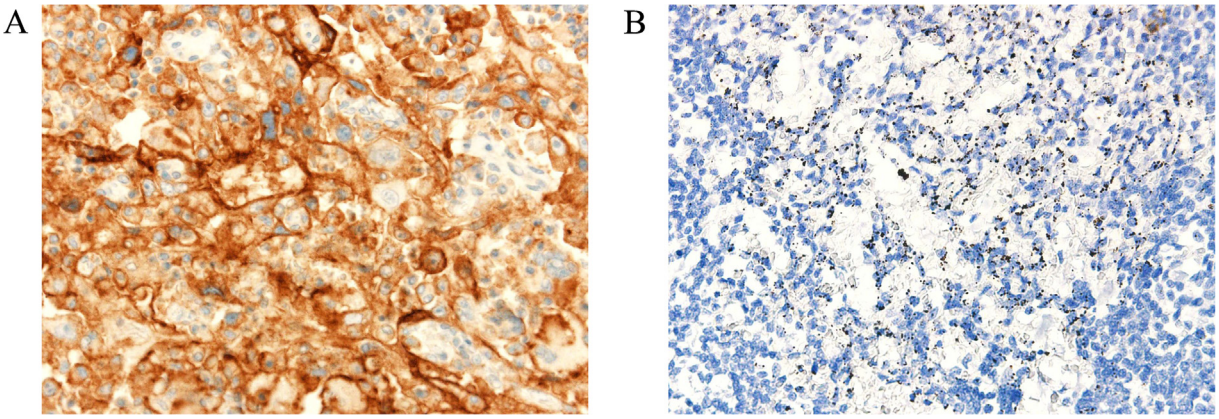

Fig. 2. PD-L1 immunohistochemical staining and PD-L2 *in situ* hybridization findings. Salivary duct carcinoma cells showing membranous staining of PD-L1 (original magnification × 400) (A). Adenoid cystic carcinoma cells showing cytoplasmic dots (original magnification × 400) (B).

Table 3
Association between PD-L2 status and distant metastasis among AdCC or non-AdCC.

		AdCC			non-AdCC		
		PD-L2		*P* value	PD-L2		*P* value
		Positive	Negative		Positive	Negative	
		5	8		6	11	
Recurrence (Distant)	Positive	4	4	N.S.	4	0	0.001
	Negative	1	4		2	11	

Abbreviation: PD-L2, programmed cell death-ligand 2; AdCC, adenoid cystic carcinoma; non-AdCC, salivary duct carcinoma, mucoepidermoid carcinoma, or carcinoma ex pleomorphic adenoma; N.S., not significant.

pleomorphic adenomas (CxPA, 13.3%). Two cases (6.7%) were low-grade tumors, 11 (36.7%) were intermediate-grade tumors, and 17 (56.7%) were high-grade tumors. Regarding surgical margins, we were able to obtain pathological diagnoses for 26 cases; 8 cases (30.8%) were positive and 18 cases (69.2%) were negative. High-TILs were detected in 10 cases (33.3%). Local recurrence and regional lymph node recurrence was detected in 5 cases (16.7%) and 3 cases (10.0%), respectively. Twelve cases (40.0%) developed distant metastasis. Of these recurrent cases, 1 case showed both local recurrence and regional lymph node recurrence, 1 case showed both local recurrence and distant metastasis, 1 case showed both lymph node recurrence and distant metastasis, and 1 case showed local recurrence, regional lymph node recurrence, and distant metastasis. At the last contact, 15 cases (50.0%) showed no evidence of disease, 4 cases (13.3%) remained alive with their disease, and 11 cases (36.7%) died with their disease.

Immunohistochemistry for PD-L1

Table 2 and Fig. 1 show the association between the results of PD-L1 and the clinicopathological findings. Immunohistochemically, PD-L1 protein overexpression was detected in 11 cases (36.7%), including 4 cases (50.0%) of SDC, 4 (57.1%) of MEC, and 3 (75.0%) of CxPA [Table 2 and Fig. 1A (blue bars)]. In PD-L1 positive cases, PD-L1 was expressed at the cell membrane (Fig. 2A). No case of AdCC showed PD-L1 protein overexpression. Regarding histological grading, 1 case (9.1%) having an intermediate-grade tumor and 10 (58.8%) having high-grade tumors showed PD-L1 protein overexpression. None of the low-grade tumors showed PD-L1 protein overexpression [Table 2 and Fig. 1B (blue bar)]. As shown in Table 2 and Fig. 1B (blue bars), PD-L1 positivity increased proportionally with histological grade, and the difference between the low- to intermediate-grade and high-grade was statistically significant ($P = 0.0067$). In addition, PD-L1 positivity was significantly associated with N-stage ($P = 0.0045$) and histopathological subtype (non-AdCC, $P = 0.0015$), whereas there were no statistical differences regarding age, sex, T-stage, pathological stage, surgical margin, TILs, local/reginal LN recurrence, or distant metastasis.

In situ hybridization for PD-L2

Table 2 and Fig. 1 show the association between the results of PD-L2 ISH and the clinicopathological findings. Eleven cases (36.7%) were PD-L2 ISH-positive, including 5 cases (45.6%) of AdCC, 4 cases (50.0%) of SDC, 1 (14.3%) of MEC, and 1 (25.0%) of CxPA [Table 2 and Fig. 1A (orange bars)]. In PD-L2 ISH-positive cases, cytoplasmic signals were detected (Fig. 2B). PD-L2 ISH-positive status was significantly associated with development of distant metastasis ($P = 0.0049$). Regarding histological grade, 1 case (50%) having a low-grade tumor, 4 (36.4%) having intermediate-grade tumors, and 6 (35.3%) having high-grade tumors were PD-L2 ISH-positive, and there was no specific tendency [Table 2 and Fig. 1B (orange bars)]. Additionally, there were no statistical differences regarding age, sex, T-stage, N-stage, pathological stage, histopathological subtypes, surgical margin, TILs, and local/regional LN recurrence.

PD-L2 ISH-positive status was significantly correlated with the development of distant metastasis (Table 3, $P = 0.001$) in the cases with non-AdCC, but not with AdCC.

PD-L1 IHC and PD-L2 ISH analysis

Four cases (13.3%) were both PD-L1 IHC-positive and PD-L2 ISH-positive, including 3 cases (37.5%) of SDC and 1 (25.0%) of CxPA, and all of these were high-grade tumors (Table 2). Both PD-L1 and PD-L2 positivity were statistically collated with sex (male, $P = 0.0252$), N-stage ($P = 0.0025$), histopathological subtype (non-AdCC, $P = 0.0356$), and high grade ($P = 0.0252$). In contrast, there was no statistical

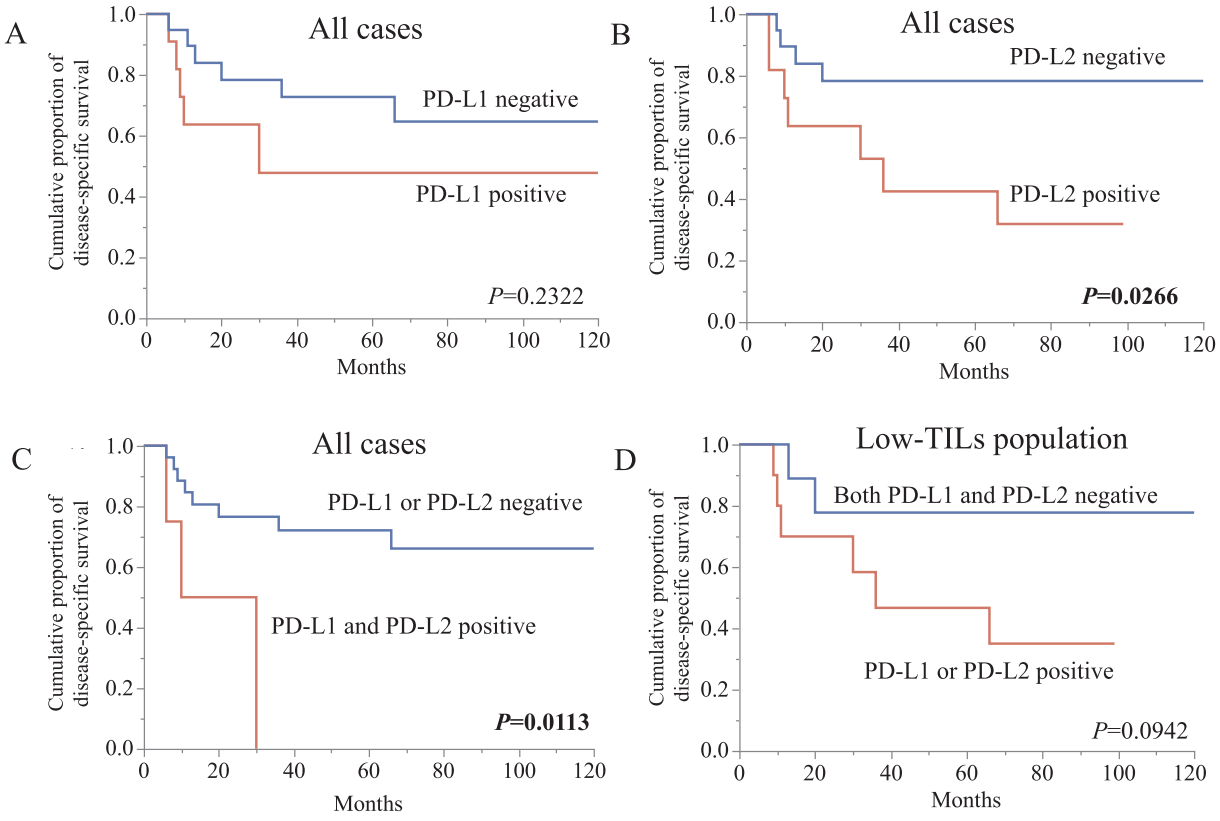

Fig. 3. The Kaplan–Meier analysis for disease-specific survival (DSS) in salivary gland carcinoma via PD-L1 immunohistochemical staining (IHC). (A). PD-L2 *in situ* hybridization (ISH) status (B), both PD-L1 IHC and PD-L2 ISH status (C), and PD-L1 IHC and PD-L2 ISH status within the low-TILs group (D). PD-L2 ISH-positive status (B) and positive status for both PD-L1 IHC and PD-L2 ISH (C) was statistically associated with reduced DSS (P = 0.0226 and P = 0.0113, respectively), whereas there was no statistical difference between PD-L1 IHC and DSS (A), and PD-L1/L2 status within the low-TILs group (D) (P = 0.2322 and P = 0.0942, respectively).

difference regarding age, T-stage, pathological stage, surgical margin, TILs, local/reginal LN recurrence, or distant metastasis.

Tumor-infiltrating lymphocytes

High levels of TILs were associated with non-AdCCs (P = 0.00323). All AdCC showed low levels of TILs. There was no statistical difference regarding age, sex, T-stage, N-stage, pathological stage, histological grade, surgical margin, local/reginal LN recurrence, or distant metastasis.

Analysis of microsatellite instability

Microsatellite instability was not detected in the present study.

Prognostic analyzes

Figs. 3 and 4 show the association of DSS and DFS with each factor in salivary gland cancer.

PD-L1 IHC-positive cases tended to show worse prognoses than PD-L1 IHC-negative cases, although differences were not statistically significant (P = 0.2322; Fig. 3A). Cases that were PD-L2 ISH-positive and cases that were both PD-L1 IHC-positive and PD-L2 ISH-positive were statistically associated with shorter DSS (P = 0.0226 and P = 0.0113, respectively; Fig. 3B and Fig. 3C).

PD-L2 ISH-positive cases were statistically correlated with reduced DFS (P = 0.0209; Fig. 4B). On the other hand, while cases that were PD-L1 IHC-positive or both PD-L1 IHC-positive and PD-L2 ISH-positive tended to be associated with reduced DFS, the differences were not statistically significant (P = 0.7358 and P = 0.2926, respectively; Fig. 4A and Fig. 4C).

Among the cases showing low-TILs, either PD-L1 IHC-positive or PD-L2 ISH-positive status were correlated with reduced DFS (P = 0.0302; Fig. 4D), but not with DSS (P = 0.0942; Fig. 3D), while among cases showing high-TILs, PD-L1 or -L2 status had no significant effects on DFS or DSS (data not shown). Between the low-TILs and high-TILs groups, there were no significant differences in the DFS or DSS curves (data not shown).

Discussion

As proposed by the 2017 World Health Organization classification system [39], SGC are classified into 24 different categories. Because rare cancers are subdivided with such great detail under this system, it is difficult to accumulate sufficient cases for examination in a single institute. Consequently, there have been only a few biological studies analyzing the immunological status of SGC, and, therefore, the scientific rationale for the use of ICI to treat SGC remains obscure. To address this issue, we comprehensively examined the prognostic values of four factors: PD-L1 and PD-L2 expression, MSI, and TILs, which are currently proposed as promising biomarkers to predict the responses of several tumors to ICI [14–17,27–29,36–38,40 15–19,29–31,38,39].

In various types of tumors, including HNSCC, the overexpression of PD-1 and its ligands, PD-L1 and -L2, is reported to correlate with poor prognosis and predict better responses to ICI [15–21,40]. As for salivary gland cancer, the reported overexpression rates of PD-L1 were 0%–30% of tumors [22–26]. In the present study, we observed relatively high PD-L1 overexpression in 37% of tumors (Table 2), which was significantly correlated with lymph node metastasis and high-grade tumors (Table 2 and Fig. 1B), but not with DSS or DFS (Fig. 3A and 3B). Consistent with our data, previous studies also demonstrated controversial results; two studies reported [23,26] the positive relevance of

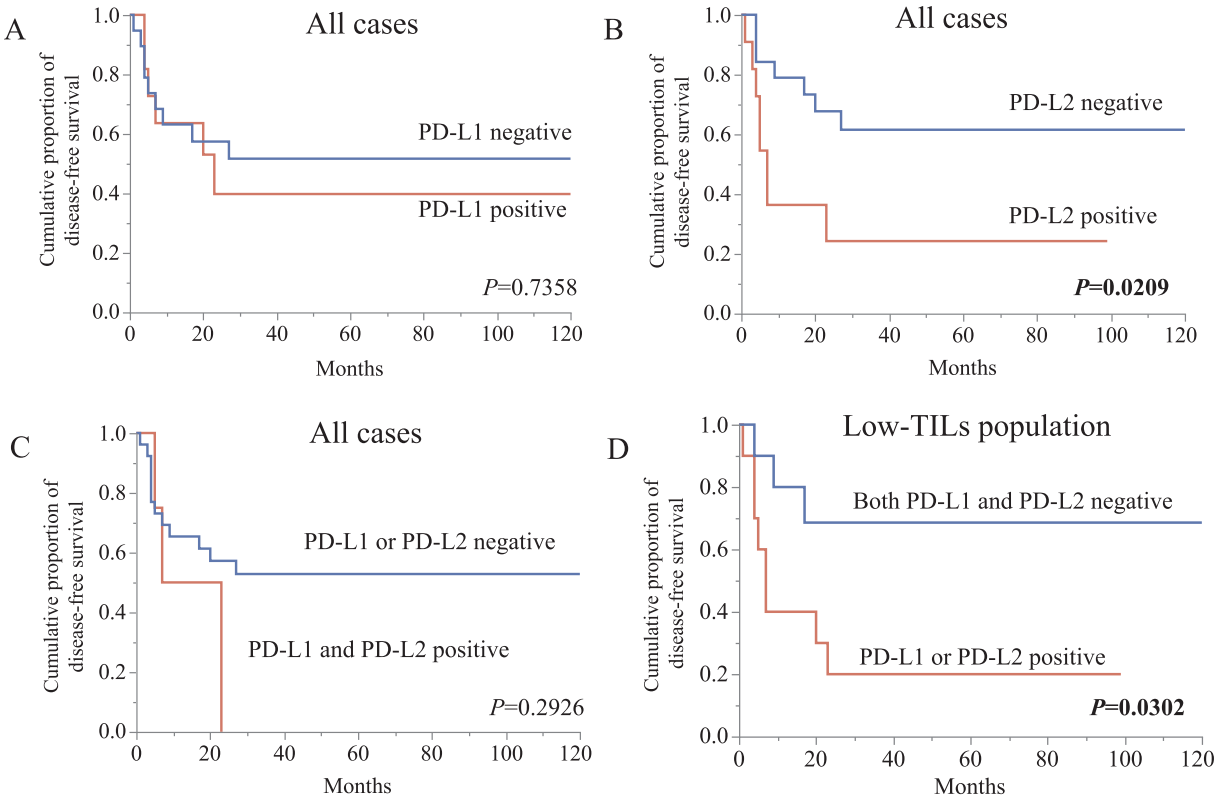

Fig. 4. The Kaplan–Meier analysis for disease-free survival (DFS) in salivary gland carcinoma via PD-L1 immunohistochemical staining (IHC). (A). PD-L2 *in situ* hybridization (ISH) status (B), both PD-L1 IHC and PD-L2 ISH status (C), and PD-L1 IHC and PD-L2 ISH status within the low-TILs group (D). PD-L2 ISH-positive status (B) and either PD-L1 IHC-positive or PD-L2 ISH-positive status within the low-TILs group (D) were statistically associated with reduced DFS (P = 0.0209 and P = 0.0302, respectively), whereas there was no statistical difference between DFS and PD-L1 IHC (A), and positive status for both PD-L1 IHC and L2 ISH (C) (P = 0.7358 and P = 0.2926, respectively).

PD-L1 expression to aggressive clinicopathological features including high T-stage (T3/T4), lymph node metastasis, human epidermal growth factor receptor 2 (HER2) status, high Ki-67 index, and high-grade tumors, as well as to worse prognosis, while another two studies [22,25] showed no association between PD-L1 expression and clinicopathological findings or prognosis (DSS and DFS). One plausible explanation for these discrepancies regarding PD-L1 expression level and its role as a biomarker appears to be the use of ununiformed antibodies and/or cutoff criteria. In addition, histopathological bias may also cause these discrepancies, because substantially different expression levels of PD-L1 were seen depending on the histological typing: 22%–45.5% in SDCs [22,23,26], 6%–25.9% in MECs [22,23], 36% in CxPAs [23], and 0%–26.7% in AdCCs [22–24]. This is consistent with our results: 50% in SDCs, 57% in MECs, 70% in CxPAs, and 0% in AdCCs (Table 2 and Fig. 1A). Taken together, non-AdCCs show relatively higher PD-L1 protein overexpression than AdCCs, indicating that non-AdCC tumors are better targets for PDL-1 inhibition than AdCC tumors. In any case, the biological roles of PDL-1 expression in SGC must be determined in a larger scale study using a standardized antibody and evaluation criteria.

For the detection of PDL-2 expression, we employed an ISH system instead of IHC. This is because while several PD-L1 IHC antibodies used in the global clinical trials were available at the time of this study, no reliable PDL-2 IHC antibody and evaluation system is established, and the antibody (clone 3G2), whose reliability was examined in comparison to PCR and ISH, was not commercially available at the time of the experiment [35]. We found that 37% of tumors demonstrated high PDL-2 mRNA expression. This figure is lower than the previously reported 60–70% in two studies that examined PDL-2 protein overexpression in SGC using IHC [22,24]. A simple comparison of the different detection systems used is not rational, and the precise reason for this difference is not known. However, our results showing that AdCCs tend to express higher levels of PDL-2 (46%) than non-AdCCs (32%) is supported by the previous IHC studies showing AdCCs at 60–90% and non-AdCCs at 60% [22,24]. Thus, the relatively high PD-L2 expression may be one of the specific features of AdCC. However, Chang and colleagues [22] reported that low PD-L2 expression was associated with reduced DFS, contradicting our result that PD-L2 overexpression is a predictor of worse prognosis (DSS and DFS) and distant metastasis (Figs. 3B, 4B, and Table 2), and that co-overexpression of PD-L1 and PD-L2 was correlated with shorter DSS (Fig. 3C). Among non-AdCCs, all distant metastases occurred specifically (100%) in PD-L2-positive cases (Table 3). Therefore, our results strongly indicate that PD-L2 expression, which appears to reflect the biological aggressiveness of SGC, may be a promising molecular target for PD-L2-inhibiting ICI (i.e., PD-1 antibody).

TILs were shown to be a predictor of favorable prognosis and sensitivity of chemotherapy in various types of cancers including SGC [22,27,37,38]. In the present study, 52.6% of non-AdCCs showed high-TILs, whereas none of the AdCCs studied showed high-TILs (Table 2). This was consistent with the results of a previous study, wherein high-TILs were detected in 89.5% of non-AdCCs and 40% of AdCCs [22]. However, in contrast to the favorable prognosis value of high-TILs reported in the previous study, our results demonstrated no relationship between high-TILs and the other clinicopathological factors or prognosis. However, when low levels of TILs were combined with either PD-L1 or PD-L2 overexpression, a correlation with reduced DFS was observed (Fig. 4D), suggesting that cold tumor conditions (i.e., a lack of TILs) [41] contribute to immune evasion in the SGC microenvironment.

In colorectal carcinoma, MSI-H has been detected in approximately 12%–15% cases, and is recognized as a biomarker for favorable prognosis [27,28] and responses to ICI [29]. Conversely, the frequency and the prognostic significance of MSI is controversial in other carcinomas,

including endometrial, gastric, hepatocellular, thyroid, ovarian, cervical [32,33], pancreatic, head and neck, and prostate carcinoma [27]. Moreover, little is known about the MSI status and its relevance to the PD-1/PD-L1 and -L2 pathway in SGC. In the present study, none of the cases studied showed MSI, although we employed a relatively larger number of cases than previous studies, which demonstrated 0%–50% MSI in SGC [30,31]. One possible explanation may be the difference in the probes used for the detection of MSI. In any case, further studies on a larger scale are required to more precisely assess the clinical significance of MSI in SGC in comparison with MMR deficiency and tumor mutation burdens.

Conclusions

To our knowledge, this is the first comprehensive study evaluating the expression levels of PD-L1 and PD-L2, MSI status, and TILs simultaneously in SGC patients. Our data suggest that PDL-2 expression alone or in combination with PD-L1 and/or level of TILs was significantly associated with unfavorable prognosis. Additionally, in non-AdCCs, PD-L2 ISH-positive status was a predictor of distant metastases with 100% accuracy. Taken together, our study provides positive evidence for the use of ICI in SGC, suggesting that among currently available ICI, the PD-1 antibody, which can inhibit both PD-L1 and PDL-2, may be a good option, and should be evaluated in further clinical studies.

Conflict of interest

None declared.

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