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Clinical Significance of Tumor and Immune Cell PD-L1 Expression in Gastric Adenocarcinoma

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Abstract. *Background/Aim: The prognostic relevance of programmed cell death ligand-1 (PD-L1) protein expression in gastric cancer (GC) remains controversial. The aims of the present study were to determine the correlations between tumor cell (TC) and immune cell (IC) PD-L1 protein levels with prognosis, and to determine the correlation between PD-L1 expression and different molecular GC subtypes. Materials and Methods: TC and IC PD-L1 protein levels were measured in 286 GC patients. The patients were classified according to the Cancer Genome Atlas and Asian Cancer Research Group guidelines using immunohistochemistry and in situ hybridization. Results: TC and IC PD-L1 protein levels were positively correlated with patient survival. TC PD-L1 expression was negatively correlated with tumor grade. TC and IC PD-L1 expression was associated with improved prognosis in Epstein-Barr virus negative (EBV$^-$), microsatellite instability (MSI) rather than microsatellite stability (MSS) subgroup GC patients. Conclusion: PD-L1 protein expression in TCs and ICs can be used as a prognostic indicator for GC patients, particularly in the EBV$^-$, MSI, and MSS subgroups.*

Gastric cancer (GC) is the fifth most common cancer and the third leading cause of cancer-related death globally (1). The standard of care for advanced GC is surgical resection combined with chemo- or radiotherapy (2). Recurrence and/or metastasis commonly occur even following successful gastrectomy, and the 5-year survival rate for GC patients with metastasis is approximately 20% (3). Recently, the concept of immunotherapy has emerged; immunotherapy treats patients by acting on their immune system and boosting the immune response to identify and destroy cancer cells (4). Still, a limited number of chemotherapeutic agents have been used for GC patients, and immunotherapy can be an alternative to classic chemotherapy (5).

Cancer cells produce programmed cell death ligand-1 (PD-L1) to bind T-cells, inhibiting their activation (6). Blocking PD-L1 or PD-1 binding is the primary target of immunotherapy, which prevents cancer cells from evading the immune system (7). Anti-PD-L1 therapy was first introduced as a cancer treatment, subsequently, many clinical trials have been conducted to assess the efficacy of PD-L1 and PD-1 inhibitors in the treatment of multiple types of solid tumors (8). Clinical trials have demonstrated the therapeutic efficacy of anti-PD-L1 monoclonal antibody in GC, and this modality has been proposed as a treatment option (9). However, the treatment was only effective in a limited number of patients, particularly those with microsatellite instability (MSI) rather than microsatellite stability (MSS) (10, 11).

The Cancer Genome Atlas (TCGA) and Asian Cancer Research Group (ACRG) studies identified distinct molecular GC subtypes. GC is classified based on the integration of 1) microsatellite status, 2) Epstein-Barr Virus (EBV) infection, 3) epithelial-to-mesenchymal transition (EMT) status, and 4) tumor protein 53 (*TP53*) expression, which are known to affect clinical outcomes of GC (12, 13). These findings provided a new subgroup classification system for GC to aid in the prediction of GC prognosis and the development of biomarkers for targeted therapy. Determining the relationship between PD-L1 expression and these newly identified molecular subgroups can

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determine which subgroups are most likely to respond to immunotherapy.

However, the prognostic relevance of PD-L1 protein expression in GC remains controversial, and prior studies have shown PD-L1 to have a promotive or suppressive role in GC (14). In the present study, we investigated the correlations between tumor cell (TC) and immune cell (IC) PD-L1 protein expression, and clinical/pathological variables and survival time in GC patients and in different GC subtypes. Measuring IC and TC PD-L1 in GC patients can be used to evaluate the potential role of PD-L1 in GC, and to classify patients with PD-L1 expression and molecular GC subtypes to predict patient prognosis.

Materials and Methods

Patients and tissue samples. This study was performed using 286 cases of gastric adenocarcinoma who underwent surgical resection at Chungnam National University Hospital (Daejeon, Republic of Korea) from January 2010 to December 2012. Patient characteristics and survival times were collected from medical records, and follow-up periods lasted up to 71 months. Patients who received preoperative chemo- or radiotherapy were excluded. Cancer stage was determined according to the American Joint Committee on Cancer TNM criteria in the Cancer Staging System, Eighth Edition (15). Hematoxylin and eosin-stained slides were reviewed by two experienced pathologists (G.E.B. and M.K.Y.), and the most representative (tumor and immune cell rich) areas were selected. To generate a tissue microarray, tissue columns (3.0 mm) were punched from original paraffin blocks and inserted into new recipient paraffin blocks. All specimens were provided by the Biobank of Chungnam National University Hospital, a member of the Korea Biobank Network. The study was approved by the Institutional Review Board of Chungnam National University Hospital (CNUHIRB No. 2020-01-073). The study was retrospective, and a waiver of consent was approved by the Institutional Review Board.

Immunohistochemical staining analysis. Tissue sections (4 μm) were cut from the tissue microarray using a microtome and mounted onto coated slides, which were then transferred to Dako (Glostrup, Denmark) and Ventana (Tucson, AZ, USA) automated immunostainers. Staining was performed according to the manufacturer's protocol using anti-PD-L1 (Ready-to-Use (RTU), clone SP263; Ventana, Tucson, AZ, USA), MLH1 (RTU, clone M1; Ventana), MSH2 (RTU, clone G219-1129; Cell Marque, Rocklin, CA, USA), MSH6 (RTU, clone 44; Ventana), PMS2 (RTU, clone EPR3947; Cell Marque), E-cadherin (M3612, 1:300; Dako), and p53 (M7001, 1:300; Dako) antibodies. *In situ* hybridization with EBV-encoded RNA was performed using Leica Biosystems (Newcastle Ltd., Newcastle Upon Tyne, UK) equipment. Formalin-fixed, paraffin-embedded PD-L1–positive NCI-H226 cell line were used as positive control for anti-PD-L1 (clone SP263) and human placental and tonsil tissues as negative controls. A normal gastric mucosa (MLH1, MSH2, MSH6, PMS2, E-cadherin, and P53) and a lymph node (EBV infected and non-infected) served as controls, and a primary antibody was omitted from the negative control.

TC PD-L1 (SP263) staining was scored with a 1% expression cut-off level. TC PD-L1 expression <1% was categorized as "negative" and ≥1% as "positive," with significant separation in survival curves between 1%, 5%, 10%, 20%, and 50% cut-offs. IC PD-L1 expression <5% was categorized as "negative" and ≥5% as "positive," with significant separation in survival curves between 1%, 5%, 10%, 20%, and 50% cut-offs. Microsatellite stability was determined based on mismatched repair protein (MMR; MLH1, MSH2, MSH6, and PMS2) expression, and GCs were categorized into MSI and MSS subtypes (16). Based on MMR protein expression and EBV *in situ* hybridization results, GC was divided into EBV$^+$, MSI, and MSS subtypes (17). p53 immunohistochemical staining patterns were divided into *TP53* mutant and *TP53* wild-type subtypes (18). E-cadherin status was evaluated based on loss of expression. Markedly decreased E-cadherin staining intensity was considered to be indicative of the EMT subtype (19, 20). Based on MMR, E-cadherin, and p53 staining, GC was divided into MSI, MSS/EMT, MSS/*TP53* mutant, and MSS/*TP53* wild-type subtypes (21).

Statistical analyses. Associations between TC and IC PD-L1 protein levels and clinic-pathological parameters of GC were examined using Spearman rank correlation coefficients and Mann-Whitney *U*-tests. For univariate analyses, overall and disease-free survival curves were generated using the Kaplan-Meier method with log-rank test. Multivariate survival analysis was performed using the Cox proportional hazard regression model. $p<0.05$ was considered statistically significant. All analyses were performed using SPSS 24.0 (SPSS Inc., Chicago, IL, USA).

Results

GC patient characteristics. IC and TC PD-L1 expression was measured in 286 GC samples. Patient age ranged from 21 to 86 years, with a mean age of 60.8 years and male/female ratio was 1.9:1. GCs were present in the upper (fundus and upper body) (n=57), mid (mid and lower body) (n=105), and lower (antrum and pylorus) (n=124) locations. GCs were divided into intestinal (n=176), diffuse (n=73), and mixed (n=37) types. GCs were diagnosed as tubular adenocarcinoma (n=176), poorly cohesive carcinoma including signet ring cell carcinoma (n=49), and other (n=37) based on WHO guidelines, 4th edition (22).

GCs were EBV$^+$, as demonstrated by *in situ* hybridization, in 17 (5.9%) patients. Sixty (21%) GCs were classified as MSI, and 226 (79%) were classified as MSS. One hundred and seven (37.4%) GCs showed a *TP53* mutant type and 179 (62.6%) showed *TP53* wild type. Ninety-seven (33.9%) were classified as EMT (marked decreased of E-cadherin) and 189 (66.1%) showed preserved E-cadherin expression. Finally, GCs were divided into MSI (n=60), MSS/*TP53* mutant (n=85), MSS/*TP53* wild-type (n=75), and MSS/EMT (n=65) subtypes according to ACRG classification.

Correlations between gastric adenocarcinoma PD-L1 expression and clinicopathologic variables. PD-L1 expression

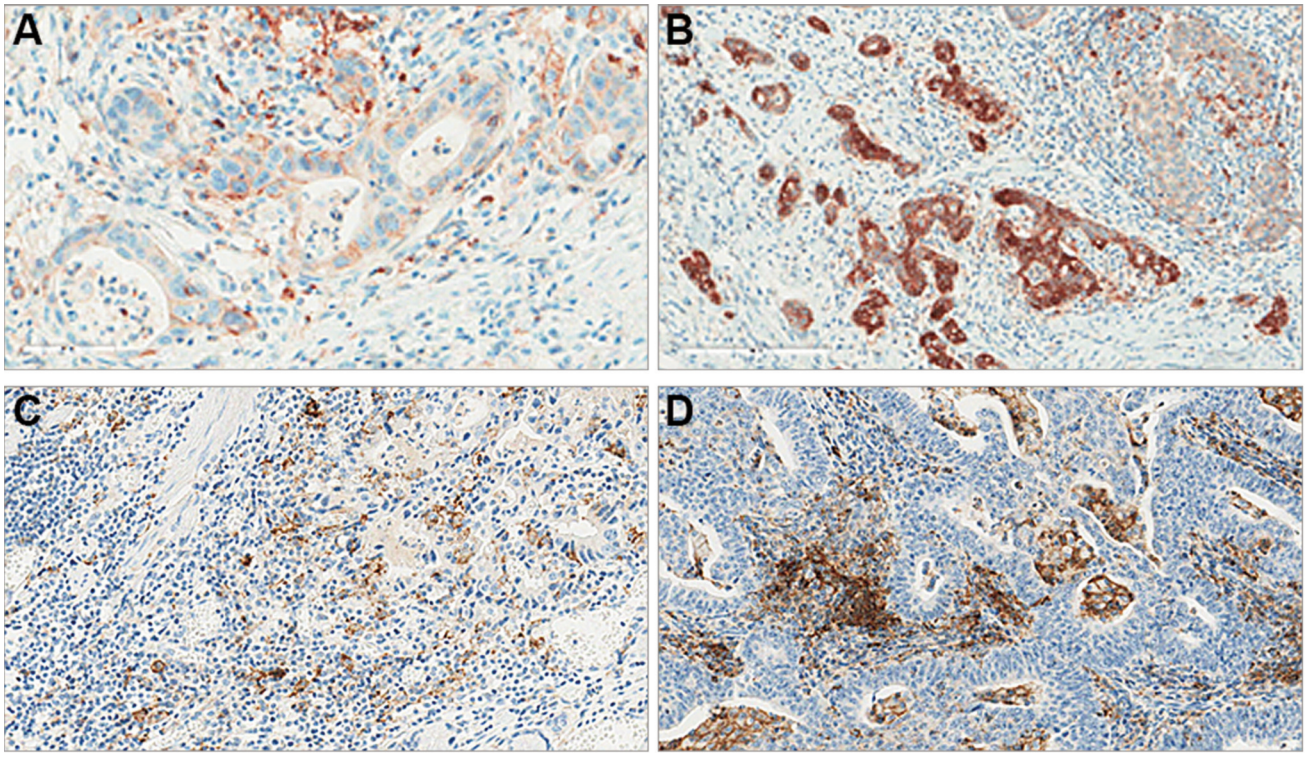

Figure 1. *Representative images of immunohistochemical staining for (A-B) tumor cell (TC) PD-L1 positivity, and (C-D) immune cell (IC) PD-L1 positivity.*

was observed in TCs and tumor-infiltrating ICs (Figure 1). The relationships between TCs and ICs with PD-L1-positive expression and selective clinicopathologic features were investigated (Table I). TC PD-L1 expression was negatively correlated with advanced GC, higher pathologic stage, lymph node metastasis, and perineural invasion ($p<0.0001$, $p=0.005$, $p=0.011$, and $p=0.011$, respectively). IC PD-L1 expression was negatively correlated with perineural invasion ($p=0.014$). IC PD-L1 expression was not significantly correlated with advanced GC, higher stage, or lymph node metastasis.

The relationship between PD-L1 expression and GC molecular subtype was investigated (Table II). IC PD-L1 expression was significantly higher in EBV^+ GCs, MSI GCs, and MSS/*TP53* wild-type GCs according to ACRG classification ($p<0.0001$ and $p=0.024$). TC PD-L1 expression was not significantly correlated with molecular classification.

Relationship between GC PD-L1 expression and patient prognosis. Both disease-free and overall survival analyses were performed in all GC patients (n=286). Kaplan-Meier survival curves and log-rank tests revealed significant positive correlations between TC PD-L1 expression and disease-free and overall survival rates ($p<0.0001$ and $p=0.009$, respectively), and between IC PD-L1 expression and disease-free and overall survival rates ($p=0.009$ and $p=0.039$, respectively) (Figure 2).

The prognostic relevance of TC and IC PD-L1 expression was assessed among molecular GC subtypes (Figure 3). In the EBV^- GC subgroup, TC PD-L1 expression was positively correlated with disease-free and overall survival rates ($p=0.001$ and $p=0.012$, respectively), and IC PD-L1 expression was positively correlated with disease-free survival ($p=0.010$). In the MSI GC subgroup, TC PD-L1 expression was positively correlated with disease-free survival ($p=0.036$), and IC PD-L1 expression was positively correlated with overall survival ($p=0.011$). In the MSS GC subgroup, TC PD-L1 expression was positively correlated with disease-free and overall survival ($p=0.003$ and $p=0.017$), and IC PD-L1 expression was positively correlated with disease-free survival ($p=0.031$). In the ACRG subgroup, TC and IC PD-L1 expression was positively correlated with overall survival time, but this relationship did not reach statistical significance (Figure 4).

Multivariate analyses using the Cox proportional hazard model for disease-free survival were performed in all GC patients (n=286) to determine the correlation between TC and IC PD-L1 expression and advanced stage GC (EGC *vs*. AGC), lymph node metastasis, distant metastasis, and younger age at onset (under 61 *vs*. over 61). TC PD-L1

Table I. *PD-L1 expression relative to clinicopathologic characteristics.*

Characteristics	Patients No. (%)	Tumor cell PD-L1			Immune cell PD-L1		
		Negative	Positive	*p*-Value	Negative	Positive	*p*-Value
Gender				0.295			0.755
Male	189 (66.1)	123 (68.3)	66 (62.3)		145 (65.6)	44 (67.7)	
Female	97 (33.9)	57 (31.7)	40 (37.7)		76 (34.4)	21 (32.3)	
Age				0.791			0.996
≤61	132 (46.2)	82 (45.6)	50 (47.2)		105 (47.5)	27 (41.5)	
>61	154 (53.8)	98 (54.4)	56 (52.8)		116 (52.5)	38 (58.5)	
EGV *vs.* AGC				0.000			0.260
EGC	172 (60.1)	93 (51.7)	79 (73.6)		129 (58.4)	43 (66.2)	
AGC	114 (39.9)	87 (48.3)	27 (26.4)		92 (41.7)	22 (33.8)	
Pathologic stage				0.005			0.107
I	192 (67.1)	139 (62.9)	53 (81.5)		143 (64.7)	49 (75.4)	
II–IV	94 (322.9)	82 (37.1)	12 (18.5)		78 (35.3)	16 (24.6)	
LN metastasis				0.011			0.067
Absent	198 (69.2)	115 (63.9)	83 (78.3)		147 (66.5)	51 (78.5)	
Present	88 (30.8)	65 (36.1)	23 (21.7)		74 (33.5)	25 (21.5)	
Perineural invasion				0.011			0.014
Absent	213 (74.5)	125 (69.4)	88 (83)		157 (71.0)	56 (86.2)	
Present	73 (25.5)	55 (30.6)	18 (17)		64 (29.0)	9 (13.8)	

EGC: Early gastric cancer; AGC: advanced gastric cancer.

Table II. *PD-L1 expression relative to gastric classification.*

Characteristics	Patients No. (%)	Tumor cell PD-L1			Immune cell PD-L1		
		Negative	Positive	*p*-Value	Negative	Positive	*p*-Value
EBV expression				0.055			0.000
Negative	269 (94.1)	173 (96.1)	96 (90.6)		215 (97.3)	54 (83.1)	
Positive	17 (5.9)	7 (3.9)	10 (9.4)		6 (2.7)	11 (16.9)	
MSI status				0.596			0.240
MSI	60 (21.0)	36 (20.0)	24 (22.6)		43 (19.5)	17 (26.2)	
MSS	226 (79.0)	144 (80.0)	82 (77.4)		178 (80.5)	48 (73.8)	
TP53 expression				0.092			0.065
Wild-type	179 (62.6)	120 (66.7)	69 (65.1)		132 (59.7)	47 (72.3)	
Mutant	107 (37.4)	60 (33.3)	37 (34.9)		89 (40.3)	18 (27.7)	
ACRG				0.774			0.024
MSI	60 (21.0)	36 (20.0)	24 (21.0)		19 (16.1)	41 (24.4)	
MSS/TP53 mutant	65 (22.7)	44 (24.4)	21 (19.8)		30 (25.4)	35 (20.8)	
MSS/TP53 wild	86 (30.1)	52 (28.9)	34 (32.1)		29 (24.6)	57 (33.9)	
MSS/EMT	75 (26.2)	48 (26.7)	27 (25.5)		40 (33.9)	35 (26.2)	
Lauren				0.975			0.167
Intestinal	176 (63.5)	109 (63.4)	67 (63.8)		65 (55.1)	111 (66.1)	
Diffuse	73 (26.7)	46 (26.7)	27 (25.7)		40 (33.9)	42 (25.0)	
Mixed	28 (10.1)	17 (9.9)	11 (10.5)		13 (11.0)	15 (8.9)	

MSI: Microsatellite instability; MSS: microsatellite stability; ACRG: Asian Cancer Research Group classification; EMT: epithelial-mesenchymal transition.

expression was a significant prognostic factor and was positively correlated with overall survival (p=0.005) (Table III), and IC PD-L1 expression was positively correlated with disease-free survival (p=0.039) (Table IV).

Discussion

PD-L1 is known to play a key role in cancer immune evasion (6). Cancer cells express the PD-L1 co-inhibitory receptor in

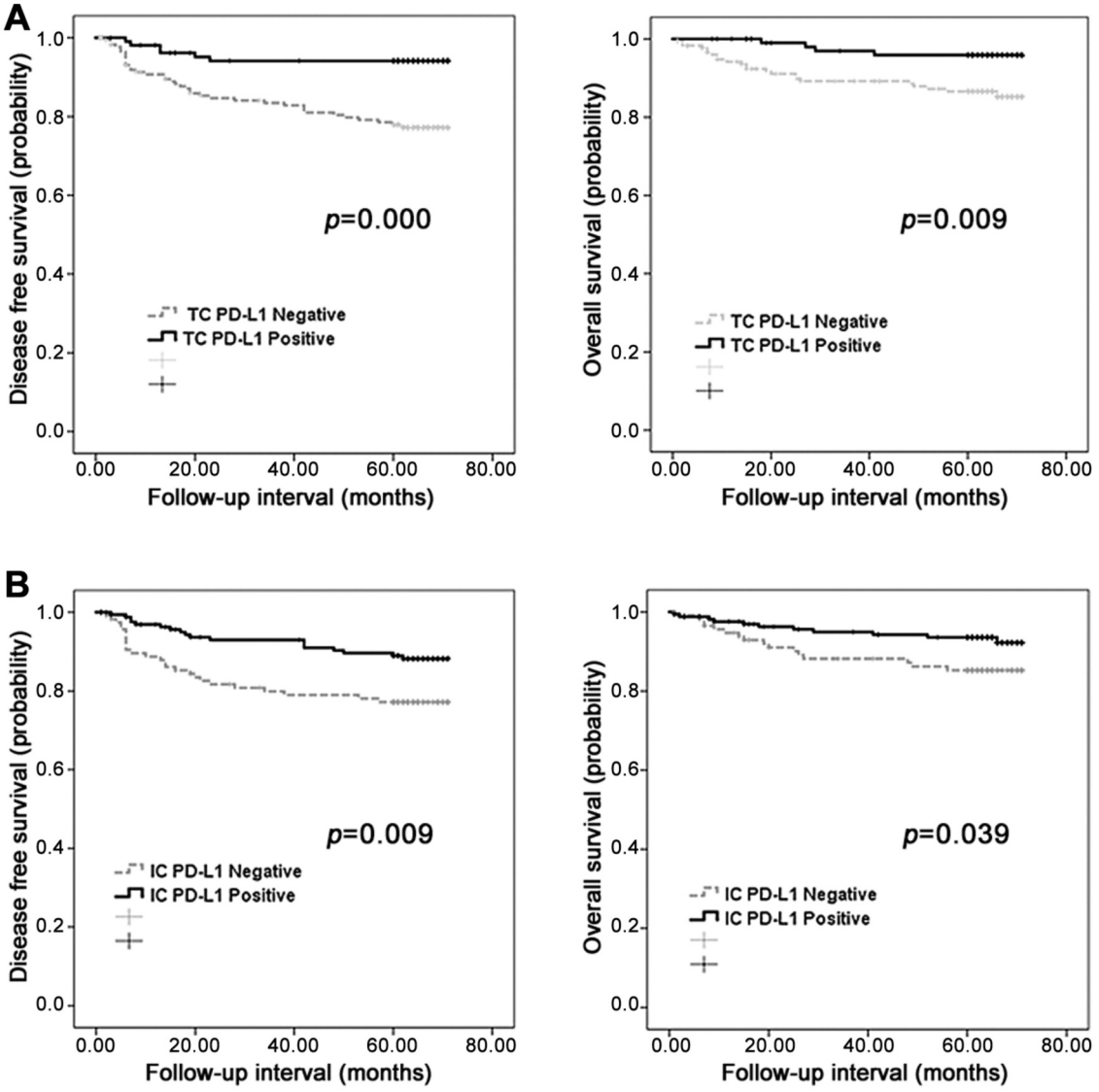

Figure 2. *Kaplan-Meier curves of disease-free and overall survival according to (A) tumor cell (TC) and (B) immune cell (IC) PD-L1 expression in all GC patients evaluated (n=286).*

response to immune attack to inhibit T-cell mediated antitumor immunity. Increased PD-L1 expression in solid cancer cells is generally thought to contribute to immune cell activation and is indicative for poor prognosis, however, increased PD-L1 expression also reveals good prognostic impacts. Prior studies have suggested that the role of PD-L1 in disease progression is dependent on cancer type, with a deleterious role in urothelial, renal, and hepatocellular carcinomas (23), despite being associated with improved clinical outcomes in breast cancer and merkel cell carcinoma (24). In addition to TCs, host ICs express PD-L1, which can contribute to tumor cell immunity (25). In prior studies, IC PD-L1 expression was associated with poor prognosis in GC, and improved prognosis in lung and colorectal cancers (26). Thus, the prognostic relevance of TC and IC PD-L1 expression remains elusive and controversial for many cancers.

In the present study, both TC and IC PD-L1 expression was associated with improved prognosis, as demonstrated by uni- and multivariate analyses of GC patients. TC PD-L1 expression was negatively correlated with advanced GC, higher pathologic stage, lymph node metastasis, and perineural invasion. Patients with TC and IC PD-L1-positive staining had improved disease-free and overall survival rates in all cases of GC. Prior studies evaluating the prognostic relevance of PD-L1 expression in GC have yielded opposing results. Prior studies in Chinese and Japanese GC patients suggested that both TC and IC PD-L1

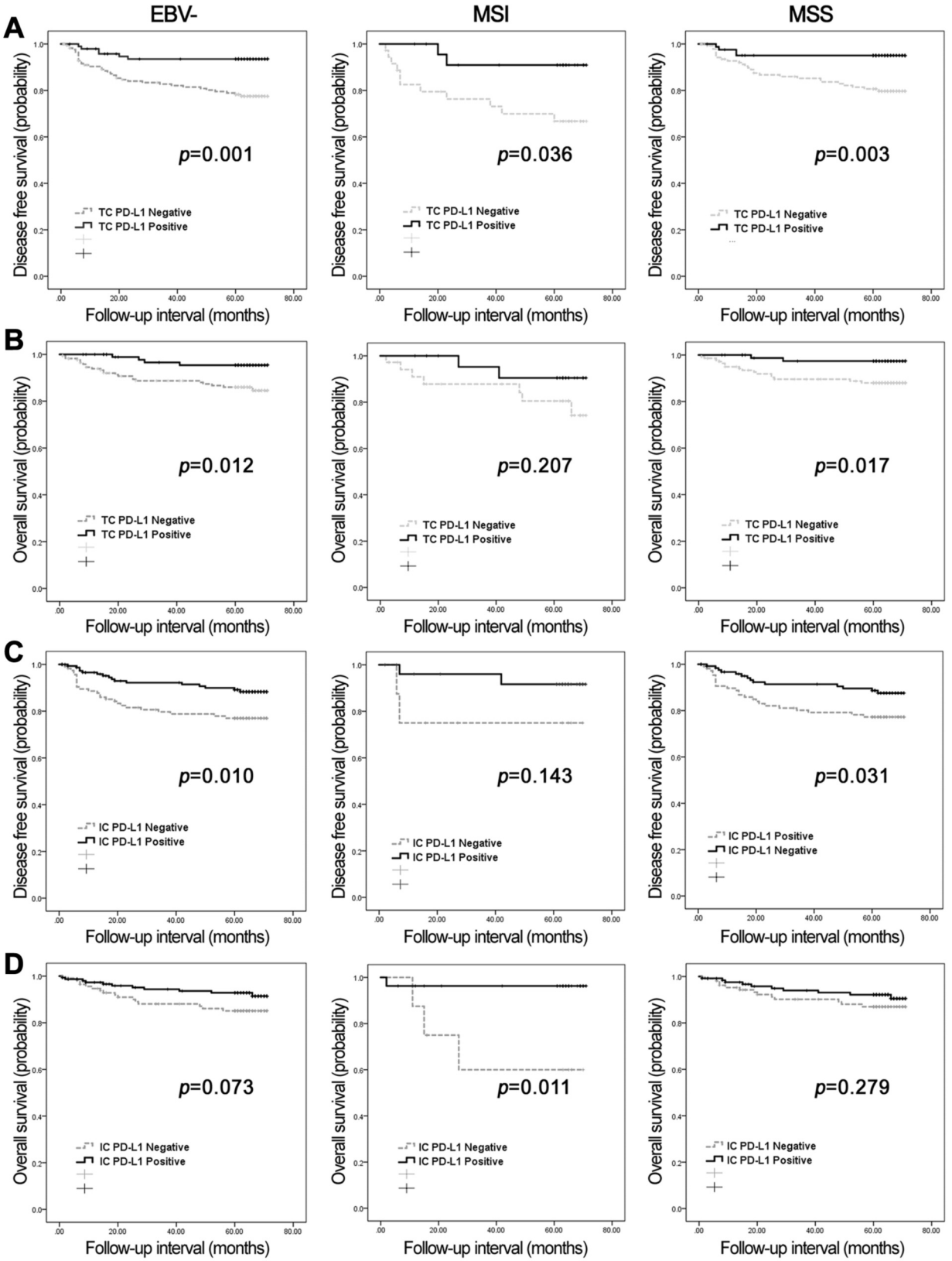

Figure 3. *Kaplan-Meier curves of disease-free and overall survival according to (A-B) tumor cell (TC) and (C-D) immune cell (IC) PD-L1 expression in the EBV negative (EBV⁻), microsatellite instability (MSI), and microsatellite-stable (MSS) subtypes.*

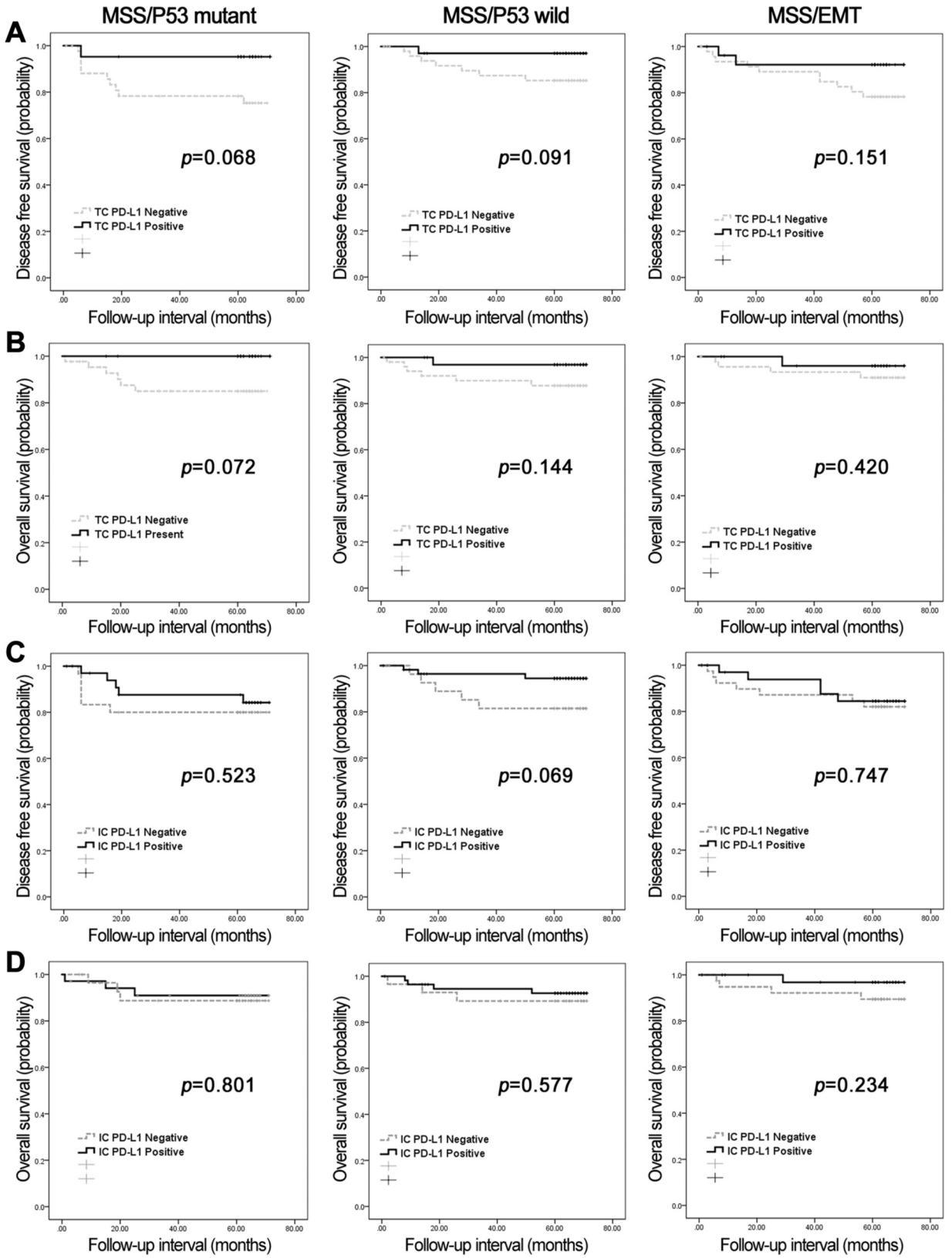

Figure 4. *Kaplan-Meier curves of disease-free and overall survival according to (A-B) tumor cell (TC) and (C-D) immune cell (IC) PD-L1 expression in the MSS/TP53 mutant, MSS/TP53 wild-type, and MSS/EMT GC subtypes.*

Table III. *Multivariate analysis results for overall survival in gastric adenocarcinoma patients.*

	Overall survival		
	HR	95%CI	*p*-Value
Tumor cell PD-L1	0.229	0.082-0.642	0.005
EGC *vs.* AGC	38.762	4.950-303.537	0.000
Lymph node metastasis	2.275	0.921-5.650	0.075
Distant metastasis	12.324	2.336-65.009	0.003
Age (under 61 *vs.* over 61)	1.121	0.519-2.422	0.771

HR: Hazard ratio; CI: confidence index; Age unit: years; EGC: early gastric cancer; AGC: advanced gastric cancer.

Table IV. *Multivariate analysis results for disease-free survival in gastric adenocarcinoma patients.*

	Disease-free survival		
	HR	95%CI	*p*-Value
Immune cell PD-L1	0.529	0.289-0.970	0.039
EGC *vs.* AGC	24.270	5.631-104.607	0.000
Lymph node metastasis	3.560	1.711-7.405	0.001
Distant metastasis	0.000	0.000	0.982
Age (under 61 *vs.* over 61)	1.232	0.674-2.250	0.498

HR: Hazard ratio; CI: confidence index; Age unit: years; EGC: early gastric cancer; AGC: advanced gastric cancer.

expression were poor prognostic indicators for GC (27-30). Contrastingly, other studies have suggested that TC and IC PD-L1 expression are positive prognostic indicators in most Western, some Korean, and some Chinese GC patients (27, 31-33). In addition, samples (tissue or serum), resources of antibody (rabbit or mouse), and type of antibody (mono- or polyclonal) were considered in relation with the prognostic significance of PD-L1 expression (30). PD-L1 immunohistochemical assays from different companies (Abcam, Cell Signaling, and DAKO) have been developed and showed different clinical significances (31, 32, 34). These suggest that the clinical significance of PD-L1 expression varies between patients and could be dependent on many factors including patient ethnicity, samples, and applied types of antibody (14).

We applied a 1% cut-off level for TC PD-L1 positivity and a 5% cut-off level for IC PD-L1 positivity to assess the prognostic significance of TC and IC PD-L1 positivity based on the results of the best and most significant separation in survival curves between the applied cut-offs of 1%, 5%, 10%, 20%, and 50%. Previous studies identified different cut-off values for TC PD-L1 of 1%, 5%, 10%, and 50% (27), and for IC PD-L1 of 1% and 5% (30). Unlike lung cancer, the prognostically significant cut-off values for PD-L1 expression in GC have not yet been validated. Immunotherapeutic drugs for GC are being developed together with diagnostic biomarkers related to PD-L1, so cut-off points to determine PD-L1 positivity will change with patient treatment response (35).

We also assessed PD-L1 expression relative to molecular TCGA and ACRG GC subtypes. IC PD-L1 was significantly upregulated in the EBV^+ group. A previous study reported the association between PD-L1 expression and CD8 cytotoxic T cell infiltration in EBV^+ GC, which is associated with high proportion of tumor-infiltrating CD8 cytotoxic T cells (36). Herein, IC and TC PD-L1 expression was associated with improved prognostic outcome in the EBV^-, MSI, and MSS subgroups. Previous studies demonstrated that PD-L1 expression is increased in the EBV^+ and MSI GC subgroups (30). Further, MSI and TC PT-L1 expression, when combined, were stronger predictive factors for GC patient prognosis (37). Because GCs are molecularly heterogeneous diseases, the TCGA and ACRG guidelines provided a new classification system for GC. Although we evaluated TCGA and ACRG classifications using immunohistochemistry and *in situ* hybridization, MSI and MSS subgroups can also be separated based on TC and IC expression of PD-L1 (38, 39). Integrated assessment of PD-L1 expression and molecular classification could better facilitate prediction of GC prognosis.

In conclusion, TC and IC PD-L1 protein levels were confirmed as biomarkers for prediction of improved prognosis in the evaluated cohort of GC patients. Molecular GC classification combined with PD-L1 expression can provide insight into patient prognosis. Additional studies are needed to determine whether PD-L1 expression is predictive of the response to immunotherapy in GC.

Conflicts of Interest

The Authors declare that there are no known conflicts of interest associated with the work presented in this manuscript. Furthermore, the Authors confirm that the funding provided for these studies did not influence the results in anyway.

Authors' Contributions

Conceptualization, K.S.Song; Funding acquisition, M.-K.Y.; Investigation, D.H.K., G.E.B and S.-I. L; Methodology, J.S.K. and D.H.K.; Supervision, K.S.Suh and M.-K. Y.; Validation, Writing-original draft, D.R and K.S.Song.; Writing-review & editing, G.E.B. and M.-K.Y.

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