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ORIGINAL ARTICLE – CLINICAL ONCOLOGY

Prognostic relevance of programmed cell death-ligand 1 expression and CD8+ TILs in rectal cancer patients before and after neoadjuvant chemoradiotherapy

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Abstract

Purpose/background Radiotherapy has been recently reported to boost the therapeutic response of immune checkpoint blockade (ICB); however, few studies have focused on programmed cell death-ligand 1 (PD-L1) expression in locally advanced rectal cancer (LARC) patients who receive preoperative neoadjuvant chemoradiotherapy (neoCRT). The aim of the present study was to investigate the PD-L1 expression status and CD8+ intra-tumoral infiltrating lymphocytes (TILs) before and after neoCRT and its association with clinicopathological characteristics in rectal cancer.

Materials and methods Immunostainings of PD-L1 and CD8+ TILs were performed in 112 pair-matched LARC patients treated by neoCRT. Tumor PD-L1 expression and CD8+ TILs within the tumor microenvironment before and after neoCRT were evaluated via immunohistochemistry.

Results High tumor PD-L1 expression was significantly increased from 50 to 63%, and high CD8+ TILs counts were also slightly increased from 32 to 35% after neoCRT treatment. High tumor PD-L1 before and after neoCRT was associated with improved disease-free survival (DFS, pre-neoCRT: $p = 0.003$ and post-neoCRT: $p = 0.003$) and overall survival (OS, pre-neoCRT: $p = 0.045$ and post-neoCRT: $p = 0.0001$). High CD8+ TILs before neoCRT was associated with improved DFS ($p = 0.057$), and it was significantly associated with improved DFS after neoCRT ($p = 0.039$). Patients with high tumor PD-L1 and CD8+ TILs before and after neoCRT were significantly associated with improved DFS (pre-neoCRT: $p = 0.004$ and post-neoCRT: $p = 0.006$).

Conclusion The present results provide evidence that tumor PD-L1 expression and recruitment of CD8+ TILs within the tumor microenvironment were increased by neoCRT treatment. Tumor PD-L1 and CD8+ TILs are prognostic biomarkers for the survival of LARC patients treated with neoCRT.

Keywords Neoadjuvant chemoradiotherapy · Programmed cell death 1 ligand 1 · CD8 · Tumor-infiltrating lymphocyte · Locally advanced rectal cancer

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Introduction

Colorectal cancer (CRC) is among the most common cancers worldwide (Jemal et al. 2009), and rectal cancer cases account for ~ 30% of all CRCs (Conde-Muino et al. 2015). However, different therapeutic strategies were required for colon and rectal cancers. Preoperative (neoadjuvant) chemoradiotherapy (neoCRT) has been reported as the most effective therapeutic strategy to control tumor growth and improve clinical outcome in patients with locally advanced rectal cancer (LARC, cT3-4 or cN+ patients) (Sauer et al. 2012; Yoon et al. 2015). After neoCRT treatment, only 15–20% of LARC patients achieve a complete response with no residual tumor, most LARC patients achieve a pathological partial response (Balko and Black 2009). neoCRT treatment not only directly induces cancer cell death but also activates anti-tumor immunity via a process called immunogenic cell death (ICD). This process triggers the release of danger-associated molecular patterns (DAMPs) and cytokines from damaged cancer cells and immune cells to create an inflammatory and immunogenic tumor microenvironment to activate T lymphocytes for anti-tumor immunity (Showalter et al. 2017; Wennerberg et al. 2017).

Programmed cell death 1 ligand 1 (PD-L1/CD274) negatively regulates T lymphocytes to cause lymphocyte "exhaustion" through the programmed cell death 1 receptor (PD-1) (Keir et al. 2008). Upregulation of PD-L1 in malignant cells leads to suppression of cytotoxic CD8+ T lymphocyte activity (Hirano et al. 2005; Topalian et al. 2012). Therefore, PD-1/PD-L1 immunotherapy via immune checkpoint blockade (ICB) has been proposed as a promising therapeutic strategy to re-activate the host immune system to eradicate tumors, which have demonstrated impressive therapeutic responses in patients with melanoma, non-small cell lung cancer (NSCLC), renal cell carcinoma (RCC), and bladder cancer (Wang et al. 2016). However, PD-L1 expression can be induced by immune cell infiltration in several malignancies, and interferon-γ secreted by the infiltrated immune cells is required for PD-L1 induction for adaptive immune resistance (Spranger et al. 2013; Taube et al. 2012). In colorectal cancer, patients with microsatellite instability (MSI) have improved therapeutic response for immune checkpoint blockade (ICB) (Lee et al. 2016; Phipps et al. 2015). But the MSI is rarely noted in rectal cancer.

Recently, classification of tumors microenvironment into subgroups on the basis of PD-L1 status and the density of tumor-infiltrating lymphocytes (TILs) were proposed as a predictive marker for the therapeutic response to ICB (Taube et al. 2012; Teng et al. 2015). Patients with high tumor PD-L1 and TILs are most likely to benefit from PD-1/PD-L1 immunotherapy. Moreover, high PD-L1 and TILs are also associated with improved survival outcome in colorectal cancer and breast cancer (Huang et al. 2018c; Schalper et al. 2014). However, there are only a few studies about PD-L1 expression and CD8+ TILs in rectal cancer treated by neoCRT (Lim et al. 2017; Ogura et al. 2018). In the present study, we aimed to evaluate the relationship between tumor PD-L1 expression and CD8+ TILs in pair-matched locally advanced rectal cancer before and after neoCRT treatment and analyze the survival outcomes according to the immune status focused on PD-L1 expression and CD8+ TILs. These results provide the basis for knowledge of immunologic impact on neoCRT, thus suggesting the potential for a combined strategy of cytotoxic therapy and immune checkpoint inhibitors.

Materials and methods

Patient characteristics, clinical staging, treatment, and pathological evaluation

Two hundred eleven patients with locally advanced rectal cancer were treated at our hospital from 2006 to 2014. Among these patients, 171 received neoCRT followed by surgery. Patients with biopsy-proven locally advanced rectal cancer [cT3-4 or cN+ by endorectal ultrasonography (EUS), computed tomography (CT), or magnetic resonance imaging (MRI)] who were treated with preoperative chemoradiotherapy followed by radical resection at China Medical University Hospital comprised the study cohort. This study was reviewed and approved by the Institutional Review Board (IRB) of China Medical University Hospital [Protocol number: CMUH105-REC2-072]. Tumors were staged based on the American Joint Committee on Cancer (AJCC) staging system. EUS, MRI or CT was used to assess the pretreatment clinical stage, and pretreatment biopsies were reviewed by pathologists as previously described (Huang et al. 2018b).

Patients were treated with chemoradiotherapy with a median radiotherapy dose of 50.4 Gy in 28 fractions and concurrent fluoropyrimidine-based chemotherapy (mainly single-agent orally administration with capecitabine, 500 mg/m^2/day b.i.d). Patients were assessed for their clinical response 6–8 weeks after the completion of neoCRT according to rigorous criteria of clinical, endoscopic, and radiologic findings. The three criteria for complete clinical response (cCR) were (a) the absence of a residual ulceration, mass, or mucosal irregularity upon clinical/endoscopic assessment; (b) whitening of the mucosa and the presence of neovasculature; and (c) radiologic imaging, such as CT, RUS, or MRI, without evidence of extrarectal residual disease.

After the chemoradiotherapy regime was completed, surgery was performed 6 to 8 weeks later. Low anterior resection, proctectomy with coloanal reconstruction, abdominoperineal resection, or multivisceral rectal resection were included according to total mesorectal excision (TME) principles. Resected specimen pathologic staging was performed after resection in accordance with the guidelines of the College of American Pathologists. Adjuvant chemotherapy was recommended for patients with metastatic lymph node(s) in surgical specimens and consisted of fluorouracil infusion or capecitabine for a period of 4–6 months. Tumor regression grade (TRG) of a primary tumor after neoCRT was semiquantitatively evaluated on hematoxylin and eosin-stained slides according to Dworak's criteria (Dworak et al. 1997): TRG 0, tumor without regression; TRG 1, dominant tumor mass with obvious fibrosis; TRG 2, dominantly fibrotic changes with few tumor cells; TRG 3, very few tumor cells in the fibrotic tissue; and TRG 4, no viable tumor cells.

Construction of tissue microarray (TMA) and immunohistochemistry

Tissue microarrays were constructed from 112 pair-matched pre-neoCRT biopsies and post-neoCRT surgical tissue from rectal cancer patients, and other specimens were not available (material not suitable for IHC) as previously described. Areas of tumor cells were marked on the hematoxylin and eosin (H&E)-stained slides. The corresponding area on the matching paraffin block (donor block) was then identified and marked. We used the AutoTiss 10C system (EverBio Technology Inc., Taipei, Taiwan) to remove the tissue core from these areas of the donor blocks into the recipient block in a precise, arrayed fashion. The punches were 2 mm in diameter, and a maximum of 60 punches were placed on a single block. Sample sections cut on a microtome were then mounted on capillary-gap slides (Dako, Hamburg, Germany).

Immunohistochemistry (IHC) was performed using 3-μm thick histological TMA sections as previously described (Huang et al. 2018a, b; Lin et al. 2015; Wang et al. 2018). TMA slides were stained individually with horseradish peroxidase-conjugated avidin–biotin complex (ABC) using the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) and NovaRed chromogen (Vector Laboratories) and counterstained with hematoxylin. The following antibodies were used in this study: anti-PD-L1 (ab205921, abcam, Cambridge, UK) and CD8 (ab4055, abcam, Cambridge, UK). The stained tissue sections were scored separately by two pathologists blinded to the clinicopathological parameters. Tumor PD-L1 immunostaining was scored in accordance with the intensity and extent of staining on a semiquantitative scale (0–3+) as follows: 0, absent; 1+, weak; 2+, moderate; 3+, strong membrane staining. The percentage of PD-L1 tumor cells was recorded as follows: a score of 0 was assigned when no staining or positive tumor cell proportion was detected in $<5\%$ of the cells; a score of 1 was assigned when membranous staining was present in $>5\%$ of the positive cell proportion. The 5% threshold was based on a previous phase I trial of anti-PD-1 agents and studies of other malignancies (Thompson et al. 2006; Topalian et al. 2012). CD8 staining was positive when detected in the cytoplasm or at the cell membrane of tumor-infiltrating lymphocytes (TILs) and was evaluated using microscopy (OLYMPUS BX53, Tokyo, Japan) according to the intensity of CD8+ TILs. Two pathologists blinded to all sample information evaluated the CD8+ TILs. With respect to the detection of CD8+ TILs, the tissue was reviewed at $40\times$ magnification, and the area with the highest density of CD8+ TILs adjacent to malignant cells was counted at $400\times$ magnification (no. of CD8+ TILs/high-power field). The average number of CD8+ TILs in five high-power fields was included in the evaluation. For CD8, a count of zero CD8+ TILs in a high-power field was given a score of 0, a count of 1–3 CD8+ TILs was given a score of 1, a count of 4–10 CD8+ TILs was given a score of 2, and a count of >10 CD8+ TILs was given a score of 3 (Chiang et al. 2018; Goode et al. 2017; Huang et al. 2018c).

Statistical analysis

SAS statistical software version PC 9.4 (SAS Institute, NC, USA) was used to perform the statistical analysis. All tests reported a two-sided p value with a significance level set at 0.05. Student's t test, Pearson Chi-square and Fisher's exact test were used for group comparisons. Cox regression analysis was used to estimate the hazard ratios (HRs) and 95% confidence intervals (CIs) for univariate and multivariate models. Influential factors that affected the rectal cancer patient survival rate were adjusted in the Cox models, including TRG ($3+4$ vs $1+2$), clinical response (complete response and partial response vs stable disease and disease progression), and pN stage (positive vs negative). The Kaplan–Meier method was used to estimate the 5-year overall survival and disease-free survival. Survival time was defined as the time from surgery until death. The univariate comparison was performed using the log-rank test.

Results

Patient characteristics

Table 1 presents the clinical pathological characteristics of these pair-matched patients (112 pre-neoCRT biopsies and 97 post-neoCRT surgical tissues). The mean age at diagnosis was 59.4 ± 12.5 years (range 31–90 years). The

Table 1 Relationship between PD-L1 and various patient characteristics ($N = 112$)

Clinicopathological parameters	Total cases (%)	Tumor PD-L1 (pre-neoCRT)			Tumor PD-L1 (post-neoCRT)			
		High (%)	Low (%)	*p* value	High (%)	Low (%)	NA (%)	*p* value
	112 (100%)	56 (50%)	56 (50%)		61 (54%)	36 (32%)	15 (13%)	
Age				0.69				0.62
< 65	72 (64%)	35 (63%)	37 (66%)		42 (69%)	23 (64%)	7 (47%)	
≥ 65	40 (36%)	21 (38%)	19 (34%)		19 (31%)	13 (36%)	8 (53%)	
Sex				0.15				0.95
Female	35 (31%)	14 (25%)	21 (38%)		19 (31%)	11 (31%)	5 (33%)	
Male	77 (69%)	42 (75%)	35 (63%)		42 (69%)	25 (69%)	10 (67%)	
pN stage				0.32				0.33
Negative	75 (67%)	40 (71%)	35 (63%)		40 (66%)	20 (56%)	15 (100%)	
Positive	37 (33%)	16 (29%)	21 (38%)		21 (34%)	16 (44%)	0 (0%)	
Clinical TNM stage (7th AJCC)				0.04*				0.04*
I	4 (4%)	3 (6%)	1 (2%)		2 (4%)	2 (6%)	0 (0%)	
II	48 (45%)	25 (46%)	23 (44%)		28 (48%)	12 (36%)	8 (53%)	
III	54 (51%)	26 (48%)	28 (54%)		28 (48%)	19 (58%)	7 (47%)	
TRG				0.67				0.61
4	15 (13%)	6 (11%)	9 (16%)		0 (0%)	0 (0%)	15 (0%)	
3	60 (54%)	33 (59%)	27 (48%)		39 (64%)	21 (58%)	0 (0%)	
2	25 (22%)	12 (21%)	13 (23%)		16 (26%)	9 (25%)	0 (0%)	
1	12 (11%)	5 (9%)	7 (13%)		6 (10%)	6 (17%)	0 (0%)	
Clinical response				0.002*				0.03*
CR	15 (13%)	5 (9%)	10 (18%)		1 (2%)	0 (0%)	14 (93)%	
PR	41 (37%)	23 (41%)	18 (32%)		25 (41%)	15 (42%)	1 (7%)	
SD	50 (45%)	24 (43%)	26 (46%)		32 (52%)	18 (50%)	0 (0%)	
PD	6 (5%)	4 (7%)	2 (4%)		3 (5%)	3 (8%)	0 (0%)	
Concurrent chemotherapy				0.3				0.01*
Capecitabine	53 (47%)	31 (55%)	22 (39%)		30 (49%)	17 (47%)	6 (40%)	
UFT	38 (34%)	15 (27%)	23 (41%)		20 (33%)	12 (33%)	6 (40%)	
5-FU	11 (10%)	6 (11%)	5 (9%)		6 (10%)	4 (11%)	1 (7%)	
Others	10 (9%)	4 (7%)	6 (11%)		5 (8%)	3 (8%)	2 (13%)	
CD8+ TILs (pre-neoCRT)				0.42				0.33
High	36 (32%)	20 (36%)	16 (29%)		21 (34%)	9 (25%)	6 (40%)	
Low	76 (68%)	36 (64%)	40 (71%)		40 (66%)	27 (75%)	9 (60%)	
CD8+ TILs (post-neoCRT)				0.69				0.25
High	34 (30%)	18 (32%)	16 (29%)		24 (39%)	10 (28%)	0 (0%)	
Low	63 (56%)	32 (57%)	31 (55%)		37 (61%)	26 (72%)	0 (0%)	
NA	15 (13%)	6 (11%)	9 (16%)		15 (100%)	0 (0%)	0 (0%)	
Local recurrence				0.04*				0.04*
Negative	99 (88%)	53 (95%)	46 (82%)		57 (93%)	29 (81%)	13 (87%)	
Positive	13 (11%)	3 (5%)	10 (18%)		4 (7%)	7 (19%)	2 (13%)	
Distant metastasis				0.004*				0.26
Negative	88 (79%)	50 (89%)	38 (68%)		50 (82%)	26 (72%)	12 (80%)	
Positive	24 (21%)	6 (11%)	18 (32%)		11 (18%)	10 (28%)	3 (20%)	
NA	15 (13%)	6 (11%)	9 (16%)		0 (0%)	0 (0%)	15 (100%)	

pN stage: positive (Stage 1a + 1b + 2) vs negative (Stage 0 + X); Tumor PD-L1: high (grade 2 + 3) vs low (grade 0 + 1); CD8+ TILs: high (grade 2 + 3) vs low (grade 0 + 1)

Chi-square test was used

Fisher's exact test was used when > 25% of the cells have expected counts less than 5

The contrast test was not including the 'NA' group

*$p < 0.05$ (statistically significant)

majority of the patients were men (69%). The median radiation dose was 50.4 Gy administered in 28 fractions (minimum dose: 44.8 Gy; maximum dose: 50.4 Gy). Concurrent chemotherapy was fluorouracil-based for 44% of the LARC patients and capecitabine for 47%. All patients underwent total mesorectal excision (TME) depending on the extent and location of the tumor after neoCRT. Surgical specimens were reviewed and scored based on the Tumor Regression Grade (TRG) system (Rodel et al. 2005). In total, 13% (15/112) of patients exhibited a pathologic complete response (pCR, TRG 4), whereas 87% (97/112) of patients exhibited a pathologic partial response (TRG 1–3). After neoCRT treatment, a pathological tumor regression grade 4 (TRG 4) sample was not included in the TMA because the sample had no residual tumor. Thirty-seven patients (33%) presented with lymph node metastases, and twenty-four patients (21%) presented with distant metastasis (Table 1).

Tumor PD-L1 expression and CD8+ intra-tumoral infiltrating lymphocytes were analyzed by immunohistochemistry (IHC) and examined in tumor tissues and adjacent normal mucosae. PD-L1 was detectable in epithelial cells from normal colonic mucosae and cancer cells (Fig. 1). The clinicopathologic characteristics of patients and the correlation with PD-L1 expression in pre-neoCRT biopsies and post-neoCRT surgical tissues are presented in Table 1.

Tumor PD-L1 expression is associated with 5-year DFS and 5-year OS in LARC patients

We found that 28 LARC patients (25%) died within the 5-year follow-up period, and the estimated 5-year disease-free survival (DFS) and overall survival (OS) rates were 67% and 75%, respectively (Table 2). In the 5-year DFS analysis, patients with negative pN stage (79% vs 59%, $p=0.004$), good clinical response (82% vs 61%, $p=0.013$), and high tumor PD-L1 expression with the pre-treatment biopsies (86% vs 57%, $p=0.003$) exhibited significantly better DFS. Moreover, patients with high CD8+ TILs within tumor microenvironment in the pre-neoCRT biopsies exhibited a tendency for better DFS (83% vs 66%, $p=0.057$). After neoCRT regimen treatment, patients with high tumor PD-L1 (82% vs 53%, $p=0.003$) and CD8+ TILs (85% vs 63%, $p=0.0039$) within the tumor microenvironment in the post-neoCRT surgical tissues exhibit better DFS. In the 5-year OS analysis, patients with high tumor PD-L1 expression in the pre-neoCRT biopsies (91 vs 73%, $p=0.045$) and post-neoCRT surgical tissues (93 vs 64%, $p=0.0001$) exhibited significantly better OS.

By Kaplan–Meier survival analysis, patients with high tumor PD-L1 expression were associated with a significantly better 5-year DFS in the pre-neoCRT biopsies (Log rank $p=0.003$, Fig. 2a) and post-neoCRT surgical tissues (Log rank $p=0.003$, Fig. 2b). Patients with high CD8+TILs exhibit a tendency for better 5-year DFS in pre-neoCRT biopsies (log rank $p=0.0586$, Fig. 2c) and are associated

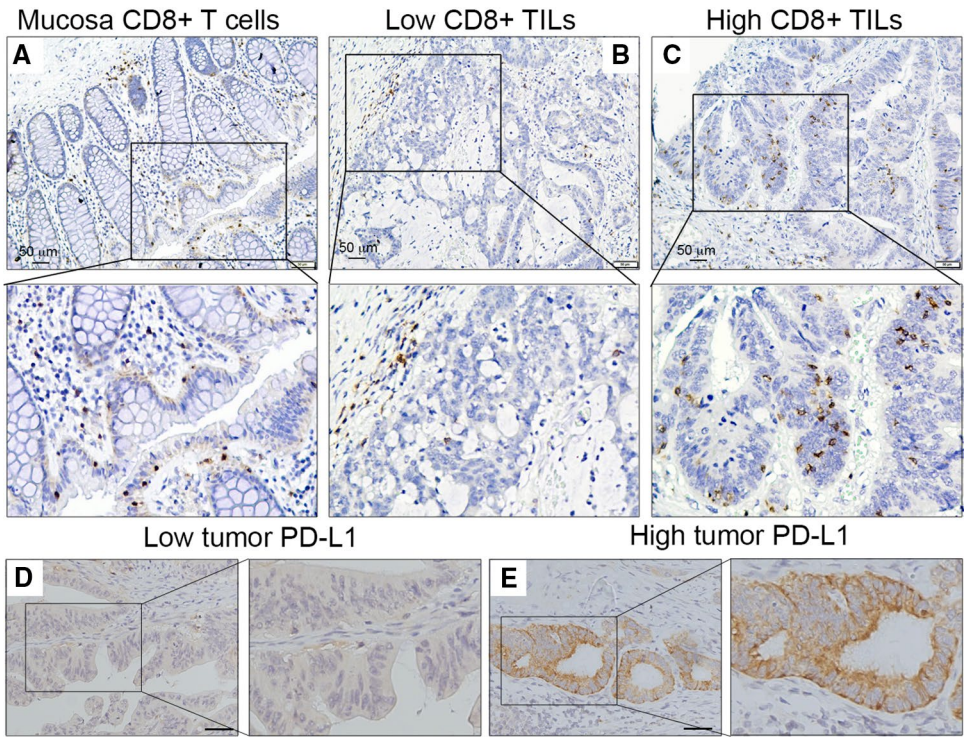

Fig. 1 Representative images of PD-L1 and CD8+ TIL immunohistochemistry in TMA patients with LARC. **a** CD8+ TIL expression in normal mucosa. **b, c** Low and high intra-tumoral CD8+ TILs within the tumor microenvironment. **d, e** Low and high tumor PD-L1 expression within the tumor microenvironment

Table 2 Clinicopathologic parameters and 5-year DFS and 5-year OS

Variable	Total case	5-year DFS, %	*p* value*	5-year OS, %	*p* value*
Pre-neoCRT ($n=112$)	112	67		75	
Sex			0.44		0.013*
Female	35	77		97	
Male	77	69		75	
pN stage			0.004*		0.089
Negative	75	79		85	
Positive	37	57		76	
Clinical response			0.013*		0.29
Good response	56	82		86	
Poor response	56	61		79	
TRG			0.13		0.11
Good response	75	76		87	
Poor response	37	62		73	
Tumor PD-L1 (pre-neoCRT)			0.003*		0.045*
High	56	86		91	
Low	56	57		73	
CD8+ TILs (pre-neoCRT)			0.057		0.21
High	36	83		89	
Low	76	66		79	
CD8+ TILs/tumor PD-L1 (pre-neoCRT)			0.004*		0.031*
High/High	20	100		100	
Low or Low	92	65		78	
Post-neoCRT ($n=97$)					
Tumor PD-L1 (post-neoCRT)			0.003*		0.0001*
High	61	82		93	
Low	36	53		64	
CD8+ TILs (post-neoCRT)			0.039*		0.18
High	34	85		91	
Low	63	63		78	
CD8+ TILs/tumor PD-L1 (post-neoCRT)			0.006*		0.022*
High/high	24	96		100	
Low or low	73	63		77	

pN stage: positive (Stage 1a + 1b + 2) vs negative (Stage 0 + X); Clinical response: good response (complete response and partial response) vs poor response (stable disease and progression disease); TRG: good response (TRG 3–4) vs poor response (TRG 1–2); Tumor PD-L1: high (grade 2 + 3) vs low (grade 0 + 1); CD8+ TILs: high (grade 2 + 3) vs low (grade 0 + 1)

Kaplan–Meier method was used for survival analysis

SE standard error

**p* value was obtained from log-rank test

with a significantly better 5-year DFS in the post-neoCRT surgical tissues (log rank $p=0.0385$, Fig. 2d). These results suggest that upregulated tumor PD-L1 reflects good clinical outcome in LARC patients.

Next, we assessed the survival differences between groups classified by these two factors. Within the combined group of CD8+ TIL and tumor PD-L1 subsets, patients with both high CD8+ TILs and tumor PD-L1 levels exhibited significantly better DFS in the pre-neoCRT biopsies (100% vs 65%, $p=0.0037$, Fig. 2e) or post-neoCRT surgical tissues (96% vs 63%, $p=0.0061$, Fig. 2f). These results suggest that combined CD8+ TILs and tumor PD-L1 expression can be a good prognostic factor for LARC patients who are receiving neoCRT treatment.

Independent risk factor for LARC patients with neoCRT treatment

In the univariate analysis of 5-year DFS, the following parameters were associated with patient survival rate: pN

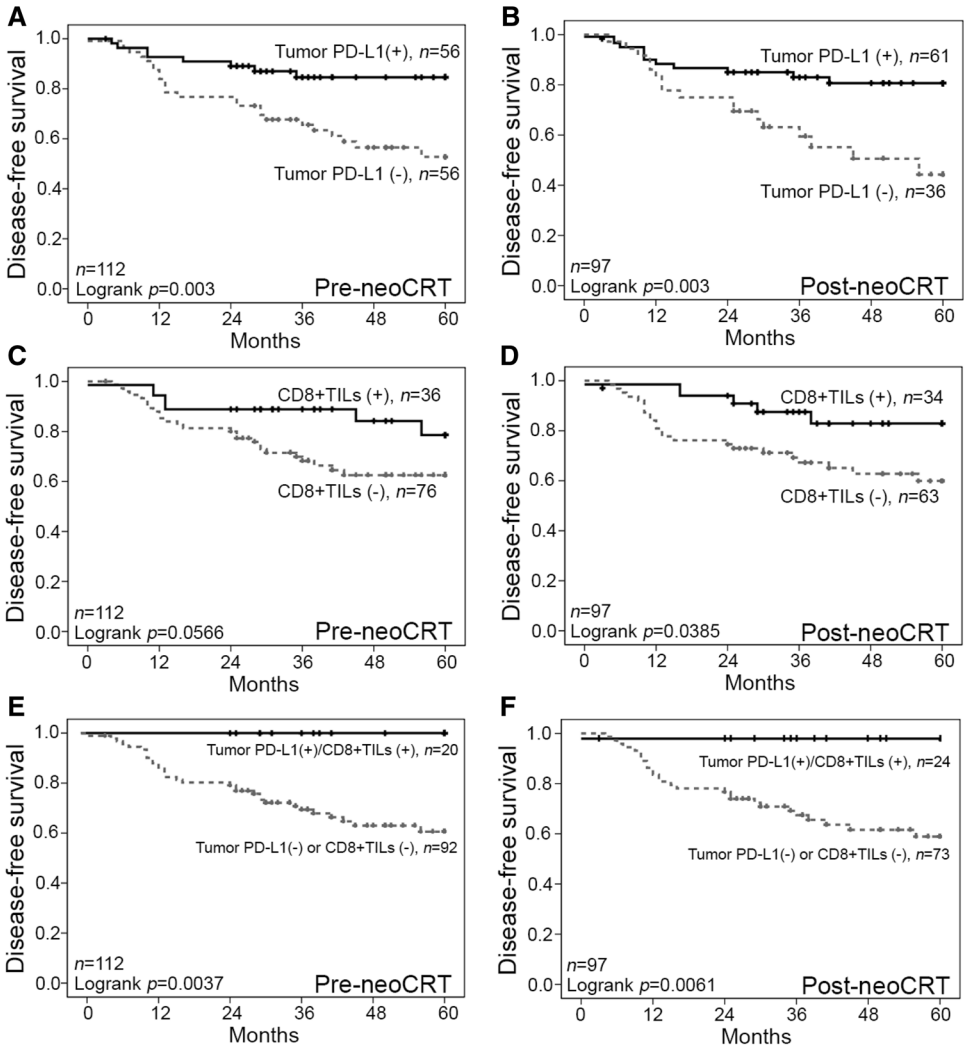

Fig. 2 Kaplan–Meier curves of DFS with tumor PD-L1 and intra-tumoral CD8+ TILs in LARC patients. **a** Kaplan–Meier curves demonstrating that tumor PD-L1 expression is associated with 5-year DFS in pre-neoCRT biopsies ($p=0.003$). **b** Kaplan–Meier curves demonstrating that tumor PD-L1 expression is associated with 5-year DFS in post-neoCRT surgical tissues ($p=0.003$). **c** High density of CD8+ TILs within the tumor microenvironment is with associated improved 5-year DFS in pre-neoCRT biopsies ($p=0.0566$). **d** High density of CD8+ TIL within the tumor microenvironment is associated with improved 5-year DFS in post-neoCRT surgical tissues ($p=0.0385$). **e** Patients with both high tumor PD-L1 and CD8+TILs within the tumor microenvironment are associated with improved 5-year DFS in pre-neoCRT biopsies ($p=0.0037$). **f** Patients with both high tumor PD-L1 and CD8+ TILs within the tumor microenvironment are associated with improved 5-year DFS in post-neoCRT surgical tissues ($p=0.0061$)

stage and clinical response. Moreover, CD8+ TIL counts and tumor PD-L1 levels were statistically associated with 5-year DFS. Patients with low tumor PD-L1 expression exhibited an increased risk for a poorer 5-year DFS (HR = 3.139, 95% CI 1.410–6.991, $p=0.005$), and those with a low density of CD8+ TILs also exhibited a tendency for increased risk of a lower 5-year DFS (HR = 2.308, 95% CI 0.950–5.611, $p=0.065$) compared with patients with a high CD8+ TIL count and high tumor PD-L1 in pre-neoCRT biopsies (Table 3). Moreover, patients with both a low CD8+ TIL count and tumor PD-L1 exhibited an increased risk in terms of 5-year DFS in the pre-neoCRT biopsies (HR = 17.01, 95% CI 2.41–2152.60, $p=0.05$). Similar results were also observed in the univariate analysis of 5-year OS (Table 3).

After neoCRT treatment, patients with low tumor PD-L1 exhibited an increased risk for reduced 5-year DFS (HR = 2.982, 95% CI 1.392–6.385, $p=0.005$), and those with a low density of CD8+ TILs also exhibited an increased risk for reduced 5-year DFS (HR = 2.663, 95% CI 1.012–7.011, $p=0.047$) compared with patients with a high CD8+ TIL count and high tumor PD-L1 in the post-neoCRT surgical tissues (Table 3). Moreover, patients with both a low CD8+ TIL count and tumor PD-L1 exhibited an increased risk in terms of 5-year DFS in the post-neoCRT surgical tissues (HR = 9.654, 95% CI 1.311–71.071, $p=0.026$). These results indicate that CD8+ TILs and tumor PD-L1 exhibit significant prognostic value for locally advanced rectal cancer patients.

Subsequently, we examined whether the inclusion of other variables affected the parameter estimate for CD8+ TILs and tumor PD-L1 (Table 4). Patients with a low tumor PD-L1 level within the tumor microenvironment presented an increased risk for poor DFS either in the pre-neoCRT biopsies (HR = 2.765, 95% CI 1.232–6.209, $p=0.01$, Table 4) or post-neoCRT surgical tissues (HR = 2.692, 95% CI 1.245–5.820, $p=0.01$, Table 4) after adjustment for age, pN stage, clinical response, and TRG. Moreover, patients with both a low density of CD8+ TILs or a low tumor PD-L1 level within the tumor microenvironment exhibited an increased risk of a poor DFS (HR = 8.132, 95% CI

Table 3 Univariate analysis of clinicopathologic parameters on 5-year DFS and 5-year OS

Variable	5-year disease-free survival (DFS)			5-year overall survival (OS)		
	HR	95% CI	*p* value	HR	95% CI	*p* value
Pre-neoCRT ($n=112$)						
Age (≥65 vs <65)	1.008	0.486–2.091	0.983	1.334	0.545–3.266	0.528
pN stage (positive vs negative)	2.676	1.333–5.369	0.006*	2.113	0.873–5.114	0.097
Clinical response (poor response vs good response)	2.501	1.184–5.285	0.016*	1.605	0.656–3.927	0.3
TRG (poor response vs good response)	1.703	0.846–3.428	0.136	2.009	0.836–4.828	0.119
Tumor PD-L1 (low vs high)	3.139	1.410–6.991	0.005*	6.56	2.14–20.18	0.001*
CD8+ TILs (low vs high)	2.308	0.950–5.611	0.065	2	0.668–5.985	0.215
Tumor PD-L1/CD8+ TILs (low vs high)	17.01	2.41–2152.60	0.05*	9.55	1.32–1214.77	0.12
Post-neoCRT ($n=97$)						
Age (≥65 vs <65)	1.236	0.570–2.678	0.592	1.54	0.59–4.05	0.38
pN stage (positive vs negative)	2.8	1.319–5.943	0.007*	2.25	0.87–5.84	0.1
Clinical response (poor response vs good response)	2.99	1.212–7.378	0.017*	1.82	0.64–5.18	0.26
TRG (poor response vs good response)	1.724	0.821–3.619	0.15	2.23	0.85–5.87	0.1
Tumor PD-L1 (low vs high)	2.982	1.392–6.385	0.005*	6.56	2.14–20.18	0.001*
CD8+ TILs (low vs high)	2.663	1.012–7.011	0.047*	2.28	0.65–7.93	0.2
Tumor PD-L1/CD8+ TILs (low vs high)	9.654	1.311–71.071	0.026*	10.79	1.47–1375.05	0.11

pN stage: positive (Stage 1a + 1b + 2) vs negative (Stage 0 + X); Clinical response: good response (complete response and partial response) vs poor response (stable disease and progression disease); TRG: good response (TRG 3–4) vs poor response (TRG 1–2); Tumor PD-L1: high (grade 2 + 3) vs low (grade 0 + 1); CD8+ TILs: high (grade 2 + 3) vs low

*$p < 0.05$ was considered statistically significant

Table 4 Multivariate analysis of cliniopathologic parameters on 5-year DFS

Variable	5-year disease-free survival (DFS)					
	Multivariate analysis			Multivariate analysis		
	HR	95% CI	*p* value	HR	95% CI	*p* value
Pre-neoCRT ($n=112$)						
Age (≥65 vs <65)	1.075	0.502–2.302	0.85	1.02	0.44–2.28	0.97
pN stage (positive vs negative)	1.97	0.866–4.481	0.11	2.33	1.04–5.28	0.04*
Clinical response (poor response vs good response)	1.645	0.680–3.978	0.27	1.44	0.61–3.53	0.41
TRG (poor response vs good response)	1.424	0.654–3.100	0.37	1.4	0.64–3.02	0.4
Tumor PD-L1 (low vs high)	2.765	1.232–6.209	0.01*	–	–	–
CD8+ TILs (low vs high)	1.657	0.657–4.126	0.29	–	–	–
Tumor PD-L1/CD8+ TILs (low vs high)	–	–	–	14.82	2.07–1881.19	0.06
Post-neoCRT ($n=97$)						
Age (≥65 vs <65)	1.205	0.542–2.684	0.65	1.198	0.527–2.724	0.67
pN stage (positive vs negative)	2.313	1.019–5.250	0.05*	2.42	1.077–5.438	0.03*
Clinical response (poor response vs good response)	1.875	0.685–5.128	0.22	1.753	0.647–4.749	0.27
TRG (poor response vs good response)	1.453	0.650–3.246	0.36	1.66	0.723–3.810	0.23
Tumor PD-L1 (low vs high)	2.692	1.245–5.820	0.01*	–	–	–
CD8+ TILs (low vs high)	2.144	0.809–5.679	0.13	–	–	–
Tumor PD-L1/CD8+ TILs (low vs high)	–	–	–	8.132	1.103–59.978	0.04*

pN stage: positive (Stage 1a + 1b + 2) vs negative (Stage 0 + X); Clinical response: good response (complete response and partial response) vs poor response (stable disease and progression disease); TRG: good response (TRG 3–4) vs Poor response (TRG 1–2); Tumor PD-L1: high (grade 2 + 3) vs low (grade 0 + 1); CD8+ TILs: high (grade 2 + 3) vs low (grade 0 + 1)

*$p < 0.05$ was considered statistically significant

1.103–59.978, $p = 0.04$, Table 4). These results demonstrate that the combination of CD8+ TILs and tumor PD-L1 levels is an independent prognostic factor (Table 4).

Discussion

This pair-matched analysis of pre-neoCRT biopsies and post-neoCRT surgical rectal cancer specimens demonstrated that both PD-L1 expression and the density of CD8+ TILs markedly increased after preoperative chemoradiotherapy (neoCRT).

Patients with a consistently high level of PD-L1 expression experienced better DFS and OS, and patients with high CD8+ TILs recruitment within tumor microenvironment by neoCRT also exhibited improved DFS. These rectal cancer patients with both high PD-L1 and CD8+ TILs profiles were associated with best survival outcomes compared with other groups as assessed by Kaplan–Meier analysis. Multivariate analysis models demonstrated that patients with low PD-L1 expression and CD8+ TILs are associated with increased risk on DFS after neoCRT treatment, suggesting that tumor PD-L1 and CD8+ TILs are prognostic factors for locally advanced rectal cancer receiving neoCRT.

To date, few studies evaluated PD-L1 expression after neoCRT in all malignancies, including rectal cancer (Hecht et al. 2016; Jomrich et al. 2016; Ogura et al. 2018; Saigusa et al. 2016). Our analysis including 112 pair-matched rectal cancer patients, 112 pre-neoCRT biopsies and 97 post-neoCRT surgical specimens (15 patients with TRG4), and we observed a significant increase in the proportion of tumor PD-L1. This matched pair analysis of rectal cancer highlights the immunologic impact of neoCRT on the level of PD-L1 checkpoint molecules and CD8+ TIL recruitment, which demonstrates their prognostic value on survival outcome in patients with locally advanced rectal cancers. Based on increased PD-L1 expression and CD8+ TIL density after neoCRT, more prominent tumor-specific immune responses after treatment could be expected. Direct irradiation on tumor tissues upregulates and releases tumor-associated antigens (TAAs) (Gameiro et al. 2014), damage-associated pattern molecules (DAMPs) (Huang et al. 2018b), and major histocompatibility complex molecules (MHC), which is an important underlying mechanism of immunogenic cell death (Pol et al. 2015). Consecutive chemoradiotherapy promotes TAA and DAMP release, increasing inflammatory cytokines and cytotoxic T lymphocytes (CTLs) maturation (Showalter et al. 2017). These effects result in the shift of immunologic equilibrium within the tumor microenvironment. Recent reports have demonstrated that tumor PD-L1 expression was strongly correlated with improved survival outcomes in several malignancies, such as breast cancer (Baptista et al. 2016; Sabatier et al. 2015; Schalper et al. 2014), NSCLC (Velcheti et al. 2014), malignant melanomas (Taube et al. 2012), and CRC (Droeser et al. 2013). However, previous studies have demonstrated that high tumor PD-L1 expression was identified in mismatch repair-deficient (MMR) colorectal cancer patients (Lee et al. 2016), who exhibit a better therapeutic response to anti-PD-1 immunotherapy (Dudley et al. 2016). Kim et al. (2016) reported that PD-L1 expression in MSI-high colorectal cancer is associated with higher CD3+ TILs. MSI-high colorectal cancer is characterized by high mutation load, a high level of TILs, and high PD-L1 expression on tumor and stromal immune cells (Xiao and Freeman 2015). However, MSI-high is rare in rectal cancer (Phipps et al. 2015), and the underlying mechanism of high tumor PD-L1 in the present study should be further investigated.

Immune system function is a dynamic balance between stimulatory and inhibitory forces and upshifting the anti-tumor immunity is a well-known trigger for inhibitory immune checkpoints. In our results, a marked neoCRT-induced immunologic response increased the expression of PD-L1 [pre-neoCRT: 50.0% (56/112) and post-neoCRT: 62.9% (61/97)] and density of CD8+ TILs [pre-neoCRT: 32.1% (36/112) and post-neoCRT: 35.1% (34/97)]. Moreover, patients with sustained high PD-L1 activity or high CD8+ TIL density before and after neoCRT exhibited better survival outcomes. In neoCRT-treated rectal cancer, the mechanisms of increased PD-L1 expression after neoCRT remain to be elucidated. However, these high CD8+ TILs after neoCRT, which could be induced by increased TAAs and DAMPs release by neoCRT (Huang et al. 2018b; Smyth et al. 2015), was associated with high PD-L1 expression.

Recent studies implied that high tumor PD-L1 expression is involved with the feedback mechanism caused by the induction of IFN-γ, especially in tumor cells and CD8+ TILs (Chiang et al. 2018; Droeser et al. 2013). Hence, our data demonstrate that PD-L1 may be upregulated by the host immune response through IFN-γ that is released from CD8+ TILs, suggesting that subsequent treatment may reinvigorate the preexisting anti-tumor immune response, leading to better responses. Therefore, these immunologic factors were used to identify patients with more prominent anti-tumor immunity, suggesting potential candidates who can benefit from further enhancement of tumor-specific immune responses. If increased PD-L1 expression after neoCRT is related to an immune-suppressive microenvironment, immune checkpoint blockade might improve the response to neoCRT in PD-L1 high rectal cancer. Future studies are warranted to establish the validity of a therapeutic strategy combining checkpoint inhibitors with conventional cytotoxic treatments or neoCRT to improve the response rate in rectal cancer.

Taken together, this study demonstrates that increased PD-L1 expression in both pre- and post-neoCRT tissues

correlate with improved prognosis for patient with LARC. Moreover, combinational PD-L1 expression and CD8+ TILs may be useful biomarkers to predict outcomes in patients receiving neoCRT treatment for LARC.

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Author contributions T-WC, S-FC and KC-YH conducted and performed the experiments; WT-LC, T-WK and T-WC enrolled the LARC patients and performed IHC evaluation; S-FC and KSCC supervised this study; S-FC, and KSCC analyzed the data and wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was reviewed and approved by the Internal Review Board (IRB) of China Medical University Hospital [Protocol number: CMUH105-REC2-072].

Informed consent Informed consents were obtained from all participants in the study.

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