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ORIGINAL ARTICLE – CANCER RESEARCH

PD-L1 is a double-edged sword in colorectal cancer: the prognostic value of PD-L1 depends on the cell type expressing PD-L1

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Abstract

Purpose To investigate the associations between programmed cell death ligand-1 (PD-L1) on tumor cells (TCs) or PD-L1 on tumor-infiltrating immune cells (TIICs) and the microsatellite instability (MSI) status in colorectal cancer (CRC).

Methods In total, 238 CRC patients were enrolled. PD-L1 expression and MSI status were studied by immunohistochemical staining and polymerase chain reaction. The χ^2 test was used to compare characteristics. The Kaplan–Meier method was used for survival analysis. Cox proportional hazards models were used to determine the prognostic influence of clinicopathological factors.

Results Eighteen patients (7.6%) were had MSI-high (MSI-H) CRC. The number of patients with PD-L1 expression on TCs, stromal TIICs and intraepithelial TIICs was 13 (5.5%), 64 (26.9%) and 45 (18.9%), respectively. The MSI-H phenotype was significantly associated with younger age, right sidedness, mucinous component, high grade, stromal TIICs expressing PD-L1 ($P=0.042$) and intraepithelial TIICs expressing PD-L1 ($P<0.001$), but not TCs expressing PD-L1. PD-L1-expressing TCs were an independent marker of poor prognosis [hazard ratio (HR) = 3.387, $P=0.003$], and PD-L1-expressing stromal TIICs were an independent marker of good prognosis (HR = 0.551, $P<0.001$).

Conclusions PD-L1-expressing TCs were a marker of poor prognosis; in contrast, PD-L1-expressing TIICs were a marker of good prognosis. The MSI-H phenotype was associated with the presence of PD-L1-expressing TIICs, but not of PD-L1-expressing TCs.

Keywords Colorectal cancer · Microsatellite instability · PD-L1 · Immunotherapy

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Introduction

Colorectal cancer (CRC) ranks as the most common cause of cancer in Taiwan. Unfortunately, approximately 40–45% of patients with CRC will progress to metastatic CRC (mCRC). Despite many advancements in mCRC treatment, such as improvements in surgery, radiation, and biochemotherapy, including anti-EGFR drugs, anti-VEGF inhibitors, and regorafenib, the majority of mCRC patients have a modest prognosis due to the high frequency of drug resistance, metastasis and recurrence. The median overall survival (OS) is approximately 30 months (Brenner et al. 2014; Elez et al. 2015; Haggar and Boushey 2009).

In the new era of cancer therapy, Postow et al. (2015) immunotherapy options are rapidly growing. In this context, negative regulators of the immune system called immune checkpoints play a key role in limiting antitumor immunologic responses. Thus, immune checkpoint-inhibiting

agents, such as those directed against programmed death-1 receptor (PD1) and its ligand PD-L1, have been developed as antitumor agents and have produced interesting results in clinical studies. Initially, anti-PD-1/anti-PD-L1 agents were successfully used to treat melanoma, gastric cancer and lung cancer (Herbst et al. 2016; Le et al. 2015; Postow et al. 2015; Robert et al. 2015; Shitara et al. 2018). In addition, these checkpoint inhibitors have been applied for mCRC treatment. In mCRC, a microsatellite instability-high (MSI-H) phenotype is a predictive marker that supports the use of pembrolizumab (anti-PD-1 antibody) and nivolumab (anti-PD-1 antibody) (Le et al. 2015; Overman et al. 2017). The mechanism is related to the high mutation rate in patients with the MSI-H phenotype (Hellmann et al. 2018; Lawrence et al. 2013; Vogelstein et al. 2013). Unfortunately, only 2–4% of mCRC patients have the MSI-H phenotype. Most patients with mCRC (96–98%) have a microsatellite stable (MSS) phenotype, and anti-PD-1 or anti-PD-L1 agents alone do not exhibit efficacy in treating MSS mCRC (Vilar and Gruber 2010). Therefore, it is critical to reexamine the roles of PD-L1/PD1 in mCRC from a clinical perspective.

In CRC, the role of PD-L1 remains unclear. In the literature (Droeser et al. 2013; El Jabbour et al. 2018; Inaguma et al. 2017; Lee et al. 2017, 2018a, b; Li et al. 2016), PD-L1 is often shown to be expressed by at least 2 cell types: tumor cells (TCs) and tumor-infiltrating immune cells (TIICs). There are conflicting reports regarding the clinical associations between the MSI phenotype, PD-L1 expression, or PD-L1 distribution (TCs or TIICs) and OS. Generally, in early studies, the focus was on TCs rather than on TIICs. The expression of PD-L1 by TCs and TIICs varies widely, and the associations between the MSI phenotype and expression of PD-L1 in TCs or TIICs are not consistent. Thus, the impact of PD-L1-expressing TCs is not consistent.

In this work, we aimed to investigate the clinical associations between PD-L1 expression on TCs and TIICs and clinical parameters and MSI status. We found that PD-L1 was a double-edged sword in CRC; the presence of TCs that express PD-L1 was a marker of poor prognosis, and the presence of TIICs that express PD-L1 was a marker of good prognosis. Moreover, the MSI-H phenotype was associated with the presence of TIICs expressing PD-L1 but not TCs expressing PD-L1.

Materials and methods

Patients and tissue blocks

A total of 238 patients diagnosed with CRC at Taipei Veterans General Hospital in Taiwan were enrolled in our study. Disease stage was assessed based on the American Joint Committee on Cancer staging system, 7th edition. Clinicopathological staging and clinical course were determined by searching a computer database containing detailed information. The medical residual samples from patients were acquired from the residual sample bank of Taipei Veterans General Hospital, and this study was approved by the Institutional Review Board of Taipei Veterans General Hospital (VGH IRB). The VGH IRB waived the requirement for inform consent.

Immunohistochemistry (IHC)

For tissue microarray (TMA) and IHC analyses, the procedures followed the manufacturer's instructions. Positive immunostaining was evaluated by the clinical physician. Sections (4-μm thick) cut from formalin-fixed, paraffin-embedded tissue blocks were deparaffinized in xylene, rehydrated through a graded ethanol series and subjected to IHC.

To measure PD-L1 expression, we performed the PD-L1 IHC 22C3 pharmaDx assay (Dako Inc. Santa Clara, CA, USA) that utilizes the mouse monoclonal antibody clone 22C3, which recognizes the extracellular domain of PD-L1, using a Dako Autostainer Link 48 platform.

For the examination of PD-L1 expression in TCs, the intensity of staining was scored as 0, 1, 2, or 3. The percentage of stained cells was also counted, and the final H-score was obtained by multiplying the staining intensity (0–3) by the percentage of positive cells (%). Thus, the H-scores represented a continuous variable that ranged from 0 to 300 points. A cutoff point of 10 was used to distinguish between the TCs without PD-L1 expression (score of less than 10) and the TCs with PD-L1 expression (score greater than or equal to 10).

For the examination of PD-L1 expression in TIICs, CRC specimens obtained from routine pathological diagnostic procedures were stained with hematoxylin and eosin (H&E) and used to assess two types of TIIC populations located in the stroma and intraepithelial cancer structures by light microscopy (magnification, 50–400×). Staining was evaluated by clinical physicians who were blinded to the clinical information. TIICs in the stroma were identified according to the recommendations of the International TILs Working Group, 2014 (Salgado et al. 2015). For statistical analysis, three levels of infiltration were defined for the stromal TILs expressing PD-L1: 1, weak (0–10% of stromal TILs); 2, moderate (20–40% of stromal TILs); and 3, strong (50–90% of stromal TILs). Our study population was classified as follows: no staining, grade 0 (G0; 0–10% of stromal TIICs); weak, grade 1 (G1); moderate, grade 2 (G2; 10–50% of stromal TIICs) and strong, grade 3 (G3; > 50% of stromal TIICs). Intraepithelial TIICs located within the tumor, with the exception of apoptotic bodies, were counted in 5 high-power fields (HPFs), and the mean number per adenocarcinoma structure was determined. For statistical

analysis, the intraepithelial TIICs expressing PD-L1 within the tumors were divided into two categories: 0, absent (no intraepithelial TIICs expressing PD-L1) and 1, present (≥ 1 intraepithelial TIICs expressing PD-L1 in the intraepithelial tube) (Jakubowska et al. 2017).

For mismatch repair (MMR) protein expression, four monoclonal antibodies, namely, anti-MLH1 (clone M1, Ventana), anti-PMS2 (clone EPR3947, Ventana), anti-MSH2 (clone G219–1129, Ventana), and anti-MSH6 (clone 44, Ventana), were used for immunostaining with a Ventana BenchMark ULTRA system according to the manufacturers' recommendations.

MSI analysis by polymerase chain reaction (PCR)

Due to limitations in specimens, MSI analysis was performed if the result of the MMR IHC was unclear. For MSI analysis, H&E-stained tissue slides were reviewed by pathologists to select non-tumor and tumor regions. The selected regions were manually microdissected from consecutive, deparaffinized tissue sections and subjected to genomic DNA extraction. Briefly, the marked regions were transferred to an Eppendorf tube containing a proteinase K solution. The tube was then incubated at 56 °C for 16 h, and then an inactivation step was performed at 95 °C for 10 min. The proteinase K-digested extract containing genomic DNA was adequate for the MSI analysis. The MSI analysis was performed using multiplex PCR assay with a commercially available MSI Analysis System (Promega). A panel of five mononucleotide repeat markers, namely, BAT25, BAT26, NR21, NR24, and MONO27, as well as two sample identification markers, PENTA C and PENTA D, were amplified by fluorescently labeled primers using PCR. After PCR amplification, the amplicons were subjected to capillary electrophoresis on an ABI 3130xl Genetic Analyzer (Applied Biosystems), and the results were analyzed using GeneMapper software version 4.1 (Applied Biosystems). The size shift of an allele peak in a tumor sample compared to that in the corresponding non-tumor sample indicated instability of the marker. Samples with alterations in ≥ 40% of the microsatellite markers (≥ 2 altered markers out of 5) were classified as MSI-H, and those with less than 40% altered markers were classified as non-MSI-H (Bacher et al. 2015).

Statistical and survival analyses

Data are expressed as the mean ± SD. Statistical comparisons were based on nonparametric tests. Correlations between clinicopathological variables and immunopositivity were analyzed using the χ^2 test or Fisher's exact test. Survival was estimated using the Kaplan–Meier method. Cox proportional hazards model were employed for univariate and multivariate analyses to determine the prognostic influence of clinicopathological factors on survival endpoints. A two-tailed P value < 0.05 was regarded as statistically significant. SPSS software (version 18.0, SPSS, Chicago, IL, USA) was used for all statistical analyses.

Results

MSI-H was significantly associated with TIICs expressing PD-L1, but not TCs expressing PD-L1

A total of 238 patients who were diagnosed with CRC were enrolled in our study. Patient characteristics, clinical parameters and PD-L1 expression were separated into two groups by MSI status (Table 1). Eighteen patients (7.6%) were in the MSI-H CRC group, and the others (92.4%) were in the MSS CRC group. The mean age at diagnosis was 57.6 years in the MSI-H CRC group and 64.7 years in the MSS CRC group. The MSI-H phenotype was significantly associated with younger age ($P = 0.027$), right-sided tumor location ($P = 0.001$), presence of a mucinous component ($P = 0.002$), high grade ($P = 0.005$), stromal TIICs expressing PD-L1 ($P = 0.042$) and intraepithelial TIICs expressing PD-L1 ($P < 0.001$). However, MSI-H was not associated with TCs expressing PD-L1. In addition, the genotype distributions of mutations regarding MMR proteins were MLH1 ($n = 7$, 38.9%), PMS2 ($n = 4$, 22.2%), MSH2 ($n = 5$, 27.8%) and MSH6 ($n = 2$, 11.1%) (Table 2). The associations between TCs expressing PD-L1, TIICs expressing PD-L1 and genotype distribution of MMR proteins are shown in Table 2.

The expression of PD-L1 was lower in TCs than in TIICs

Only thirteen patient samples (5.5%) contained TCs that stained positive for PD-L1. However, sixty-four patient samples (26.9%) had > 10% of the stromal TIICs expressing PD-L1. Moreover, 45 patient samples (18.9%) displayed an accumulation of intraepithelial TIICs expressing PD-L1. We found that PD-L1 staining was stronger in TIICs than in TCs. Representative images of IHC staining are shown in Fig. 1.

TIICs expressing PD-L1 acted as a marker of good prognosis according to the Kaplan–Meier analysis

Based on the Kaplan–Meier analysis, the MSI-H status was not a prognostic marker ($P = 0.075$, MSI-H OS: not reached; MSS OS: 43.0 months) (Fig. 2a). Furthermore, the presence of TCs expressing PD-L1 was not a prognostic marker ($P = 0.111$, OS of patients with TCs expressing PD-L1: 14.3 months; OS of patients with TCs without PD-L1 expression: 45.3 months) (Fig. 2b). In Fig. 2a, b,

Table 1 The comparison of the baseline demographics of patients with colorectal cancer stratified into MSS or MSI-H groups ($n=238$)

	MSS $N=220$ (%)	MSI-H $N=18$ (%)	P Value
Age (years)			
Mean (SD)	64.7 (12.7)	57.6 (16.6)	0.027*
Gender			
Female	73 (33.2)	9 (50.0)	0.149
Male	147 (66.8)	9 (50.0)	
Sidedness			
Left	103 (46.8)	1 (5.6)	0.001*
Right	117 (53.2)	17 (94.4)	
AJCC 7th Stage			
I	16 (7.3)	1 (5.6)	0.063
II	56 (25.5)	9 (50.0)	
III	63 (28.6)	6 (33.3)	
IV	85 (38.6)	2 (11.1)	
Pathology			
Adenocarcinoma	197 (89.5)	13 (72.2)	0.071
Carcinoma	1 (0.5)	0 (0.0)	
Mucinous adenocarcinoma	22 (10.0)	5 (27.8)	
Mucinous component			
No	143 (65.0)	5 (27.8)	0.002*
Yes	77 (35.0)	13 (72.2)	
T4			
No	145 (65.9)	13 (72.2)	0.586
Yes	75 (34.1)	5 (27.8)	
Grade			
Low	203 (92.3)	13 (72.2)	0.005*
High	17 (7.7)	5 (27.8)	
LVSI status			
No	167 (75.9)	15 (83.3)	0.475
Yes	53 (24.1)	3 (16.7)	
TCs expressing PD-L1			
No	208 (94.5)	17 (94.4)	0.986
Yes	12 (5.5)	1 (5.6)	
Stromal TIICs expressing PD-L1			
Grade 0	133 (60.5)	6 (33.3)	0.042*
Grade 1	31 (14.1)	4 (22.2)	
Grade 2	29 (13.2)	2 (11.1)	
Grade 3	27 (12.3)	6 (33.3)	
Intraepithelial TIICs expressing PD-L1			
No	184 (83.6)	9 (50.0)	< 0.001*
Yes	36 (16.4)	9 (50.0)	

AJCC American Joint Committee on Cancer, *LVSI* lymph-vascular space invasion, *MSI-H* microsatellite instability high, *MSS* microsatellite stable, *PD-L1* programmed cell death ligand-1, *SD* standard deviation, *TCs* tumor cells, *TIICs* tumor-infiltrating immune cells

*Significant

the 2 lines are dramatically separated, but the P value did not meet the significance threshold, which might be due to the small number of samples with the MSI-H phenotype (7.6%) and TCs expressing PD-L1 (5.5%). The presence of stromal TIICs expressing PD-L1 was a marker of good prognosis ($P<0.001$, OS of patients with stromal TIICs expressing PD-L1 G0: 28.7 month; G1, not reached; G2: not reached; and G3: not reached) (Fig. 2c). Similarly, the presence of intraepithelial TIICs expressing PD-L1 was a marker of good prognosis ($P=0.006$, OS of patients with intraepithelial TIICs expressing PD-L1: not reached; OS of patients with intraepithelial TIICs without PD-L1 expression: 36.5 months) (Fig. 2d).

The presence of TCs expressing PD-L1 was an independent marker of poor prognosis, and the presence of stromal TIICs expressing PD-L1 was an independent marker of good prognosis after controlling for confounding factors in a multivariate model.

To clarify the role of PD-L1 in TCs and TIICs, we employed Cox proportional hazards models (Table 3). In the univariate model, stromal TIICs expressing PD-L1, intraepithelial TIICs expressing PD-L1, disease stage, T4 stage, presence of a mucinous component and lymphovascular space invasion (LVSI) were prognostic factors, whereas TCs expressing PD-L1, MSI-H phenotype, sidedness, and high grade were not. Subsequently, the factors identified in the univariate model were used in a multivariate model. After controlling for potential confounding factors, TCs expressing PD-L1 were determined to be an independent marker of poor prognosis [hazard ratio (HR) = 3.387, $P=0.003$], and stromal TIICs expressing PD-L1 were identified as an independent marker of good prognosis (HR = 0.551, $P<0.001$).

Discussion

To the best of our knowledge, the impact of TCs expressing PD-L1 on CRC patient survival and the associations between TCs expressing PD-L1 or TIICs expressing PD-L1 and the MSI-H phenotype are not well clarified, whereas the impact of TIICs expressing PD-L1 on CRC patient survival is consistently considered positive (Table 4) (Droeser et al. 2013; El Jabbour et al. 2018; Inaguma et al. 2017; Lee et al. 2017, 2018a, b; Li et al. 2016). In our study, we found opposing impacts of TCs expressing PD-L1 and TIICs expressing PD-L1 on survival; the presence of TCs expressing PD-L1 was a marker of poor prognosis; in contrast, the presence of TIICs expressing PD-L1 was a marker of good prognosis. Moreover, the MSI-H phenotype was associated with the presence of TIICs expressing PD-L1, but not of TCs expressing PD-L1.

In our study, the presence of TCs expressing PD-L1 was identified as a marker of poor prognosis by the multivariate

Table 2 The associations between TCs expressing PD-L1, TIICs expressing PD-L1 and genotype distribution of MMR proteins ($n = 18$)

	Genotype of MMR (Inactivated gene)				
	MLH1	MSH2	MSH6	PMS2	*P* value
	n (%)	*n* (%)	*n* (%)	*n* (%)	
TCs expressing PD-L1					
No	6 (85.7)	5 (100.0)	2 (100.0)	4 (100.0)	0.645
Yes	1 (14.3)	0 (0.0)	0 (0.0)	0 (0.0)	
Stromal TIICs expressing PD-L1					
Grade 0	2 (28.6)	1 (20.0)	2 (100.0)	1 (25.0)	0.666
Grade 1	2 (28.6)	1 (20.0)	0 (0.0)	1 (25.0)	
Grade 2	0 (0.0)	1 (20.0)	0 (0.0)	1 (25.0)	
Grade 3	3 (42.9)	2 (40.0)	0 (0.0)	1 (25.0)	
Intraepithelial TIICs expressing PD-L1					
No	3 (42.9)	2 (40.0)	2 (100.0)	2 (50.0)	0.504
yes	4 (57.1)	3 (60.0)	0 (0.0)	2 (50.0)	

	MMR type		
	MLH1-PMS2	MSH2-MSH6	
	n (%)	*n* (%)	
TCs expressing PD-L1			
No	10 (90.9)	7 (100.0)	0.412
Yes	1 (9.1)	0 (0.0)	
Stromal TIICs expressing PD-L1			
Grade 0	3 (27.3)	3 (42.9)	0.845
Grade 1	3 (27.3)	1 (14.3)	
Grade 2	1 (9.1)	1 (14.3)	
Grade 3	4 (36.4)	2 (28.6)	
Intraepithelial TIICs expressing PD-L1			
No	5 (45.5)	4 (57.1)	0.629
Yes	6 (54.5)	3 (42.9)	

MMR, mismatch repair, *PD-L1* programmed cell death ligand-1, *TCs* tumor cells, *TIICs* tumor-infiltrating immune cells

Cox regression analysis. Published reports, including our study, remain inconclusive, as both good and poor prognoses have been found to be associated with TCs expressing PD-L1. Two studies reported this factor as a marker of good prognosis (Droeser et al. 2013; Li et al. 2016), one found this factor non-significant (Lee et al. 2018b), and two found this factor to be a marker of poor prognosis (Lee et al. 2018a). Our report is consistent with that from Lee at al. (2018a) and interestingly, our antibody was the same as the antibody used in their study (22C3, although we did not select this antibody by consulting their report). In addition, the proportion of TCs expressing PD-L1 was similar in both reports: 5.5% of all TCs in our report and 4.5% in the report from Lee et al. The proportion in other reports ranged from 12.0 to 66.4% (TCs expressing PD-L1 > 5–10%), which is higher than the proportion observed in our report. The antibodies recognizing PD-L1 in these other reports included SP142, E1L3 N, ab174838, 27A2 and ab82059. In the report from Rimm et al., the antibody 22C3 showed slightly, but statistically significantly lower staining of TCs expressing PD-L1 than either the 28-8 or E1L3 N antibody (Rimm et al. 2017). This staining difference might partially explain why the results differ from those of our report. Considering the clinical situation, PD-L1/PD-1 blocking strategies only have a 10% response rate in MSS mCRC (Le et al. 2015), and the MSI status is not associated with PD-L1-expressing TCs. It will be interesting to examine the efficacy of anti-PD-L1 or anti-PD1 agents in patients with TCs that stain positive for PD-L1 expression with the 22C3 antibody (the antibody-defined rare subgroup).

The rationale for identifying TCs expressing PD-L1 as a marker of poor prognosis might be as follows: PD-L1 expressed by cancer cells is part of the adaptive immune resistance mechanism whereby tumor and stromal cells suppress T-cell infiltration in the tumor microenvironment (Hamada et al. 2018; Le et al. 2015; Overman et al. 2017;

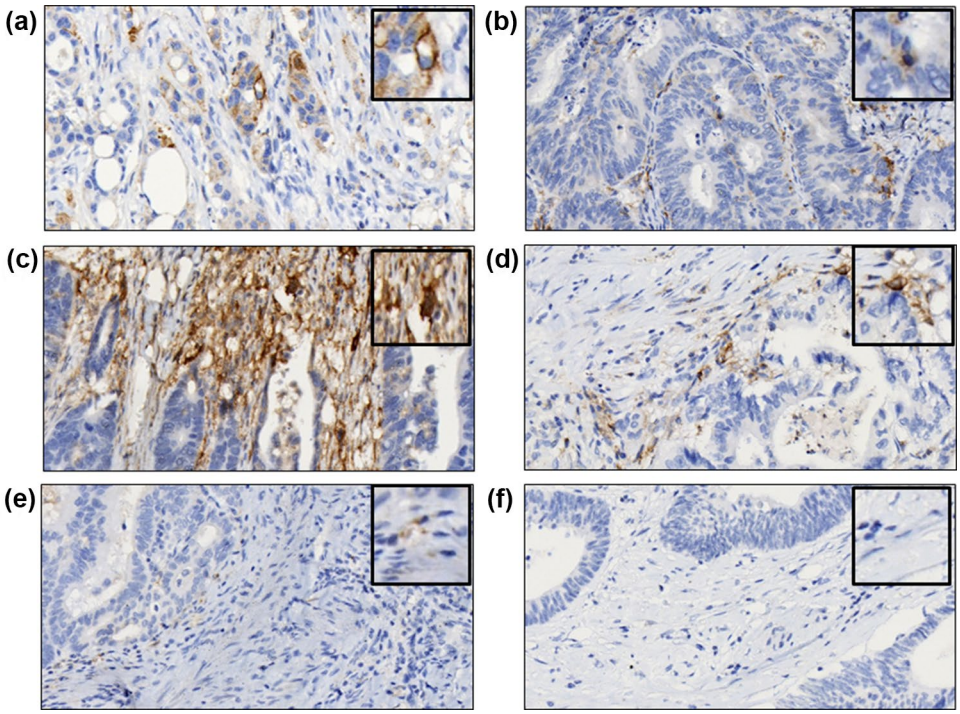

Fig. 1 Representative images of PD-L1 immunostaining in tumor cells (TCs) (**a**), intraepithelial tumor-infiltrating immune cells (TIICs) (**b**), and stromal TIICs of grade 3 (**c**), grade 2 (**d**), grade 1 (**e**) and grade 0 (**f**) samples. (Original magnification × 400; Inset: higher magnification)

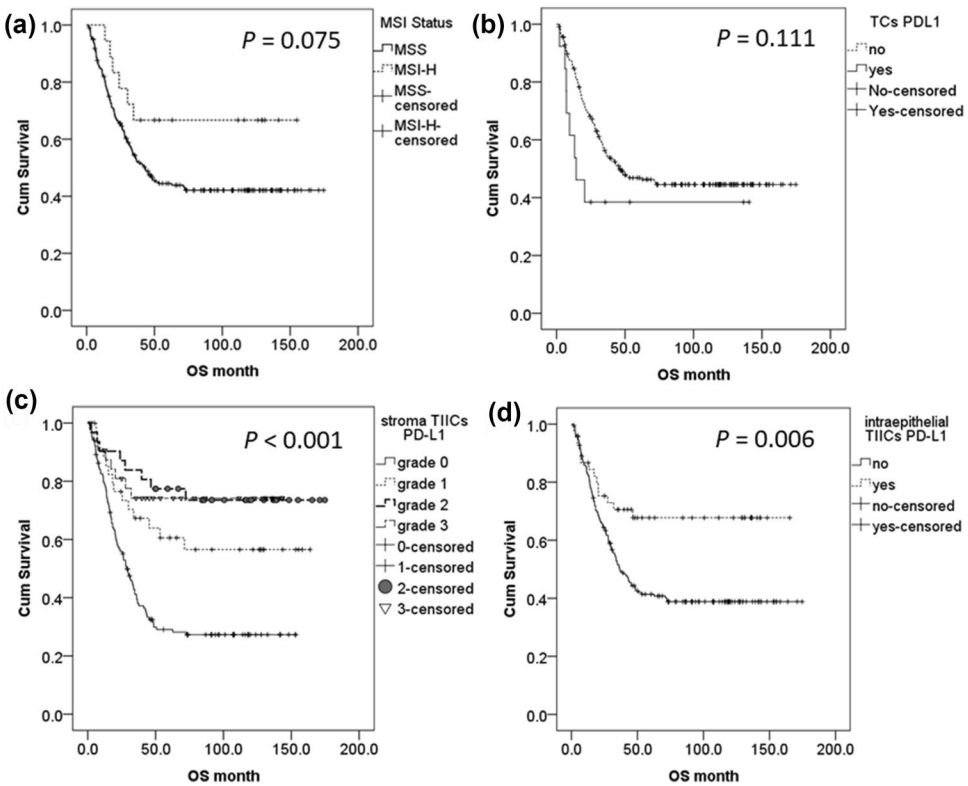

Fig. 2 **a** Overall survival (OS) of patients with colorectal cancer (CRC) stratified by the microsatellite instability (MSI) status; **b** OS of patients with CRC stratified by PD-L1 expression on tumor cells (TCs); **c** OS of patients with CRC stratified by PD-L1 expression on stromal tumor-infiltrating immune cells (TIICs); **d** OS of patients with CRC stratified by PD-L1 expression on intraepithelial TIICs

Tauriello et al. 2018). In previous evaluations, the presence of TCs with PD-L1 expression was found to be associated with poor prognosis in several tumor types, and these cells were considered to play a major role in regulating the immune response against the tumor.

With respect to TIICs expressing PD-L1, most previous studies, including ours, have reported that these cells are associated with good prognosis in CRC (Droeser et al. 2013; Lee et al. 2017, 2018a, b). We did not discuss the intraepithelial and stromal TIICs expressing PD-L1 individually

Table 3 Univariate and multivariate Cox regression analyses of prognostic factors for overall survival in patients with colorectal cancer ($n = 238$)

	Univariate			Multivariate		
	P value	HR	(95.0 CI)	*P* value	HR	(95.0 CI)
TCs expressing PD-L1	0.116	1.778	(0.868–3.643)	0.003*	3.387	(1.511–7.590)
Stromal TIICs expressing PD-L1	< 0.001*	0.565	(0.456–0.702)	<0.001*	0.551	(0.413–0.734)
Intraepithelial TIICs expressing PD-L1	0.007*	0.467	(0.268–0.814)	0.218	1.611	(0.755–3.438)
MSI-H	0.082	0.483	(0.213–1.096)	0.356	0.669	(0.285–1.571)
Sidedness	0.168	0.778	(0.545–1.111)	0.559	0.894	(0.615–1.300)
AJCC 7th Stage	< 0.001*	3.862	(2.944–5.065)	< 0.001*	3.336	(2.480–4.488)
T4	< 0.001*	3.312	(2.322–4.722)	0.017*	1.652	(1.092–2.498)
High grade	0.201	1.438	(0.825–2.506)	0.412	0.783	(0.437–1.404)
Mucinous component	0.003*	1.694	(1.191–2.409)	< 0.001*	2.389	(1.647–3.466)
LVSI	< 0.001*	2.529	(1.749–3.658)	0.094	1.417	(0.942–2.133)

AJCC American Joint Committee on Cancer, *LVSI* lymph-vascular space invasion, *MSI-H* microsatellite instability high, *PD-L1* programmed cell death ligand-1, *SD* standard deviation, *TCs* tumor cells, *TIICs* tumor-infiltrating immune cells

*Significant

because they both had the same impact on clinical survival. It is important to disclose our finding, and attempts have been recently made in clinical trials to use anti-PD-L1 agents to treat MSS mCRC using an immunomodulation strategy. It has been assumed that the cornerstone of immunotherapy in treatments for MSS mCRC is the concept of immunomodulation by other methods, such as chemotherapy, radiation or kinase inhibitors (Le et al. 2015). This concept comes from the initial success of the combination of cotellic and atezolizumab in treating MSS mCRC, but this combination (IMblaze370) ultimately failed. Thus, further detailed investigations (such as on TIICs expressing PD-L1) should be designed to examine subgroup differences. However, we could not determine whether this strategy was correct because the presence of TIICs expressing PD-L1 was a marker of good prognosis in our study. Whether the inhibition of TIICs expressing PD-L1 would compromise survival remains unclear.

The reason behind why TIICs expressing PD-L1 act as a marker of good prognosis remains unclear. Some hypotheses have been proposed, but the results are still inconclusive. PD-L1 expression is also associated with chronic viral infection and chronic inflammatory diseases (Kassem et al. 2008; Xie et al. 2009). Droeser et al. demonstrated that PD-L1 mRNA expression is correlated with interferon (IFN)-γ gene expression in CRC (Droeser et al. 2013). It has been reported that IFN-γ increases the number of CD8+ cytotoxic T lymphocytes (CTLs) during viral infections through JAK-STAT1 signaling (Whitmire et al. 2005). Moreover, Daniel et al. reported that the blockade of the PD-L1-PD-1 interaction restores the ability of exhausted CD8 + T cells to kill infected cells in lymphocytic choriomeningitis virus-infected mice (Barber et al. 2006). This finding might indirectly provide evidence for our observations. However, further studies are necessary to clarify the role of PD-L1 expressing TIICs (Spranger et al. 2013).

With respect to the associations between the MSI-H phenotype and TIICs expressing PD-L1 or TCs expressing PD-L1, MSI-H CRC was associated with the presence of both stromal and intraepithelial TIICs expressing PD-L1 but not the presence of TCs expressing PD-L1. Our report is similar to previous studies showing that the MSI-H phenotype is associated with the presence of TIICs expressing PD-L1. This association can partially be explained by the MSI-H phenotype leading to the production of more neoantigens, which lead to more TIICs (Vilar and Gruber 2010; Vogelstein et al. 2013; Xiao and Freeman 2015; Yu 2018). Nevertheless, further studies on the role of PD-L1 in TIICs are warranted. Additionally, conflicting results regarding the association between TCs expressing PD-L1 and the MSI-H phenotype have been reported in the literature, and more basic studies are needed to elucidate this story.

Our study has several limitations. First, our report was based on a relatively small patient number with regard to the presence of TCs expressing PD-L1 (5.5%) and the MSI-H phenotype (7.6%), and a larger scale study should be conducted to confirm this finding. Second, our study was based on staining with one antibody; thus, we could not address the impact of different antibodies on the detection of PD-L1 expression. Nevertheless, our results were similar to those of Lee et al. (2018a), indicating that our data is reproducible. Finally, our IHC staining was performed using a tissue array; thus, some selection bias existed, as some studies have shown regional heterogeneity in PD-L1 expression in various cancer types (Callea et al. 2015; Ilie et al. 2016; Madore et al. 2015). Furthermore, we did not separate TIIC populations located in the stroma and intraepithelial structures based on the invasive front and the tumor center because our

Table 4 The characteristics of published reports and the associations between TCs expressing PD-L1, TIICs expressing PD-L1 and MSI status in these reports

Year/Ref	Cohort	Antibody	TCs			TIICs		
			Expression	MSI *P* value	Survival	Expression	MSI *P* value	Survival
2018/(Our data)	$n=238$; CRC; I-IV	22C3	5.50	NS ($P=0.986$)	OS (bad, P = 0.003)	26.9) (Cutoff 10))	Sig. ($P=0.042$)	OS (good, $P<0.001$)
2018/(Le et al. 2018a)	$n=336$; CRC; I-IV	assay 3, 22C3	4.5 (Assay 3)	Sig. ($P=0.026$)	OS and PFS (bad, $P=$NR)	45.2 by Assay 3 (cutoff 5)	NS ($P=0.057$)	OS and PFS (good, $P=$NR)
2018/(Lee et al. 2018b)	$n=89$[a]; MSI-H; CC; I-III	Abcam (NR)	43.8) > 5 percentage; 13.5) grade 3 intensity	NR	NS ($P=$NR)	76.4) > 5 Percent; 37.1) Grade 3 intensity	NR	DFS (good, P = 0.028)
2017/(El Jabbour et al. 2018)	$n=104$[a]; MSI = 52; MSS = 52); CRC; I-IV	SP142	Percentage [< 1) (72)); 1–4) (11)); 5-49) (14)); > 50) (3))]	Sig. ($P=0.013$)	NR	Percentage [< 1) (28)); 1–9) (60)); > 10) (12))]	Sig. (P < 0.001)	NR
2017/(Lee et al. 2017)	$n=186$[a]; (MSI = 186; MSS = 153); CC; Stage I-IV	E1L3 N	65.6 in TCs at center > 5; 66.4 in TCs at periphery > 5)	NR	NR	59.9) in IC > 5; 59.8) in IP > 5	NR	OS at MSI-H (Good, IC, $P=0.007$; Good, IP, $P=0.011$); OS at MSS(Good, IC, $P=0.019$; Good, IP, $P=0.001$)
2017/(Inaguma et al. 2017)	$n=454$; CRC; I to IV	E3 N	12	Sig. ($P<0.001$)	NR	NR	NR	NR
2016/(Li et al. 2016)	$n=276$; CRC; I-IV; FUSCC cohort	ab174838(EPR1161(2))	50	NS ($P=0.21$)	DFS (good, HR = 0.558, $P=0.005$); OS (good, HR = 0.606, $P=0.027$)	NR	NR	NR
2013/(Droeser et al. 2013)	$N=1420$; CRC; I-III	Clone 27A2; ab82059	37 in MSS/ 29 in MSI	NR	OS (good, HR = 0.85, $P=0.0003$)	High expression in 2.5)	NR	OS (good, HR = 0.78, $P=0.001$)

CC colon cancer, *CRC* colorectal cancer, *DFS* disease free survival, *IC* immune cells at center, *IP* immune cells at periphery, *MSI-H* microsatellite instability high, *MSS* microsatellite instability stable, *MMR* mismatch repair, *NR* no report, *NS* not significant, *OS* overall survival, *Sig.* significant, TCs, tumor cells, *TIICs* tumor-infiltrating immune cells, *ref* references

[a]Selected group

sample was assessed by a tissue array. In the future, we need to conduct studies with large-scale samples and large-size specimens. Additionally, basic studies on the role of TIICs expressing PD-L1 are needed.

Conclusion

In this study, we found opposing impacts of TCs expressing PD-L1 and TIICs expressing PD-L1 on survival; TCs expressing PD-L1 acted as a marker of poor prognosis; in contrast, TIICs expressing PD-L1 acted as a marker of good prognosis. Moreover, the MSI-H phenotype was associated with the presence of TIICs expressing PD-L1, but not of TCs expressing PD-L1.

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Compliance with ethical standards

Conflict of interest The authors Hsiang-Ling Ho, Teh-Ying Chou, Shung-Haur Yang, Jeng-Kai Jiang, Wei-Shone Chen, Yee Chao, and Hao-Wei Teng declare that they have no conflict of interest.

Ethical approval This retrospective study was conducted using data from Taipei Veterans General Hospital, Taipei, Taiwan and under the guidelines of the Declarations of Helsinki; it was approved by the Human Subjects Protection Offices at Taipei Veterans General Hospital. The medical residual samples of the patients with CRC were acquired from the residual sample bank of Taipei Veterans General Hospital. VGHIRB waived the requirement for the use of an informed consent form.

Informed consent Informed consent was not obtained prior to analysis in this study (VGHIRB waived the requirement for the use of an inform consent form).

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