Original Article

PD-L1 immunohistochemistry comparison of 22C3 and 28-8 assays for gastric cancer

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Background: Nivolumab and pembrolizumab are promising therapies for gastric adenocarcinoma. The 22C3 and 28-8 pharmDx immunohistochemistry assays for programmed death ligand-1 scoring criteria have been developed. This study compared the programmed death ligand-1 staining patterns of gastric adenocarcinoma evaluated by the 22C3 and 28-8 pharmDx assays.

Methods: Tissue microarray analysis was performed for 226 patients with gastric adenocarcinoma who underwent curative surgery. Interobserver concordance between the 22C3 and 28-8 pharmDx assays was assessed to compare the dichotomized expression values. Programmed death ligand-1 positivity was assessed by combined positive score and tumor proportion score. Immunohistochemistry for deficient mismatch repair proteins and Epstein-Barr virus-encoded RNA *in situ* hybridization was examined.

Results: Programmed death ligand-1 positivity with a combined positive score ≥5 was detected in 63 patients (28%) by the 22C3 pharmDx assay, and in 45 patients (20%) by the 28-8 pharmDx assay. A pairwise comparison of the 22C3 and 28-8 pharmDx assays showed 87% of pairs were concordant and 11% higher expressions for the 22C3 pharmDx assay, with strong concordance (kappa score =0.881 with a combined positive score cutoff of 5). The programmed death ligand-1 positivity rate (range, 3–5%) of the tumor proportion score was markedly lower than that of the combined positive score in the two assays. Programmed death ligand-1 positivity of the combined positive score in these two assays was associated with mismatch repair proteins and Epstein-Barr virus status. There was no significant difference in the overall survival between programmed death ligand-1, mismatch repair proteins, and Epstein-Barr virus status.

Conclusions: The study findings suggest the potential interchangeability of the 22C3 and 28-8 pharmDx assays to determine programmed death ligand-1 expression levels in gastric adenocarcinoma patients.

Keywords: Combined proportion score (CPS); gastric cancer; immune checkpoint inhibitor; programmed death ligand-1 (PD-L1)

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Introduction

Gastric cancer is the fifth most common cancer and the third most common cause of cancer-related deaths worldwide (1). Although advanced gastric cancer is treated with platinum and fluoropyrimidine doublet as first-line treatment, its prognosis remains poor at 8–15 months after initial treatment (2-4).

Recently, immune checkpoint inhibitors targeting programmed death-1 (PD-1) or programmed death ligand 1 (PD-L1) showed improved survival of patients with various solid tumors compared with standard treatment options (5,6). PD-L1 and its partner PD-L2, transmembrane proteins expressed by normal tissues, inhibit T-cell activation and prevent autoimmunity. The binding of PD-1/PD-L1 on tumor cells (TCs) or tumor-infiltrating immune cells (ICs) was reported to induce T-cell tolerance. Therefore, antibodies that block this interaction showed benefit in clinical trials of patients with refractory malignancies (7). A multicenter, double-blind, randomized phase III trial (ATTRACTION 2) reported nivolumab, a fully human IgG4 monoclonal antibody against PD-1, improved survival as a third-line treatment for advanced gastric cancer compared with placebo (8). The phase 2 non-randomized KEYNOTE 059 trial reported radiological response rates were improved in gastric cancer patients with overexpressed PD-L1 proteins in TCs and ICs who were treated with pembrolizumab, another anti-PD-1 monoclonal antibody (9). In the CheckMate 649 trial, nivolumab significantly improved the overall survival (OS) and progression-free survival (PFS) of patients with a positive PD-L1 combined proportion score (CPS) using the Dako PD-L1 immunohistochemistry 28-8 pharmDx assay (10). However, the KEYNOTE-061 and KEYNOTE-062 trials evaluated tumors expressing high levels of PD-L1 (CPS ≥10) by the PD-L1 IHC 22C3 pharmDx assay (11,12). Therefore, several PD-L1 immunohistochemistry IHC assays using scoring criteria, including the tumor proportion score (TPS), have been developed in parallel (13).

Recent genomic and molecular characterization studies of gastric adenocarcinoma (GAC), such as the Cancer Genome Atlas (TCGA), have characterized PD-L1 positivity and four different subtypes (14). PD-L1 expression is high in microsatellite instability (MSI) gastric cancer and Epstein-Barr virus (EBV)-positive gastric cancer, which are susceptible to treatment with immune checkpoint inhibitors (15).

Based on these results, we planned a single-center, non-intervention, retrospective, observational study to investigate the clinicopathological features of PD-L1 expression using three different tissue microarrays (TMA) in patients with esophagogastric cancer.

We present the following article in accordance with the REMARK reporting checklist (available at https://dx.doi.org/10.21037/jgo-21-505).

Methods

Case selection

The patient selection criteria were as follows: (I) histological diagnosis of esophagogastric adenocarcinoma (stage I–IV); (II) underwent gastrectomy at a single institution from 2009 to 2010; (III) sufficient tumor content in formalin-fixed paraffin-embedded (FFPE) samples; and (IV) no systemic chemotherapy before surgery. The clinicopathological patient characteristics, including age, sex, tumor location, tumor-node-metastasis (TNM) stage (the 7th edition of the American Joint Committee on Cancer staging manual), and tumor histology were collected. This study was approved by the Institutional Review Board (IRB) at Aichi Cancer Center Hospital (IRB reference number: 2020-1-496) and conformed to the provisions of the Declaration of Helsinki (as revised in 2013). Informed consent was taken from all individual participants.

Construction of TMA

Tumor gastric FFPE samples were collected from gastrectomy specimens. The representative tumor regions were selected and marked in hematoxylin and eosin (H&E)-stained slides of each case by two pathologists. Briefly, four representative cores (2-mm diameter) with sufficient tissue quality and amount of tissue were stamped out of the donor block and transferred to a recipient paraffin block. Serial 4-μm sections were used for H&E staining, IHC, and in situ hybridization (ISH).

Immunohistochemistry

The PD-L1 expression of TCs and ICs, mismatch repair (MMR) (MLH1/MSH2/MSH6/PMS2) and HER2 were evaluated by IHC. PD-L1 positivity was evaluated by the CPS, which was defined as the number of PD-L1 stained cells (TCs, lymphocytes, macrophages) as a proportion of the total number of TCs multiplied by 100 (16). The TPS

was also evaluated by membrane staining as follows: score 3+, ≥50%; score 2+, ≥25, <50%; and score 1+, ≥1, <25%. Lacking MLH1, MSH2, MSH6, or PMS2 was defined as defective MMR (dMMR), and maintaining MLH1, MSH2, MSH6, and PMS2 was defined as MMR proficient (pMMR). EBV positive was defined by chromogenic encoded ribonucleic acid in situ hybridization (INFORM EBER probe, Ventana, Tucson, USA). HER2 positivity was defined as an IHC score of 3+ or an IHC score of 2+. ISH positivity was determined by fluorescence ISH or dual-color ISH. HER2 and ISH positivity are considered indications for using trastuzumab as in the ToGA trial (17,18). Antibodies used for IHC analysis were summarized in Table S1. IHC was performed with a Dako Autostainer Link48. ES and YY evaluated the immunostaining as board certified pathologists.

Statistical analysis

The statistical significance of differences in proportions and medians were compared using independent χ^2 tests or Fisher's exact test for categorical variables. Interobserver concordance was assessed to compare the dichotomized expression values between each assay using Cohen's Kappa (non-weighted) method. Kappa scores of 0.9 or higher were considered near perfect, scores of 0.80 to 0.89 were considered strong, scores of 0.60 to 0.79 were considered moderate, and scores of 0.40 to 0.59 were considered weak (19). The OS was defined as the time from the date of gastrectomy until death from any cause or censored at the last follow-up date. Median OS was estimated by the Kaplan-Meier method. The hazard ratio (HR) and 95% confidence interval (CI) were estimated using the Cox proportional hazards model. Statistical analyses were performed using R software version 4.1.0 (R Project for Statistical Computing, Vienna, Austria). All tests were two-sided and P values <0.05 were considered statistically significant.

Results

Patient characteristics and prevalence of PD-L1 expression

Of 331 patients who underwent gastrectomy at Aichi Cancer Center Hospital during the study period, 105 were excluded for receiving neoadjuvant chemotherapy (N=26) or insufficient tumor content (N=79). Finally, 226 patients were selected for this study. Patient demographics are shown in *Table 1*. The median age was 65 years (range, 32–86 years), and the pathological TNM stage included 100 cases of stage I (44%), 39 cases of stage II (17%), 58 cases of stage III (26%), and 29 cases of stage IV (13%). EBV was detected in 13 patients (6%) and dMMR was observed in 29 cases (13%). Of 79 patients (35%) who received adjuvant chemotherapy, 69 were treated with S-1 (tegafur, gimeracil, and oteracil potassium) monotherapy.

The 22C3 pharmDx assay demonstrated the numbers of patients with PD-L1 expression CPS ≥1, ≥5, and ≥10 were 63 (28%), 25 (11%), and 17 (8%), respectively (*Figure 1*). Numbers of patients with PD-L1 expression CPS ≥1, ≥5, and ≥10 by the 28-8 pharmDx assay were 45 (20%), 22 (10%), and 16 (7%), respectively. Higher levels of PD-L1 expression at a PD-L1 CPS ≥5 by the 22C3 pharmDx assay were more frequently observed in older patients (P=0.032), those with a tumor in the upper or lower stomach (P=0.017), or who were EBV positive (P=0.008), or dMMR (P=0.001) (*Table 2*). In contrast, using the 28-8 pharmDx assay, PD-L1 expression with CPS ≥5 was significantly associated with a tumor location in the upper or lower stomach (P=0.017), stage II or III (P=0.049), EBV positive (P=0.004), and dMMR (P=0.012). The clinicopathological features obtained from the analysis of PD-L1 positivity evaluated by CPS in the E1L3 assay and examined by TPS in each assay are presented in Tables S2-S7. The PD-L1 positivity rate (range, 3–5%) of TPS was markedly lower than that of CPS in all three assays.

Comparison of 22C3 and 28-8 PD-L1 pharmDx assays

To quantify potential differences in PD-L1 expression, a pairwise comparison of the 22C3 and 28-8 pharmDx assays was plotted: 87% of pairs were concordant and 11% had a higher PD-L1 expression by the 22C3 assay (*Table 3*). With a CPS cutoff of 5, concordance between the 22C3 and 28-8 assays was strong (kappa score =0.881) (*Table 4*). However, various kappa scores were observed when using different cutoff points for CPS: kappa score =0.735 at a cutoff point of 1; and kappa score =0.837 at a cutoff point of 10. Conversely, the analysis of TPS within all three assays showed higher concordant rates (range, 94–97%) (Tables S8-S10).

Representative images of the PD-L1 immunohistochemical staining of the 22C3 and 28-8 pharmDx assays are shown in *Figure 1*. In most cases, the assays had equivalent relative staining. However, in a few cases, the 28-8 pharmDx assay had a weaker staining of TC and IC membranes compared

Table 1 Patient characteristics

Characteristics	Categories	N=226	%
Age, years	Median [range]	65 [32–86]	–
	<65/≥65	113/113	50/50
Sex	Male/female	162/64	72/28
Tumor location	EGJ/U/M/L	12/47/85/82	5/21/38/36
Depth of invasion	T1/T2/T3/T4	94/25/32/75	42/11/14/33
Lymph node metastasis	N0/N1/N2/N3	111/32/29/54	49/14/13/24
TNM stage	I/II/III/IV	100/39/58/29	44/17/26/13
Tumor histology	Diffuse/intestinal	87/139	39/61
EBV	Positive/negative	13/213	6/94
MMR	pMMR/dMMR	197/29	87/13
HER2	Positive/negative	24/202	11/89
Adjuvant chemotherapy	Yes/no	79/147	35/65
PD-L1 CPS (22C3)	≥1/≥5/≥10	63/25/17	28/11/8
PD-L1 CPS (28-8)	≥1/≥5/≥10	45/22/16	20/10/7
PD-L1 CPS (E1L3)	≥1/≥5/≥10	79/33/23	35/15/10

CPS, combined positive score; dMMR, defective mismatch repair; EBV, Epstein-Barr virus; GEJ, esophagogastric junction; HER2, human epidermal growth factor 2; L, lower third; M, middle third; MMR, mismatch repair; PD-L1, programmed death ligand-1; pMMR, mismatch repair proficient; U, upper third.

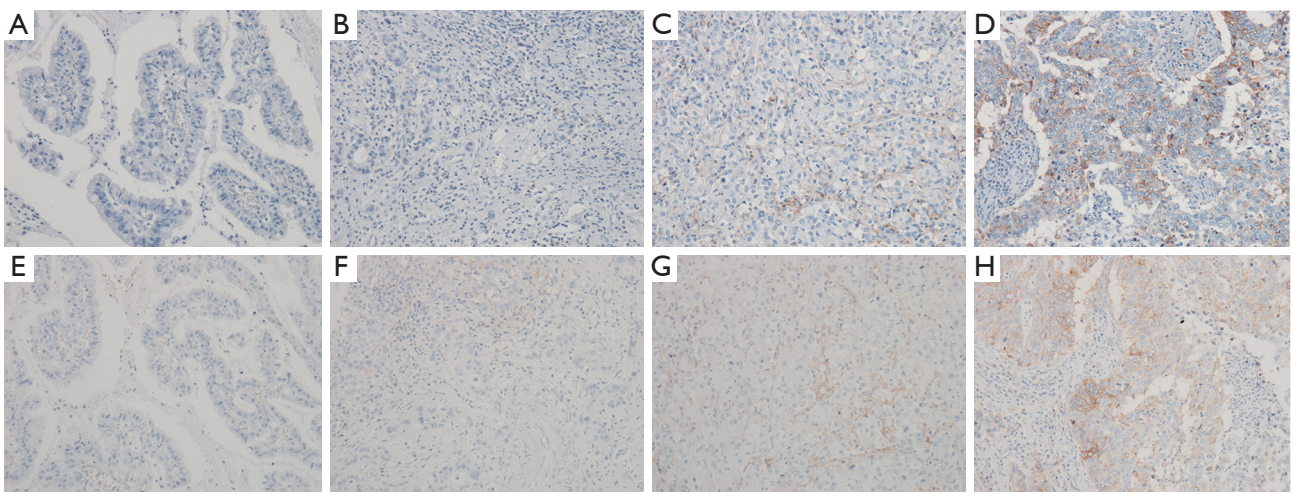

Figure 1 Representative images of PD-L1 immunohistochemical staining using the 22C3 and 28-8 pharmDx assays. (A) PD-L1 CPS <1 in the 22C3 pharmDx assay; (B) PD-L1 CPS ≥1, <5 in the 22C3 pharmDx assay; (C) PD-L1 CPS ≥5, <10 in the 22C3 pharmDx assay; (D) PD-L1 CPS ≥10 in the 22C3 pharmDx assay; (E) PD-L1 CPS <1 in the 28-8 pharmDx assay; (F) PD-L1 CPS ≥1, <5 in the 28-8 pharmDx assay; (G) PD-L1 CPS ≥5, <10 in the 28-8 pharmDx assay; (H) PD-L1 CPS ≥10 in the 28-8 pharmDx assay. The photo shows magnification ×200. CPS, combined positive score; PD-L1, programmed death ligand-1.

Table 2 Patient characteristics according to PD-L1 expression between 22C3 and 28-8 pharmDx assays

Characteristics	Categories	PD-L1, N [%]					
		22C3 pharmDx			28-8 pharmDx		
		CPS ≥5 (N=25)	CPS <5 (N=201)	P	CPS ≥5 (N=22)	CPS <5 (N=184)	P
Age, years	<65	7 [28]	106 [53]	0.032	7 [32]	106 [52]	0.115
	≥65	18 [72]	95 [47]		15 [68]	98 [48]	
Sex	Male	18 [72]	144 [72]	1.000	16 [73]	146 [72]	1.000
	Female	7 [28]	57 [28]		6 [27]	58 [28]	
Tumor location	GEJ	0	12 [6]	0.017	0	12 [6]	0.017
	U	10 [40]	37 [18]		9 [41]	38 [19]	
	M	4 [16]	81 [40]		3 [14]	82 [40]	
	L	11 [44]	71 [35]		10 [45]	72 [35]	
Depth of invasion	T1	6 [24]	88 [44]	0.064	5 [23]	89 [44]	0.087
	T2	1 [4]	24 [12]		1 [5]	24 [12]	
	T3	6 [24]	26 [13]		4 [18]	28 [14]	
	T4	12 [48]	63 [31]		12 [55]	63 [31]	
Lymph node metastasis	Absent	10 [40]	101 [50]	0.399	7 [32]	104 [51]	0.116
	Present	15 [60]	100 [50]		15 [68]	100 [49]	
TNM stage	I	7 [28]	93 [46]	0.053	6 [27]	94 [46]	0.049
	II	6 [24]	33 [16]		4 [18]	35 [17]	
	III	11 [44]	47 [23]		11 [50]	47 [23]	
	IV	1 [4]	28 [14]		1 [5]	28 [14]	
Tumor histology	Intestinal	11 [44]	76 [38]	0.664	11 [50]	76 [37]	0.257
	Diffuse	14 [56]	125 [62]		11 [50]	128 [63]	
EBV	Positive	5 [20]	8 [4]	0.008	5 [23]	8 [4]	0.004
	Negative	20 [80]	194 [96]		17 [77]	196 [96]	
MMR	dMMR	9 [36]	20 [10]	0.001	7 [32]	22 [11]	0.012
	pMMR	16 [64]	182 [90]		15 [68]	182 [89]	
HER2	Positive	3 [12]	21 [10]	0.734	3 [14]	21 [10]	0.713
	Negative	22 [88]	181 [90]		19 [86]	183 [90]	

CPS, combined positive score; dMMR, defective mismatch repair; EBV, Epstein-Barr virus; GEJ, esophagogastric junction; HER2, human epidermal growth factor 2; L, lower third; M, middle third; MMR, mismatch repair; PD-L1, programmed death ligand-1; pMMR, mismatch repair proficient; U, upper third.

with the 22C3 assay.

Survival analysis

During the median follow-up time of 60.3 months, 54 patients (24%) died (*Figure 2*). Using the 22C3 pharmDx assay, the median OS was not reached (NR) with PD-L1 CPS ≥5 *vs.* NR with PD-L1 CPS <5 (HR, 0.94; 95% CI: 0.40–2.20). Using the 28-8 pharmDx assay, no survival difference was observed with a CPS cutoff point of 5 (HR,

0.70; 95% CI: 0.22–2.25). In addition, PD-L1 expression was not a prognostic factor for OS when using other CPS cutoff points in the 22C3 pharmDx, 28-8 pharmDx, and E1L3 assays (Figure S1). Overall, 38 patients (44%) who were stage III or IV died, and patients who were PD-L1 positive tended to have a better prognosis than those who were PD-L1 negative by all three assays (Table S11). Furthermore, MMR and EBV status were not independent prognostic factors for OS (Figure S2).

Discussion

The present study of gastric cancer diagnostic samples demonstrated high concordance for PD-L1 expression between the 22C3 and 28-8 pharmDx assays when using a CPS cutoff of 5. In addition, the PD-L1 positivity rate evaluated by CPS was higher than that evaluated by TPS in all three assays.

The independent development of PD-L1 assays for the clinical use of pembrolizumab or nivolumab makes it difficult to determine whether the interchangeability of the 22C3 and 28-8 pharmDx assays is useful in clinical settings. In the present cohort, comparisons of PD-L1 CPS between the 22C3 and 28-8 pharmDx assays showed a relatively high concordance rate with a CPS cutoff point of 5 or 10. However, a lower concordant rate of 1 was also observed. These results are consistent with data for other cancer types including lung cancer, bladder cancer, urothelial cancer, and triple-negative breast cancer (20-22). Many studies have reported the PD-L1 expression of TPS but not that of CPS. The data of 1,930 patients with lung cancer or other malignancies demonstrated strong concordance (Cohen's kappa of 0.90–0.95) between the PD-L1 IHC 22C3 and 28-8 assays when evaluating the percentage tumor-cell membrane PD-L1 expression (23). Regarding the PD-L1 CPS when comparing the 22C3 and 28-8 pharmDx assays, two small sample sized studies of breast cancer and gastric cancer reported the Kappa coefficient was moderate to strong (kappa values, 0.80–1.00) (21,24). These results provide evidence for the interchangeability of these two assays to determine PD-L1 expression levels in gastric cancer patients.

In our cohort, the PD-L1 expression using the three CPS cutoff values in the 22C3 pharmDx assay was higher than that in the 28-8 pharmDx assay, with a difference of 1.8–13.3%. These results are in contrast with previous reports. The PD-L1 positivity rate with a CPS cutoff of 1 using the 28-8 pharmDx assay was slightly higher than that of the 22C3 pharmDx assay for breast cancer (43% *vs.* 35%) (21). In addition, in gastric cancer, a CPS cutoff of 10 in the 28-8 pharmDx assay showed a modestly higher PD-L1 positivity rate than that in the 22C3 assay (25.5% *vs.* 21.8%) (24). According to our study, a PD-L1 CPS cutoff of 5 in the E1L3 assay had a higher PD-L1 positivity rate than the 22C3 (11% *vs.* 15%) and 28-8 (10% *vs.* 15%) pharmDx

Table 3 Pairwise comparison of PD-L1 CPS between 22C3 and 28-8 pharmDx assays

28-8 pharmDx	22C3 pharmDx			
	<1	≥1, <5	≥5, <10	≥10
<1	161	19	1	0
≥1, <5	2	18	2	1
≥5, <10	0	1	3	2
≥10	0	0	2	14

Concordant, 0.87; 22C3 higher, 0.11; 28-8 higher, 0.02. CPS, combined positive score; PD-L1, programmed death ligand-1.

Table 4 Comparison of PD-L1 CPS with the clinical cutoff between 22C3 and 28-8 pharmDx assays

28-8 pharmDx	22C3 pharmDx, N (%)						Kappa value
	PD-L1 CPS <1	PD-L1 CPS ≥1	PD-L1 CPS <5	PD-L1 CPS ≥5	PD-L1 CPS <10	PD-L1 CPS ≥10	
PD-L1 CPS <1	161 (71.2)	20 (8.8)					0.735
PD-L1 CPS ≥1	2 (0.9)	43 (19.0)					
PD-L1 CPS <5			200 (88.5)	4 (1.8)			0.881
PD-L1 CPS ≥5			1 (0.4)	21 (9.3)			
PD-L1 CPS <10					207 (91.6)	3 (13.3)	0.837
PD-L1 CPS ≥10					2 (0.9)	14 (6.2)	

CPS, combined positive score; and PD-L1, programmed death ligand-1.

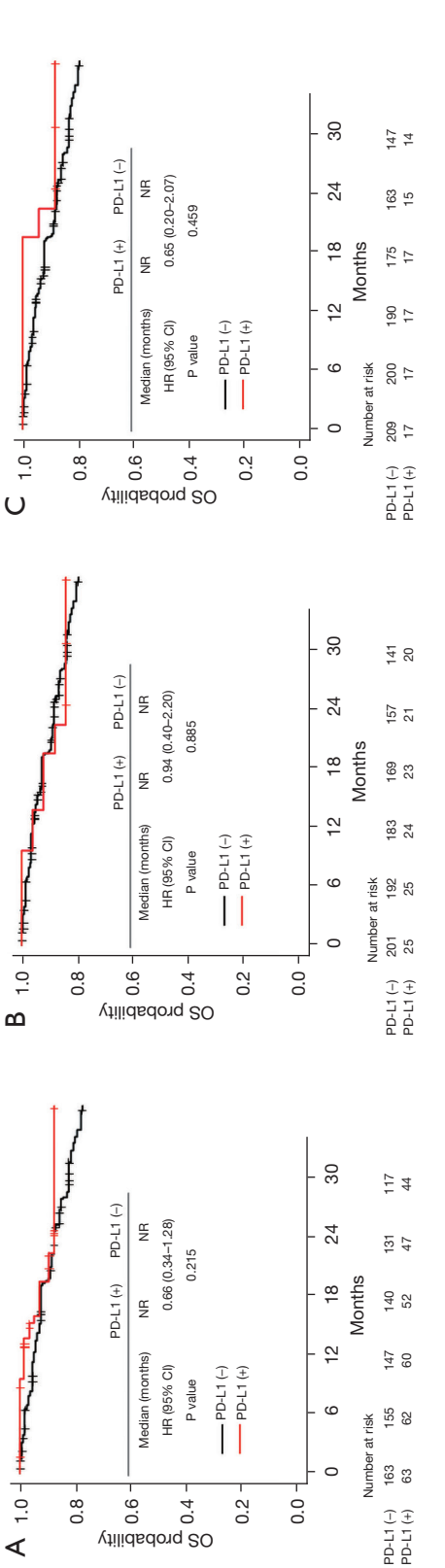

Figure 2 Kaplan-Meier plots of overall survival according to PD-L1 expression between 22C3 and 28-8 pharmDx assays. (A) PD-L1 CPS ≥1 versus <1 in the 22C3 pharmDx assay; (B) PD-L1 CPS ≥5 versus <5 in the 22C3 pharmDx assay; (C) PD-L1 CPS ≥10 versus <10 in the 22C3 pharmDx assay; (D) PD-L1 CPS ≥1 versus <1 in the 28-8 pharmDx assay; (E) PD-L1 CPS ≥5 versus <5 in the 28-8 pharmDx assay; (F) PD-L1 CPS ≥10 versus <10 in the 28-8 pharmDx assay. CPS, combined positive score; PD-L1, programmed death ligand-1; NR, not reached.

assays. The Blueprint IHC Assay Comparison Project of four PD-L1 IHC assays (22C3, 28-8, SP142, and SP263) in lung cancer reported the comparability of the 22C3, 28-8, and SP263 assays whereas the SP142 assay had the lowest levels of agreement, when evaluating the TPS. Based on these conflicting results, different IHC assays and scoring algorithms suggest that caution should be taken when selecting patients who will benefit from immune-checkpoint inhibitors. A practical next step will be to compare the staining patterns using the 22C3 and 28-8 pharmDx assays in a larger cohort of gastric cancer patients.

In the current study, the PD-L1 positivity rate determined by CPS was higher than that by TPS (10–15% *vs.* 3–5%). Our results are in accord with a previous study reporting that PD-L1 expression on TCs and ICs was positive in 8.4% and 65.3% of cases (25). The prospective CheckMate 649, KEYNOTE-061, and KEYNOTE-062 clinical trials evaluating the efficacy of immune checkpoint inhibitors as chemotherapy reported that as the CPS cutoff increased, the HR tended to improve (10-12). However, in the ATTRACTION-2 trial, PD-L1 positivity examined by TPS was not a predictive marker for nivolumab therapy. These findings suggest that the PD-L1 CPS is more useful than PD-L1 TPS in clinical settings when selecting cases that are likely to derive benefit from immune checkpoint inhibitor treatment.

It is well known that dMMR and EBV positive gastric cancers are associated with the overexpression of PD-L1 (14,15,25,26). In addition, the impact of PD-L1 expression, MMR status, and EBV status on prognosis remains controversial in gastric cancer (26-28). Our results are in line with these previous reports. Furthermore, dMMR and EBV positive are associated with responses to immune checkpoint inhibitors. A prestigious meta-analysis reported that MSI was a robust prognostic marker for resectable gastric cancer (29). Further studies are needed to clarify the significance of immune checkpoint blockade in an adjuvant setting stratified by PD-L1 expression, MSI status, and EBV status.

There were some limitations in this study. This was a retrospective study with a small number of cases from a single institution. Although there may be a lack of correction for multiple comparisons in statistics, it still remains difficult to select a proper method suitable for the various experimental properties. The CPS was classified into three categories without assessing the individual patient scores. However, to the best of our knowledge, this is the largest cohort study to provide information on the PD-L1 CPS evaluated by the 22C3 and 28-8 assays.

In conclusion, our study demonstrated that the PD-L1 CPS in gastric cancer patients was highly concordant between the 22C3 and 28-8 pharmDx assays using various CPS cutoffs. This study suggests the potential interchangeability of these two assays to determine PD-L1 expression levels in gastric cancer patients.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and was approved by Institutional Review Board (IRB) at Aichi Cancer Center Hospital (IRB No. 2020-1-496). Informed consent was taken from all individual participants.

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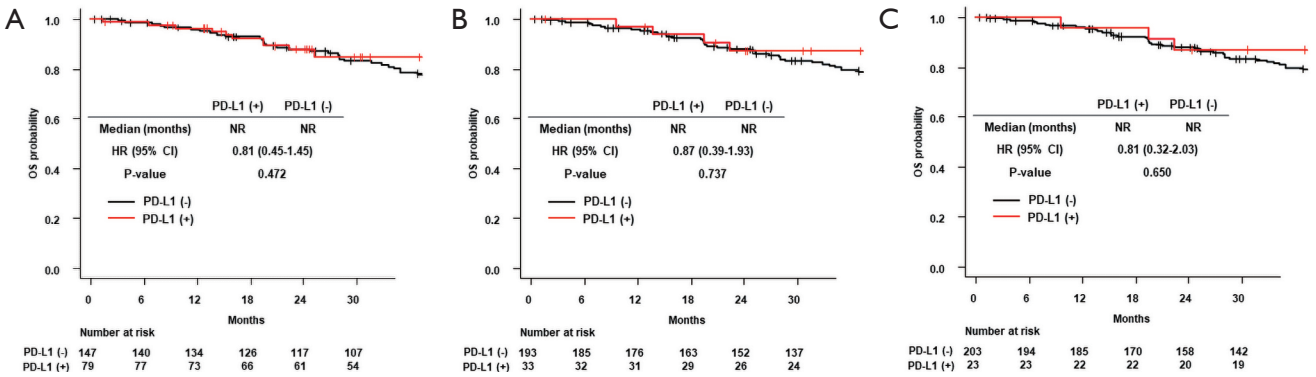

Figure S1 Kaplan-Meier plots of overall survival according to PD-L1 expression in the E1L3 assay. (A) PD-L1 CPS ≥1 versus <1; (B) PD-L1 CPS ≥5 versus <5; (C) PD-L1 CPS ≥10 versus <10. CPS, combined positive score; PD-L1, programmed death ligand-1; NR, not reached.

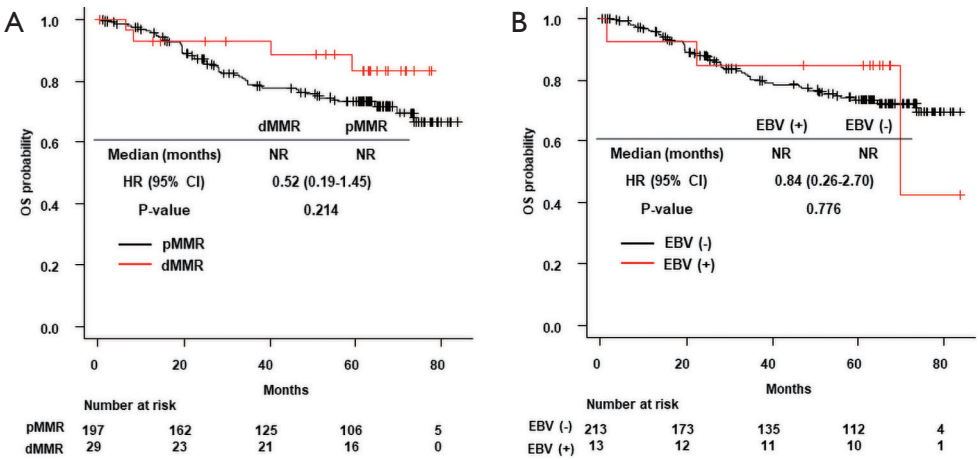

Figure S2 Kaplan-Meier plots of overall survival according to MSI status (A) and EBV status (B). OS, overall survival; HR, hazard ratio; CI, confidence interval; NR, not reached; pMMR, mismatch repair proficient; dMMR, defective mismatch repair; MSI, microsatellite instability; EBV, Epstein-Barr virus.

Table S1 Antibodies used for immunohistochemical studies and in situ hybridization probes

Antibody	Clone	Manufacturer	Platform	Detection	Dilution
PD-L1	22C3	Dako	Dako Autostainer Link48	Dako pharmDx kit	RTU
PD-L1	28-8	Dako	Dako Autostainer Link48	Dako pharmDx kit	RTU
PD-L1	E1L3N	Cell signaling	Dako Autostainer Link48	Envision Flex	1:200
MLH1	G168-15	Dako	Dako Autostainer Link48	Envision Flex	RTU
MSH2	FE11	Dako	Dako Autostainer Link48	Envision Flex	RTU
MSH6	Sp93	Roche	Ventana Benchmark XT	OptiView	RTU
PMS2	A16-4	Roche	Ventana Benchmark XT	OptiView	RTU
HER2	neu	Dako	Dako Autostainer Link48	HercepTest kit	RTU

RTU, ready-to-use; NA, not applicable.

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Table S2 Patient characteristics according to PD-L1 expression in the E1L3 assay

Characteristics	Categories	PD-L1, N [%]		
		E1L3		
		CPS ≥5 (N=33)	CPS <5 (N=193)	P
Age, years	<65	14 [42]	99 [51]	0.45
	≥65	19 [58]	94 [49]	
Sex	Male	24 [73]	138 [72]	1.00
	Female	9 [27]	55 [28]	
Tumor location	GEJ	1 [3]	11 [6]	0.24
	U	11 [33]	36 [19]	
	M	9 [27]	76 [39]	
	L	12 [36]	70 [36]	
Depth of invasion	T1	10 [30]	84 [44]	0.23
	T2	2 [6]	23 [12]	
	T3	7 [21]	25 [13]	
	T4	14 [42]	61 [32]	
Lymph node metastasis	Absent	14 [42]	97 [50]	0.52
	Present	19 [58]	96 [50]	
pTNM stage	I	11 [33]	89 [46]	0.25
	II	6 [18]	33 [17]	
	III	13 [39]	45 [23]	
	IV	3 [9]	26 [13]	
Tumor histology	Intestinal	15 [45]	72 [37]	0.44
	Diffuse	18 [55]	121 [63]	
EBV	Positive	9 [27]	4 [2]	<0.01
	Negative	24 [63]	189 [98]	
MMR	dMMR	8 [24]	21 [11]	0.05
	pMMR	25 [66]	172 [89]	
HER2	Positive	4 [12]	20 [10]	0.76
	Negative	29 [88]	173 [90]	

CPS, combined positive score; dMMR, defective mismatch repair; EBV, Epstein-Barr virus; GEJ, esophagogastric junction; HER2, human epidermal growth factor 2; L, lower third; M, middle third; MMR, mismatch repair; PD-L1, programmed death ligand-1; pMMR, mismatch repair proficient; U, upper third.

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Table S3 Pairwise comparison of PD-L1 CPS in 22C3 pharmDx and E1L3 assays

E1L3	22C3 pharmDx			
	<1	≥1, <5	≥5, <10	≥10
<1	142	5	0	0
≥1, <5	20	25	1	0
≥5, <10	1	7	2	0
≥10	0	1	5	17

Concordant, 0.82; 22C3 higher, 0.03; E1L3 higher, 0.15. CPS, combined positive score; PD-L1, programmed death ligand-1.

Table S4 Pairwise comparison of PD-L1 CPS in 28-8 pharmDx and E1L3 assays

E1L3	28-8 pharmDx			
	<1	≥1, <5	≥5, <10	≥10
<1	145	2	0	0
≥1, <5	33	13	0	0
≥5, <10	3	5	1	1
≥10	0	3	5	15

Concordant, 0.77; 28-8 higher, 0.01; E1L3 higher, 0.22. CPS, combined positive score; PD-L1, programmed death ligand-1.

Table S5 Comparison of PD-L1 CPS with the clinical cutoff according to 22C3 pharmDx and E1L3 assays

E1L3	22C3 pharmDx						Kappa value
	PD-L1 CPS <1	PD-L1 CPS ≥1	PD-L1 CPS <5	PD-L1 CPS ≥5	PD-L1 CPS <10	PD-L1 CPS ≥10	
PD-L1 CPS <1	142	5					0.735
PD-L1 CPS ≥1	21	58					
PD-L1 CPS <5			192	1			0.803
PD-L1 CPS ≥5			9	24			
PD-L1 CPS <10					203	0	0.836
PD-L1 CPS ≥10					6	17	

CPS, combined positive score; PD-L1, programmed death ligand-1.

Table S6 Comparison of PD-L1 CPS with the clinical cutoff according to 22C3 pharmDx and E1L3 assays

E1L3	28-8 pharmDx						Kappa value
	PD-L1 CPS <1	PD-L1 CPS ≥1	PD-L1 CPS <5	PD-L1 CPS ≥5	PD-L1 CPS <10	PD-L1 CPS ≥10	
PD-L1 CPS <1	145	2					0.589
PD-L1 CPS ≥1	36	43					
PD-L1 CPS <5			196	8			0.827
PD-L1 CPS ≥5			0	22			
PD-L1 CPS <10					202	1	0.748
PD-L1 CPS ≥10					8	15	

CPS, combined positive score; PD-L1, programmed death ligand-1.

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Table S7 Patient characteristics according to PD-L1 expression by TPS

Characteristics	Categories	PD-L1, N [%]								
		22C3			28-8			E1L3		
		Positive (N=11)	Negative (N=215)	P	Positive (N=7)	Negative (N=219)	P	Positive (N=12)	Negative (N=214)	P
Age, years	<65	6 [55]	107 [50]	1.00	3 [43]	110 [50]	1.00	5 [42]	108 [50]	0.77
	≥65	5 [45]	108 [50]		4 [57]	109 [50]		7 [58]	106 [50]	
Sex	Male	10 [91]	152 [71]	0.19	7 [100]	155 [71]	0.20	10 [83]	152 [71]	0.52
	Female	1 [9]	63 [29]		0 [0]	64 [29]		2 [17]	62 [29]	
Tumor location	GEJ	0 [0]	12 [6]	0.92	0 [0]	12 [5]	0.74	0 [0]	12 [6]	0.93
	U	4 [36]	78 [36]		4 [57]	78 [36]		5 [42]	77 [36]	
	M	4 [36]	81 [38]		2 [29]	83 [38]		4 [33]	81 [38]	
	L	3 [27]	44 [20]		1 [14]	46 [21]		3 [25]	44 [21]	
Depth of invasion	T1	3 [27]	91 [42]	0.21	1 [14]	93 [42]	0.19	1 [8]	93 [43]	0.03
	T2	0 [0]	25 [12]		0 [0]	25 [11]		1 [8]	24 [11]	
	T3	1 [9]	31 [14]		2 [29]	30 [14]		4 [33]	28 [12]	
	T4	7 [64]	68 [32]		4 [57]	71 [32]		6 [50]	69 [32]	
Lymph node met.	Absent	3 [27]	108 [50]	0.22	2 [29]	109 [50]	0.45	3 [25]	108 [50]	0.14
	Present	8 [73]	107 [50]		5 [71]	110 [50]		9 [75]	106 [50]	
pTNM stage	I	3 [27]	97 [45]	0.21	1 [14]	99 [45]	0.10	1 [8]	99 [46]	0.03
	II	1 [9]	38 [18]		2 [29]	37 [17]		3 [25]	36 [17]	
	III	6 [55]	52 [24]		4 [57]	54 [25]		5 [42]	53 [25]	
	IV	1 [9]	28 [13]		0 [0]	29 [13]		3 [25]	26 [12]	
Tumor histology	Intestinal	5 [45]	82 [38]	0.75	2 [29]	85 [39]	0.71	3 [25]	84 [39]	0.38
	Diffuse	6 [55]	133 [62]		5 [71]	134 [61]		9 [75]	130 [61]	
EBV	Positive	3 [27]	10 [5]	0.02	3 [43]	10 [5]	<0.01	3 [25]	10 [5]	0.02
	Negative	8 [73]	205 [95]		4 [57]	209 [95]		9 [75]	204 [95]	
MMR	dMMR	3 [27]	26 [12]	0.15	3 [43]	26 [12]	0.04	4 [33]	25 [12]	0.06
	pMMR	8 [73]	189 [88]		4 [57]	193 [88]		8 [67]	189 [88]	
HER2	Positive	2 [18]	22 [10]	0.33	0 [0]	24 [11]	1.00	0 [0]	24 [11]	0.62
	Negative	9 [82]	193 [90]		7 [100]	195 [89]		12 [100]	190 [89]	

dMMR, defective mismatch repair; EBV, Epstein-Barr virus; GEJ, esophagogastric junction; HER2, human epidermal growth factor 2; L, lower third; M, middle third; MMR, mismatch repair; PD-L1, programmed death ligand-1; pMMR, mismatch repair proficient; TPS, tumor proportion score; U, upper third.

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Table S8 Pairwise comparison of PD-L1 TPS between 22C3 pharmDx and 28-8 pharmDx assays

28-8 pharmDx	22C3 pharmDx			
	0	1+	2+	3+
0	213	6	0	0
1+	0	1	0	0
2+	0	0	0	0
3+	2	0	2	2

Concordant, 0.95; 22C3 higher, 0.02; 28-8 higher, 0.03. PD-L1, programmed death ligand-1; TPS, tumor proportion score.

Table S9 Pairwise comparison of PD-L1 TPS between 22C3 pharmDx and E1L3 assays

E1L3	22C3 pharmDx			
	0	1+	2+	3+
0	209	5	0	0
1+	1	1	0	0
2+	1	0	1	0
3+	4	1	1	2

Concordant, 0.94; 22C3 higher, 0.03; E1L3 higher, 0.02. PD-L1, programmed death ligand-1; TPS, tumor proportion score.

Table S10 Pairwise comparison of PD-L1 TPS between 28-8 pharmDx and E1L3 assays

E1L3	28-8 pharmDx			
	0	1+	2+	3+
0	214	2	1	2
1+	0	0	0	1
2+	0	0	0	0
3+	0	0	1	5

Concordant, 0.97; 28-8 higher, 0.00; E1L3 higher, 0.03. PD-L1, programmed death ligand-1; TPS, tumor proportion score.

Table S11 Overall survival according to PD-L1 expression in the cases of stage III or IV

Assays	Categories	Overall survival			
		Median OS (months)	HR	95% CI	P
22C3 pharmDx	CPS ≥1 (*vs.* <1)	NR *vs.* 48.6	0.51	0.23–1.11	0.091
	CPS ≥5 (*vs.* <5)	NR *vs.* 59.1	0.75	0.29–1.91	0.54
	CPS ≥10 (*vs.* <10)	NR *vs.* 55.9	0.38	0.09–1.57	0.18
28-8 pharmDx	CPS ≥1 (*vs.* <1)	NR *vs.* 48.6	0.49	0.21–1.10	0.08
	CPS ≥5 (*vs.* <5)	NR *vs.* 59.1	0.75	0.29–1.92	0.55
	CPS ≥10 (*vs.* <10)	NR *vs.* 55.9	0.39	0.09–1.61	0.19
E1L3	CPS ≥1 (*vs.* <1)	NR *vs.* 48.6	0.61	0.31–1.21	0.16
	CPS ≥5 (*vs.* <5)	NR *vs.* 55.9	0.54	0.21–1.40	0.21
	CPS ≥10 (*vs.* <10)	NR *vs.* 59.1	0.59	0.21–1.67	0.32

CI, confidence interval; CPS, combined positive score; HR, hazard ratio; NR, not reached; OS, overall survival.

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