J Gastric Cancer. 2025 Jan;25(1):177-191
https://doi.org/10.5230/jgc.2025.25.e4
pISSN 2093-582X·eISSN 2093-5641

Journal of Gastric Cancer

Review Article

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PD-L1 as a Biomarker in Gastric Cancer Immunotherapy

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OPEN ACCESS

Received: Oct 31, 2024
Accepted: Nov 29, 2024
Published online: Dec 23, 2024

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Funding
This study was supported by the Bio and Medical Technology Development Program of the National Research Foundation (NRF), funded by the Korean Government (MSIT) (Grant No. RS-2023-00222838 to S.A).

Conflict of Interest
No potential conflict of interest relevant to this article was reported.

ABSTRACT

Combining chemotherapy with immune checkpoint inhibitors (ICIs) that target the programmed death-1 (PD-1) protein has been shown to be a clinically effective first-line treatment for human epidermal growth factor receptor 2 (HER2)-negative and -positive advanced or metastatic gastric cancer (GC). Currently, PD-1 inhibitors combined with chemotherapy are the standard treatment for patients with HER2-negative/positive locally advanced or metastatic GC. Programmed death-ligand 1 (PD-L1) expression, as assessed using immunohistochemistry (IHC), is a crucial biomarker for predicting response to anti-PD-1/PD-L1 agents in various solid tumors, including GC. In GC, the PD-L1 IHC test serves as a companion or complementary diagnostic test for immunotherapy, and an accurate interpretation of PD-L1 status is essential for selecting patients who may benefit from immunotherapy. However, PD-L1 IHC testing presents several challenges that limit its reliability as a biomarker for immunotherapy. In this review, we provide an overview of the current practices of immunotherapy and PD-L1 testing in GC. In addition, we discuss the clinical challenges associated with PD-L1 testing and its future use as a biomarker for immunotherapy. Finally, we present prospective biomarkers currently under investigation as alternative predictors of immunotherapy response in GC.

Keywords: Gastric cancer; Immune checkpoint inhibitors; Immunohistochemistry

INTRODUCTION

Immunotherapy has significantly improved the efficacy of gastric cancer (GC) treatment. Immune checkpoint inhibitors (ICIs), particularly those targeting programmed death-1 (PD-1) or programmed death-ligand 1 (PD-L1), have shown long-term efficacy in a subset of patients with GC. Currently, PD-1 inhibitors combined with chemotherapy are the standard first-line treatment for both human epidermal growth factor 2 (HER2)-negative and -positive locally advanced or metastatic GC [1,2]. PD-L1 expression, assessed using immunohistochemistry (IHC), serves as a predictive biomarker for immunotherapy in several tumors, including GC, and functions as a companion or complementary diagnostic test for immunotherapy in patients with GC [3,4]. The combined positive score (CPS) is used to evaluate PD-L1 expression in GC and offers the advantage of a comprehensive assessment of

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Author Contributions

Conceptualization: A.S.; Data curation: C.Y., A.S.; Funding acquisition: A.S.; Supervision: A.S., K.K.M.; Writing - original draft: C.Y., S.A; Writing - review & editing: C.Y., A.S., K.K.M.

PD-L1 expression in both tumor and immune cells in a single reading [5]. However, several challenges remain regarding the use of PD-L1 as a biomarker in IHC.

In this review, we present the current practices of immunotherapy and the associated PD-L1 assays in patients with GC. We provide a detailed overview of the guidelines for interpreting PD-L1 IHC results and discuss the related clinicopathological factors. In addition, we discuss the clinical challenges associated with PD-L1 assays and outline future considerations for PD-L1 as a biomarker and an alternative prospective biomarker of immunotherapy responses in patients with GC.

CURRENT PRACTICE OF ANTI-PD1/PD-L1 AGENTS AS A FIRST LINE TREATMENT IN GC

Recent phase III clinical trials have shown the effectiveness of immunotherapy in combination with chemotherapy as first-line treatment for advanced or metastatic GC (**Table 1**). The efficacy of incorporating PD-1 antibodies as a first-line therapy for GC was first established in the CHECKMATE 649 trial [6]. Nivolumab (a PD-1 inhibitor) treatment combined with chemotherapy significantly improved overall survival (OS) and progression-free survival in patients with PD-L1 CPS ≥5, as assessed using the 28-8 pharmDx assay [6]. Additional results showed therapeutic effects in patients with PD-L1 CPS ≥1 and in all randomly assigned patients [6], leading to geographical variations in regulatory approvals and international guidelines regarding the addition of nivolumab to chemotherapy [2]. The U.S. Food and Drug Administration (FDA) approved the use of nivolumab without restrictions based on the PD-L1 CPS [2]. In contrast, the European Medicines Agency (EMA) restricted approval to patients with PD-L1 CPS ≥5 [2]. The Korean Ministry of Food and Drug Safety (MFDS) also approved nivolumab without PD-L1 CPS restriction; however, nivolumab reimbursement for GC treatment is limited to patients with PD-L1 CPS ≥ 5.

After the initial disappointing results of the KEYNOTE-062 trial, pembrolizumab (a PD-1 inhibitor) may become a new front-line treatment option for GC [7]. The KEYNOTE-859 trial demonstrated the efficacy of pembrolizumab in combination with chemotherapy as a first-line treatment for patients with locally advanced or metastatic HER2-negative GCs, irrespective of the PD-L1 results [8]. However, the treatment effects were enhanced in patients with CPS ≥1 or ≥10, as assessed using the 22C3 pharmDx assay [8]. The U.S. FDA approved pembrolizumab for HER2-negative GC without PD-L1 CPS restriction, whereas the EMA recommended pembrolizumab for the PD-L1 CPS ≥1 population [9,10]. The Korean MFDS approved pembrolizumab without PD-L1 restrictions. For HER2-positive

Table 1. PD-L1 assays in GC for first line treatment setting*

Variables	CheckMate-649	KEYNOTE-811	KEYNOTE-859	RATIONALE-305	ORIENT-16
Candidates	HER2-negative GC	HER2-positive GC	HER2-negative GC	HER2-negative GC	HER2-negative GC
Drug	Nivolumab	Pembrolizumab	Pembrolizumab	Tislelizumab	Sintilimab
PD-L1 assay	28-8 pharmDx	22C3 pharmDx	22C3 pharmDx	SP263	22C3 pharmDx
Antibody supplier	Dako (Agilent)	Dako (Agilent)	Dako (Agilent)	Ventana (Roche)	Dako (Agilent)
Scoring	CPS	CPS	CPS	TAP	CPS
Cutoff	5	1	PD-L1 not regarded	5%	PD-L1 not regarded
US Food and Drug Administration	Approved (Apr 16, 2021)	Approved (Aug 29, 2023)	Approved (Nov 16, 2023)	Not yet	Not yet
Korean Ministry of Food and Drug	Approved (Sep 1, 2023)	Approved (Dec 19, 2023)	Approved (Mar 6, 2024)	Not yet	Not yet

PD-L1 = programmed death-ligand 1; GC = gastric cancer; HER2 = human epidermal growth factor receptor 2; CPS = combined positive score; TAP = tumor area positivity.
*Modified from [5].

https://doi.org/10.5230/jgc.2025.25.e4

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GCs, the KEYNOTE-811 study indicated that pembrolizumab in combination with first-line trastuzumab and chemotherapy significantly improved progression-free survival in patients with PD-L1 CPS ≥1 [11]. Currently, the U.S. FDA, EMA, and Korean MFDS approved pembrolizumab with trastuzumab and chemotherapy as a first-line treatment for HER2-positive GC with PD-L1 CPS ≥1, as assessed using the 22C3 pharmDx assay [10].

In China, tislelizumab (a PD-1 inhibitor) in combination with chemotherapy was approved for patients with locally advanced/metastatic HER2-negative GC with PD-L1 tumor area positivity (TAP) ≥5%, as assessed using the SP263 assay based on the RATIONALE 305 trial [12]. In addition, sintilimab (a PD-1 inhibitor) in combination with chemotherapy was approved for patients with locally advanced or metastatic HER2-negative GC, irrespective of PD-L1 status, based on the ORIENT-16 trial [13]. The treatment effect was more pronounced in patients with CPS ≥5, as assessed using the 22C3 pharmDx assay [13]. However, these agents have not been approved outside China.

CURRENT PRACTICE OF PD-L1 TESTING IN GC

To guide treatment plans, PD-L1 testing is considered in patients with locally advanced, recurrent, or metastatic GC who are candidates for PD-1 inhibitor therapy [3,4,9] A companion or complementary diagnostic test should be performed on formalin-fixed paraffin-embedded tissues [9]. Currently, three standardized PD-L1 IHC assays (22C3 pharmDx, 28–8 pharmDx, and SP263) are used to specifically predict responses to pembrolizumab, nivolumab, and tislelizumab [5]. For adequate evaluation, a minimum of 100 tumor cells must be present on PD-L1-stained slides [3,4]. Accurate assessment of the PD-L1 CPS is crucial for reporting the exact CPS score or specifying clinically meaningful intervals (that is CPS <1; 1–4; 5–9; ≥10), which helps in selecting the best therapeutic option for patients based on their specific needs [3,4,14]. The report should also specify the type of assay performed [3,4]. In addition, it is recommended that information regarding the control tissue and sample adequacy should also be included.

Currently, two approaches are used for evaluating PD-L1 expression: CPS and TAP [15]. The 22C3 pharmDx and 28-8 pharmDx assays used the CPS scoring system, which is calculated using the following equation [16] (**Fig. 1**):

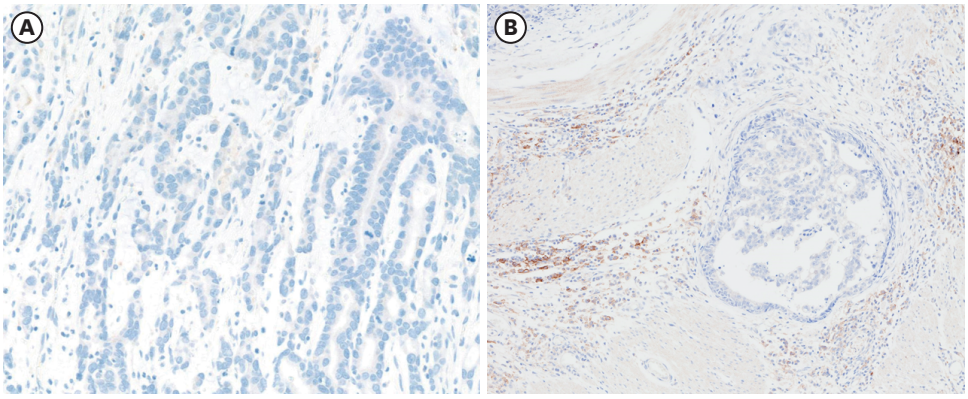

Fig. 1. Representative images of the programmed death-ligand 1 28-8 pharmDx assay. (A) CPS 0 and (B) CPS 5 (A and B: 10× magnification).
CPS = combined positive score.

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$$CPS = \frac{Number\ of\ Positive\ Tumor\ and\ Immune\ Cells}{Number\ of\ Viable\ Tumor\ Cells} \times 100$$

For tumor cells, convincing partial linear or complete membrane staining is considered PD-L1 positive, irrespective of staining intensity [16]. Tumor cells exhibiting only cytoplasmic or membranous staining of the apical surfaces within the glands are not considered positive [17]. For immune cells, membrane and/or cytoplasmic staining of lymphocytes and macrophages within tumor nests and adjacent stroma are counted irrespective of the staining intensity [16]. In some instances, macrophages within the gland lumen are highly positive with no staining in tumor cells [17]. However, this is not generally considered positive result [17]. For the CPS, a 20× field-of-view rule is applied to define tumor-associated areas [16]. Only immune cells within the 20× magnification field and areas directly related to the tumor response are scored [16]. Notably, other stromal cells such as fibroblasts, neutrophils, plasma, and necrotic cells are excluded [16]. If the calculation result exceeds 100, it is presented as a maximum score of 100 [3]. If PD-L1 staining is heterogeneous, the final CPS is estimated by calculating the CPS results for each area within the entire tumor [16]. This counting method for CPS is challenging and time-consuming [18]. The responses recorded in a recent PD-L1 quality assessment survey conducted by the College of American Pathologists (2021B) indicated that only <3% of pathologists attempt to count each cell and calculate a score [18,19], preferring visual evaluation.

Therefore, TAP assessment through visual evaluation has been suggested [15]. In the RATIONALE 305 trial for tislelizumab, TAP using the SP263 assay was used instead of CPS [12]. TAP is a simple visual method for scoring tumors and immune cells together [15]. It uses the percentage of PD-L1 expression in tumors and immune cells, evaluated as the proportion of the tumor area occupied by all viable tumor cells and the tumor-associated stroma containing tumor-associated immune cells [15]. The following equation applies:

$$TAP = \frac{\%PD-L1\ Positive\ Tumor\ And\ Immune\ Cells}{Tumor\ Area}$$

The method of counting only membranous staining for tumor cells, as well as membranous and/or cytoplasmic staining for immune cells, is the same as that used in CPS [15]. However, the TAP method involves all types of immune cells, including neutrophils, which reduces the need to confirm the cell types at high magnification [15]. Another distinction from the CPS is the application of a 10× rule to define the tumor area [15].

Liu et al. [15] compared TAP and CPS in GC and esophageal squamous cell carcinoma (ESCC) samples using the SP263 assay to assess concordance and time efficacy. The agreement between TAP and CPS was ≥85%, with a TAP score at 5% cutoff showing improved concordance with CPS 1 compared to a TAP score at 1% cutoff [15]. These findings suggested that the TAP and CPS can potentially be used to identify the same patient population [15]. In addition, the average scoring time for TAP was 5 min compared to 30 min for CPS, suggesting that TAP is less time-consuming [15]. The agreement rate for TAP among pathologists was also high [15]. Although TAP and CPS appear to be largely similar, further studies are required to validate their use of TAP in GC.

https://doi.org/10.5230/jgc.2025.25.e4

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CLINICOPATHOLOGICAL FACTORS ASSOCIATED WITH PD-L1

The positivity of PD-L1 in GC varies with different assays, specimen types, and other factors. In recent clinical trials, PD-L1 positivity at a cutoff of CPS ≥1 was reported to be >70% [6,8,11]. In the CHECKMATE 649 trial for HER2-negative GC, the positivity for CPS ≥1 was 82.0% (1,296/1,581), whereas the positivity for CPS ≥5 was 60.4% (955/1,581) [6]. In the KEYNOTE 859 trial for HER2-negative GC, the positivity for CPS ≥1 was 78.2% (1,235/1,579), and the positivity for CPS ≥10 was 34.9% (551/1,579) [8]. In the KEYNOTE 811 trial for HER2-positive GC, the positivity rate at a CPS cut-off of 1 was 85.1% (594/698) [11].

Several studies reported that PD-L1 positivity was associated with Epstein-Barr virus (EBV) positivity and microsatellite instability (MSI)-high status [20,21]. In particular, high PD-L1 (≥ CPS 10 or 50) is frequently observed in EBV-positive and MSI-high GCs (**Fig. 2**). One meta-analysis indicated that PD-L1 expression showed no correlation with sex, age, cancer location, differentiation, or tumor stage [22].

Another notable clinical issue is the overlap of PD-L1 expression with that of other biomarkers, which is crucial for optimal treatment planning [23]. Notably, zolbetuximab, an anti-Claudin 18.2 monoclonal antibody, has recently been approved as a first-line treatment for GC [24,25] and is expected to soon be integrated into routine practice. Kubota et al. [26] reported a numerically lower rate of PD-L1 CPS ≥5 among Claudin 18.2-positive patients, although this finding was not statistically significant. In contrast, Kwak et al. [27] recently reported that Claudin 18.2 positivity was higher in patients with GC with PD-L1 CPS ≥5. Future studies correlating these findings with treatment outcomes are required.

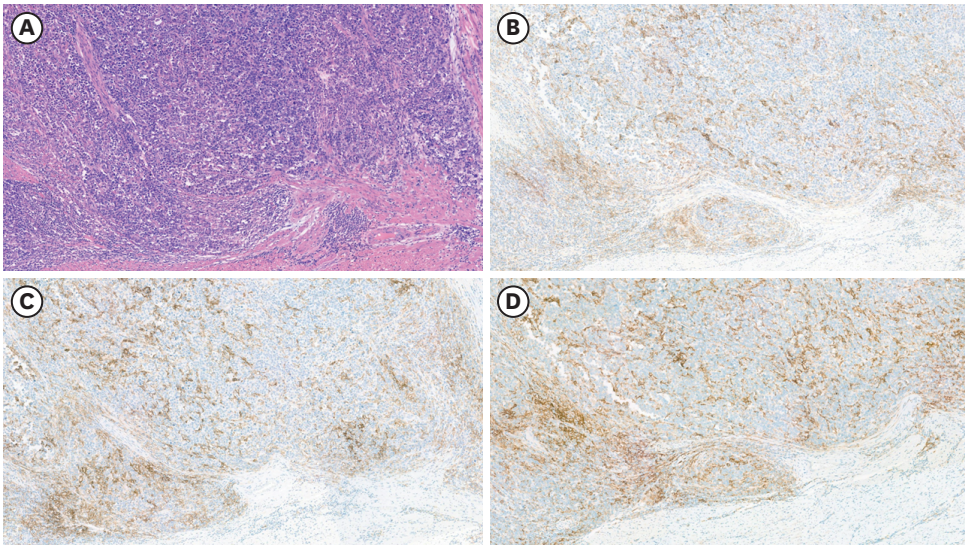

Fig. 2. Representative images of high PD-L1 CPS in microsatellite instability-high gastric cancer (CPS 30, whole area). (A) Tumor histology showing dense infiltration of immune cells (hematoxylin and eosin staining). (B) PD-L1 28-8 pharmDx, (C) PD-L1 22C3 pharmDx, and (D) PD-L1 SP263. All three assays show comparable PD-L1 staining patterns (A, B, C, and D: 4× magnification).
PD-L1 = programmed death-ligand 1; CPS = combined positive score.

https://doi.org/10.5230/jgc.2025.25.e4

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CLINICAL ISSUES ASSOCIATED WITH PD-L1 ASSAYS

CPS cutoff

The CPS cutoff value for predicting patients who will benefit the most from immunotherapy remains a topic of debate. These cutoff values are subject to change as results from new clinical trials and studies become available. Moreover, the CPS cutoff values for the approval of immunotherapy vary by country or approval agency [2]. However, the benefits of additional immunotherapy in populations with low PD-L1 expression require further investigation [28].

Intra-tumoral heterogeneity

Heterogeneous PD-L1 expression across areas within a tumor is an inherent issue that can influence its role as a predictive biomarker [29]. Heterogeneity of PD-L1 expression within and between tumor sites has been described in other solid tumors [30-34]. Colarossi et al. [35] reported a change in PD-L1 status in only one out of 53 cases of GC, suggesting consistency in PD-L1 tumor expression between primary and metastatic tumors. However, Gao et al. [36] observed that PD-L1 positivity was significantly higher in metastatic lymph nodes (45.4%) than in primary gastric tumors (38.7%). Zhou et al. [29] reported marked spatial heterogeneity between primary gastric and metastatic tumors (61% concordance). In the same study, temporal heterogeneity in PD-L1 expression was noted between tumors before and after chemotherapy (63% concordance) [29]. Elevated PD-L1 expression after neoadjuvant chemotherapy has been reported in various solid tumors, including GC [37-39]. The mechanism underlying the role of chemotherapy in the variation of PD-L1 expression in GC has not been fully elucidated [37].

In addition, the question of whether a small biopsy is representative of the PD-L1 status of the entire tumor remains to be addressed [40]. Ye et al. [40] reported that PD-L1 expression in tissue microarray samples had varying degrees of relevance to the corresponding surgical specimens, suggesting that at least five biopsies are required to accurately assess PD-L1 status.

Inter-observer concordance

Inter-observer variability among pathologists in PD-L1 assessment poses a challenge when using PD-L1 as a biomarker in GC. **Table 2** summarizes the studies that evaluated the inter-observer concordance of PD-L1 evaluation in GC. Previous studies have reported excellent interobserver agreement with overall percentage agreements (OPAs) >95% among pathologists [41,42]. Recently, Kim et al. [44] evaluated the inter-observer variability of the CPS in 143 clinical GC samples. Inter-observer variability, as represented by the intra-class correlation coefficient (ICC), was 0.89 and 0.88 for the 28-8 pharmDx and 22C3 antibody concentrates, respectively.

In contrast, two recent studies reported high interobserver variability among pathologists for CPS in cases of GC [18,45]. Fernandez et al. [18] evaluated the concordance of PD-L1 CPS between 14 pathologists using 112 biopsy samples stained using the 22C3 pharmDx assay [18]. At a CPS cutoff of 1, the OPA reached only 31.48%, and the ICC was 0.484 [5,18]. Higher cutoffs performed better than a CPS cutoff of 1 [5,18]. Robert et al. [45] evaluated the interobserver agreement of 12 pathologists using 100 biopsies stained with PD-L1 28-8 and 22C3 pharmDx assays [5]. Inter-observer agreement for CPS for 100 biopsies was poor, with only fair agreement for both pre- (ICC range, 0.45–0.55) and post-training (ICC range, 0.56–0.57) for both assays [5,45]. Next, they evaluated the inter-observer agreement for

https://doi.org/10.5230/jgc.2025.25.e4

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Table 2. Summary of studies evaluating inter-observer concordance of PD-L1 assays in gastric cancer*

Reference	PD-L1 assay	Cut-off	Number of observers	Number of cases	Sample type	Inter-observer agreement	Fleiss kappa value
Nuti et al. [41]	22C3 pharmDx	CPS≥1	120	20 (day 1), 25 (day 2)	Resection	OPA 90.6%	0.828
Kulangara et al. [42]	22C3 pharmDx	CPS≥1	3	68	Not mentioned	OPA 96.6%	
Park et al. [43]	22C3 pharmDx	CPS value	5	55	Tissue microarray	ICC 0.387 (lower 95% CI 20.9%)	CPS≥1 0.389
	Sp263	CPS value	5	55	Tissue microarray	ICC 0.349 (lower 95% CI 13.5%)	CPS≥1 0.256
Fernandez et al. [18]	22C3 pharmDx	CPS≥1	14	112	Biopsy	OPA 31.48% (95% CI, 22.72–40.24) ICC 0.484 (95% CI, 0.403–0.571)	0.477
		CPS≥10	14	112	Biopsy	OPA 67.59% (95% CI, 58.77–76.42) ICC 0.604 (95% CI, 0.584–0.624)	0.607
		CPS≥20	14	112	Biopsy	OPA 83.33% (95% CI, 76.3–90.36) ICC 0.629 (95% CI, 0.562–0.698)	0.626
Robert et al. [45]	28-8 pharmDx	CPS value	12	100	Biopsy	ICC 0.45 (95% CI, 0.38–0.53)	
	22C3 pharmDx	CPS value	12	100	Biopsy	ICC 0.55 (95% CI, 0.47–0.63)	
Kim et al. [44]	28-8 pharmDx	CPS value	3	143	Biopsy and Resection	ICC 0.89 (95% CI, 0.89–0.92)	
	22C3 concentrate	CPS value	3	143	Biopsy and Resection	ICC 0.88 (95% CI, 0.85–0.91)	

PD-L1 = programmed death-ligand 1; CPS = combined positive score; OPA = overall percentage agreement; ICC = intraclass correlation coefficient; CI = confidence interval.
*Modified from [5].

the elements comprising the CPS in 35 biopsy fragments [5,45]. Poor or fair agreements were observed for the number of PD-L1-positive immune cells (ICC, 0.19), PD-L1-positive tumor cells (ICC, 0.54), total number of viable tumor cells (ICC, 0.09), calculated CPS (ICC, 0.14), and calculated tumor cell score showed excellent agreement (ICC, 0.82) [5,45]. This aligns with the results from other tumors, indicating that the interobserver concordance in immune cells is significantly lower than that in tumor cells [5,45]. This high interobserver variability raises questions about PD-L1 CPS as a biomarker for GC [5]. However, because of the increasing reliance on specific PD-L1 CPS cut-offs for clinical indications, efforts such as education and standardized guidelines are required to address this issue [5].

Inter-assay concordance

Discordant results from different PD-L1 assays pose challenges in the assessment of PD-L1 expression. Additionally, not all assays are available in all areas [37]. Notably, limited samples such as endoscopic or peritoneal biopsies are available for certain patients with GC [37]. Therefore, there is an increasing need to harmonize PD-L1 assays for GC [37]. **Table 3** summarizes comparative studies on PD-L1 assays in patients with GC. Ahn and Kim [37] reported that 22C3 and 28-8 pharmDx were highly comparable at various CPS cutoffs. The OPA was 96.4% at the CPS 1 cutoff, with higher OPA at the CPS 10 and 50 cutoffs [37]. However, nonspecific staining is often observed in the 28–8 pharmDx assay, which requires caution in interpretation [37]. Park et al. [43] reported high concordance between the 22C3 pharmDx and SP263 assays for CPS evaluation, with OPA >90% at various CPS cutoffs. In a recent study, Klempner et al. [46] compared the 22C3, 28-8 pharmDx, and SP263 assays using both CPS and TAP scoring systems. Moderate to strong ICCs (≥0.70) in pairwise assay comparisons between the scoring algorithms were reported, thereby highlighting analytical concordance in the three major PD-L1 assays when TAP and CPS are used.

However, whether inter-assay results can be compared between the 22C3 and 28-8 assays is still unclear because discordant results have also been reported [44,47]. In a recent study, Kim et al. [44] reported suboptimal agreement between the 28–8 pharmDx and 22C3 antibody

https://doi.org/10.5230/jgc.2025.25.e4

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Table 3. Summary of studies evaluating PD-L1 inter-assay concordance in gastric cancer

Reference	Assay	Scoring system	Number of cases	Specimen type	Result
Ahn and Kim [37]	28-8 pharmDx and 22C3 pharmDx	CPS	55	Surgical resection (n=49) and biopsy (n=6)	Cohen's kappa value=0.927 (for CPS ≥1) Cohen's kappa value=0.899 (for CPS ≥10) Cohen's kappa value=1.000 (for CPS ≥50)
Park et al. [43]	22C3 pharmDx and SP263	CPS, TPS	379	Tissue microarray	Spearman correlation coefficient 0.943 for TPS Spearman correlation coefficient 0.914 for CPS
Klempner et al. [46]	28-8 pharmDx, 22C3 pharmDx and SP263	CPS, TAP	100	Surgical resection	22C3 vs. 28-8: ICC 0.81 (95% CI, 0.73–0.87) SP263 vs. 22C3: ICC 0.87 (95% CI, 0.81–0.91) SP263 vs. 28-8: ICC 0.70 (95% CI, 0.58–0.79)
Kim et al. [44]	28-8 pharmDx and 22C3 concentrate	CPS	143	Surgical resection (n=25) and biopsy (n=118)	Cohen's kappa value=0.56 (for CPS ≥1) Cohen's kappa value=0.60 (for CPS ≥5) Cohen's kappa value=0.66 (for CPS ≥10)
Narita et al. [47]	28-8 pharmDx and 22C3 pharmDx	CPS	226	Tissue microarray	Cohen's kappa value=0.735 (for CPS ≥1) Cohen's kappa value=0.881 (for CPS ≥5) Cohen's kappa value=0.837 (for CPS ≥10)

PD-L1 = programmed death-ligand 1; CPS = combined positive score; TPS = tumor proportion score; TAP = tumor area positivity; ICC = intraclass correlation coefficient; CI = confidence interval.

concentrate (not pharmDx), with Cohen's kappa values and OPA between the two assays being 78.3% and 0.56 for the CPS 1 cutoff, 81.8% and 0.60 for the CPS 5 cutoff, and 88.8% and 0.66 for the CPS 10 cutoff. However, this variability should be resolved in future studies.

FUTURE CONSIDERATIONS AND OTHER BIOMARKERS

Novel immunotherapy and biomarkers

The phase II EDGE-Gastric trial evaluated several novel immunotherapy-based regimens in GC patients, some of which target both PD-1 and T-cell immunoreceptors with immunoglobulin and ITIM domains (TIGIT) [48]. Preliminary data showed that treatment-naïve patients who received both zimberelimab (a PD-1 inhibitor) and domvanalimab (a TIGIT inhibitor) in combination with chemotherapy, showed a promising outcome in all patients, especially those with TAP ≥5% using the SP263 assay. A phase III trial comparing combination therapy with zimberelimab and dombanalimab, and chemotherapy with nivolumab is ongoing.

DKN-01 exhibits immunomodulatory activity, stimulates a pro-inflammatory tumor microenvironment (TME), and upregulates PD-L1 levels. A phase II study (DisTinGuish) of DKN-01 in combination with tislelizumab and chemotherapy as the first-line therapy demonstrated a prolongation of progression-free survival and OS, especially in patients with low PD-L1 expression [49]. For this study, DKK1 was assessed by central laboratories using RNA scope and PD-L1 expression.

Image analysis-assisted PD-L1 interpretation

Among the issues in incorporating PD-L1 expression as a biomarker for immunotherapy, major methodological difficulties include the interpretation of PD-L1 expression. Efforts have been made to overcome these difficulties; the application of computer image analysis algorithms is one such endeavor. Several previous studies have identified image analysis algorithms as potential tools for improving the accuracy and reproducibility of PD-L1 scoring by pathologists for other solid tumors [50,51]. Kim et al. [52] generated PD-L1 CPS scores for 39 cases of GC using the Aperio IHC membrane image analysis algorithm (ScanScopeTM; Aperio Technologies, Vista, CA, USA) with additional input from manual annotation and

https://doi.org/10.5230/jgc.2025.25.e4

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computation, showing that PD-L1 CPS scores supported by image analysis were concordant with manual scoring performed by pathologists. Notably, PD-L1 scores derived from image analysis were comparable to manual scoring in predicting patient responses to pembrolizumab [52]. In a recent study, an artificial intelligence (AI)-aided PD-L1 image analysis algorithm demonstrated clinical efficacy as a diagnostic aid for other tumors such as lung cancer [53,54]. However, AI-aided PD-L1 assessment has not yet been attempted for GC; however, developments in this area are expected [55].

Other predictive biomarkers for immunotherapy in GC

Another potential limitation of PD-L1 IHC is the controversy regarding its efficacy as an accurate biomarker of immunotherapy. Several other biomarkers predicting immunotherapy outcomes have also been identified.

MSI-high or defective mismatch repair (dMMR) (MSI-H/dMMR) has emerged as a significant biomarker for predicting response to immunotherapy in various types of solid tumors [56]. Several clinical studies have examined the response of patients with MSI-H/dMMR GC to immunotherapy [57,58]. Among patients with MSI-high, 57.1% experienced an objective response, whereas only 9.0% of patients with non-MSI-high samples achieved an objective response [57]. In a meta-analysis of clinical trials, including the KEYNOTE-062, CheckMate-649, JAVELIN Gastric 100, and KEYNOTE-061 trials, the hazard ratios for OS benefit with anti-PD-1-based treatment was 0.34 for patients with MSI-H GC, compared to 0.85 for patients with non-MSI-H GC [58].

EBV-associated GC accounts for approximately 10% of all cases of GC [59]. This subtype typically exhibits distinct histological features and is characterized by significant immune cell infiltration within the tumor. Several studies have reported a positive correlation between EBV-associated GC and immunotherapy. Kim et al. [60] reported that all six patients with EBV-associated GC treated with pembrolizumab achieved an objective response. In another study, among nine patients with EBV-positive GC treated with various ICIs, including nivolumab, three showed a partial response and five had stable disease [61]. Notably, all seven patients who exhibited an objective response also exhibited positive PD-L1 expression [61]. However, Wang et al. [62] reported that only one of four patients with EBV-associated GC treated with toripalimab (a PD-1 inhibitor) achieved partial remission, whereas the remaining patients had two cases of stable disease and one case of disease progression [62]. Another study examined the clinical response of patients with EBV-associated GC treated with camrelizumab (a PD-1 inhibitor); however, none of the patients showed an objective response [63]. The predictive value of EBV positivity for response to immunotherapy remains uncertain, and further investigation using a larger sample size is required.

Tumor mutation burden (TMB) is another biomarker that has been investigated. Tumors with high TMB (TMB-H) levels are believed to possess a higher number of neoantigens, thereby increasing the likelihood of detection by the immune system [64,65]. As part of the KEYNOTE-062 study, the clinical effectiveness of pembrolizumab combined with chemotherapy as the first-line treatment of advanced GC was investigated [66]. Improved clinical outcomes were observed in patients treated with pembrolizumab (either as monotherapy or in conjunction with chemotherapy) for GC and TMB >10 [66]. In a phase Ib/II clinical trial that evaluated toripalimab (a PD-1 inhibitor) response in GC, the TMB-H group (defined as TMB >12 mutations/Mb) showed significantly better OS than those with low TMB (14.6 vs. 4.0 months, hazard ratio=0.48) [62]. In another clinical trial that tested lenvatinib

https://doi.org/10.5230/jgc.2025.25.e4

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in combination with pembrolizumab in patients with GC, either in the first- or second-line therapeutic settings, patients with TMB-H (TMB >10) showed an 82% objective response rate, whereas those with low TMB showed a 60% objective response rate.

The TME directly influences immunotherapy effectiveness. Several studies have focused on tumor-infiltrating lymphocytes (TILs) as predictive biomarkers of ICI response. Tong et al. [67] hypothesized that intratumoral $CD8^+$ TILs may be a positive predictive factor for clinical response to immunotherapy in PD-L1-negative advanced GC. Boku et al. [68] examined the TILs of 91 patients with advanced GC and suggested that patients with a high proportion of CTLA-4 and $LAG3^+$ myeloid cells before nivolumab treatment had a poor prognosis. Other studies have focused on tertiary lymphoid structures, which are another component of TME. Tertiary lymphoid structures have also been reported to correlate with positive immunotherapy responses in various cancer types [69]. In patients with GC, the tertiary lymphoid structure score, calculated using tertiary lymphoid structure-related genes through principal component analysis, correlated with a superior response to PD-1 blockade therapy [70]. CD73, a novel immune checkpoint protein, has also been proposed as a potential immunotherapy biomarker, and its overexpression in GC indicates better chemotherapeutic responsiveness to fluorouracil but a poorer objective response rate to pembrolizumab [71]. However, likely because of the complex nature of the TME, a single biomarker may not be adequate to identify patients with GC who will most benefit from immunotherapy. Chen et al. [72] investigated the density and spatial patterns of immune cells and determined that the density of $CD4^+FoxP3^-PD\text{-}L1^+$ T cells and the effective score of $CD8^+PD\text{-}1^+LAG\text{-}3^-$ T cells were closely associated with a positive response to anti-PD-1/PD-L1 therapy; however, $CD8^+PD\text{-}1^-LAG\text{-}3^-$ T cells and $CD68^+STING^+$ macrophages were closely associated with a negative response. This result highlights the need for a multidimensional approach to the TME analysis.

Several studies have shown that gut microbiota may be associated with tumor progression and can potentially impact the efficacy of ICIs [73,74]. In the DELIVER study investigating whether gut microbiomes serve as predictors of the efficacy of nivolumab in GC, upregulation of the bacterial invasive epithelial cell pathway was associated with disease progression after nivolumab treatment [75]. Moreover, certain bacterial species, such as those of the *Odoribacter* and *Veillonella* genera, have been associated with the nivolumab response [75].

Helicobacter pylori is a well-known infectious microorganism that is closely associated with the development of GC. In a previous study, *H. pylori* seropositivity was associated with poorer survival outcomes in patients with non-small cell lung cancer undergoing anti-PD-1 therapy [76]. A retrospective study on patients with GC showed that patients in the *H. pylori*-positive group had a higher risk of a poor response to anti-PD-1 antibodies than those in the *H. pylori*-negative group [77]. Further research is required to explore the role of *H. pylori* regarding its effect on in GC immunotherapy.

Among the genomic mutations, *POLE/POLD1* mutation is a promising marker for ICI treatment [78-80]. Based on the findings of a study that examined mutational data from 47,221 malignant tumors of various origins, including 185 esophagogastric cancers, *POLE/POLD1* mutations were associated with longer OS in patients treated with ICIs [81].

https://doi.org/10.5230/jgc.2025.25.e4

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CONCLUSION

In recent years, the combination of anti-PD-1 agents and chemotherapy has been shown to be clinically effective as a first-line treatment for advanced and metastatic GC, thereby establishing it as standard therapy. Currently, the selection of candidates who will most likely benefit from this treatment relies on the PD-L1 expression level, as determined through IHC testing. However, the PD-L1 test has limitations, and efforts are underway to minimize its negative effects. In addition, novel biomarkers are being investigated as alternative methods for predicting the efficacy of immunotherapy. Future studies are required to refine the prediction of biomarkers for immunotherapy in patients with GC.

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