$CD8^+$ T cell densities and PD-L1 associated with favorable prognosis in schistosomiasis-associated Colorectal Cancer

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Research

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Abstract

Backgroud: The expression of programmed cell death-ligand 1 (PD-L1) was correlated with CD8+ T cells, which could producing IFNγ. The effect of infection of *Schistosoma japonicum* on CD8+ tumour-infiltrating lymphocytes (TILs) and then on PD-L1 expression has rarely been reported and the utility of CD8+ TILs as a biomarker for colorectal cancer (CRC), especially for schistosomal CRC, are still controversial and needing to be determined in CRC.

Methods: A total of 338 patients with CRC were enrolled in this study. Immunohistochemical analysis were performed to evaluated the expression of PD-L1 within tumor cells (tPD-L1) and within stromal cells (sPD-L1), infiltration by CD8+ T cells.

Results: In the whole cohort, results showed that $CD8^+$ TIL density was positively correlated with tumoral and stromal PD-L1 expression ($p<0.05$). But there were no correlation between schistosomiasis and PD-L1 and CD8+ TILs. Furthermore, $CD8^+$ TIL density, schistosomiasis, TNM stage, lymph nodes positive for CRC and gender were significantly independent predictive factors for overall survival (OS)($p<0.05$). Stromal PD-L1 but not tPD-L1 expression was correlated with OS but was not an independent predictor (*p=0.046*). In patients without schistosomiasis, sPD-L1 was significantly associated with better OS but was not an independent predictor($p =0.004$).However, there were no association between schistosomiasis and OS in patients with schistosomal ifection.

Conclusions: Our analysis indicated that $CD8^+$ was an independent predictive factor for OS. And the expression of PD-L1 was positively associated with CD8+ TILs density. There were no correlation between schistosomiasis and PD-L1 and CD8+ TILs. Stromal PD-L1 but not tPD-L1 was significantly associated with OS, but was not an independent prognostic factor. It is proposed that PD-L1 expression in combination with $CD8^+$ TIL could be a useful predictive biomarker in CRC patients.

Introduction

Colorectal cancer is one of the most common malignant disease worldwide. Although a variety of anticancer drugs have been developed, the death rates of CRC have not been obviously decreased [1, 2]. Antibody blockade of the programmed death-1 receptor/programmed death-ligand 1(PD-1/PD-L1) signaling pathway have been used for the treatment of malignant cancers, showing promising outcomes[3, 4]. Expression of PD-L1 in intratumoral compartment has been suggested to influence immune response[5]. Binding of PD-L1 to its receptors PD-1 and B7.1 suppresses T cell migration, proliferation and secretion of cytotoxic mediators, ultimately restricting tumour killing by diminishing effector T cell functioning[6, 7], showing immunosuppressive effect. However, PD-L1 is not solely considered as a result of an increased immune inhibiting PD/PD-L1 interplay but rather is viewed as a reflection of adaptive antitumor immunity, where tumor-infiltrating lymphocytes are activated in response to tumor antigens[8]. Thus, the role of PD-L1 in tumor microenvironment remains obscure. Recent study showed that tumoral PD-L1 is a favorable prognostic factor in early stage non-small cell carcinoma[9]. It was also reported that there were differences in outcome in triple-negative breast cancer depending on the expression of PD-L1 in the tumour cell membrane, cytoplasm and

stromal cellular compartments[10]. Yaqi Li *et al* reported that tumoral PD-L1 correlates with better prognosis of CRC patients[11]. Whereas some studies found that PD-L1 was associated with deleterious effect on survival[12, 13], but these studied did not distinguish PD-L1 expression in tumoral or stromal cells. Therefore, PD-L1 expression used as a predictor factor is also controversial.

On the other hand, studies reported that CD8 + TIL induces PD-L1 expression in tumour cells by producing IFNγ[14, 15, 16]. CD8 + T cells are thought to have antitumor functions during tumor development in a tumor microenvironment. Evidence has shown that activated $CD8^+$ cytotoxic T lymphocytes were correlates with favorable survival of CRC patients and gastric cancer patients[17, 18,19, 20]. Therefore, further detailed analysis is needed to confirm the prognostic significance of PD-L1 and CD8 + TILs in CRC and to investigate the relationship between PD-L1 and CD8+, .

Intriguingly, we discovered schistosome eggs in almost 38% of cases in HE-stained slides under the microscope. Qingpu District of Shanghai in China was ever one of endemic areas. Schistosomiasis, which is an infectious disease[21], is considered a risk factor for CRC[22]. Schistosomiasis is correlated with inflammation[23–25]. CD8 + TILs are the main force involved in inflammatory response. In addition, PD-L1 was involved in immune microenvironment and upregulated by CD8 + TILs. With these considerations, we wonder to investigate the relationship between schistosomiasis and CD8 + TILs and PD-L1.

In short, this study aimed primarily to investigate the effect of schistosome infection on CD8 + TILs and PD-L1 expression and the relationship between schistosomiasis and CD8 + TILs and PD-L1 expression. Besides, we proposed to further to evaluate the prognostic role of PD-L1 and CD8 + TILs in CRC, especially in schistosomal CRC.

Methods And Materials

Patients

This retrospective analysis includes 338 patients with resected primary CRC at Qingpu Branch of Zhongshan Hospital affiliated to Fudan University, from January 2008 to August 2016. All of the operations followed the principle described previously[26]. Briefly, adequate resection margins, all circumferential margins were cleared. The number of positive lymph nodes and total number of retrieved lymph nodes were recorded. The inpatient medical records and pathological reports were reviewed from the pathological system and Qingpu District Center for Disease Control and Prevention, and the patients were followed up by telephone. OS is defined as the interval from the surgical operation date to the last follow-up or death caused by CRC. Inclusion criteria included the following: (i) patients with CRC as primary focus, (ii) none of these patients had received any prior anti-tumor therapy, and (iii) patients were diagnosed as adenocarcinoma by pathology after resection of CRC. Exclusion criteria included the following: (i) Tis tumors, (ii) patients who lacked complete information, (iii) patients with synchronous malignancy, and (iv) patients with survival time less than 1 month. Two expert pathologists reviewed HE-stained slides to determine the diagnosis and to restage the tumors according to the eighth edition of American Joint Committee on Cancer (AJCC). This study approved by the medical ethics

committee of Fudan University, in accordance with the Helsinki Declaration of 1975. Prior written informed consent was obtained from all patients.

Tissue microarrays(TMA)

The TMA blocks were manufactured from the most representative areas of individual paraffin blocks, as previously described[27]. Briefly, reviewed HE-stained slides and marked the represented areas in tumor tissues, and the single core (2 mm wide and 6 mm long) for each case was p

recisely arrayed in to a new recipient paraffin block. The cores containing more than 20% tumor cells were considered as valid cores.

Immunohistochemical (IHC)

All the tissue slides were stained by the fully automated Bond-□ system (Leica Microsystems, Newcastle-upon-Tyne, UK) according to the manufacturer`s instructions. The following primary antibodies were used: PD-L1 (MXR003; 1:750; MXB Biotechnologies, Fuzhou, China) and CD8 (clone NCL-L-CD8-4B11; 1 : 100; DAKO, Minneapolis, MN, USA). In brief, IHC labeling was performed on 4-mm-thick unstained sections. Slides were deparaffinized with serial xylene treatments and subjected to antigen retrieval using onboard heat-induced antigen retrieval with epitope retrieval solution (pH 9.0) at 100°C for 10 minutes, and incubated with the antibody for 30 min at room temperature. This automated system used a Refine polymer detection kit with horseradish peroxidase-polymer as a secondary antibody and DAB, and incubation with a secondary antibody was performed for 30 min at room temperature.

Pathological assessment of PD-L1 expression and CD8 + T cell density

PD-L1 IHC was analyzed independently by two experienced pathologists, who were unware to the clinical data. The results were evaluated according to the percentage of the stained cells. Scoring was assessed in both the tumoral and stromal compartments: tumoral membranous and stromal immune cell membranous compartments. Tumors were classified as PD-L1 positive if there was ≥ 1% tumoral membranous PD-L1 expression (tPD-L1$^+$), or ≥ 1% stromal PD-L1 expression (sPD-L1$^+$).

The TMA slides were scanned using a scanner system (PRECICE 500B) at 40×magnification. For CD8, the densities of positively stained cells were evaluated on whole section slides using an image analysis system (Image J software, USA) (cells per square millimeter) (Fig. 1C). At least half of the core area was selected randomly, and the results of the calculated densities were extracted and put into an Excel file. Measurements were recorded as the mean number of positive cells per tissue unit in square millimetres as well as the number of positive cells among each 1-mm^2 tissue units.

Detection of schistosome ova and assessment of tumor budding

Schistosome ova were observed in all of original HE stained formalin-fixed paraffin-embedded (FFPE) sections (usually 4–6 slides), which were examined at × 10 and × 40 magnification fields using a

conventional light microscope by two pathologists who were blinded to the clinical data. The diagnosis of schistosomiasis was done by finding schistosome eggs in HE-stained slides.

Tumor budding was defined as the presence of dedifferentiated single cells or small clusters of up to 5 cells at the invasive front of CRC[28]. The assessment of tumor budding was conducted as described previously[26]. Briefly, the 10-HPF method was used[29], the invasive front is first scanned at low magnification (×4 to ×10) to identify areas of highest budding density. Tumor buds are then counted under high magnification (×40), and the tumor budding count is reported. The evaluation of tumor budding was conducted by two pathologists who were blinded to the clinical data. Five tumor budding counts were used as breakthrough point. In brief, tumor bud counts greater than or equal to 5 were defined as the high group, otherwise as the low group.

Statistical analysis.

Data were analyzed using SPSS (version 20.0; IBM Corp.) and Graphpad 5.0. Every variable was analyzed using univariate analysis to identify all potentially important predictors and then variables with $P \leq 0.05$ in the univariate analysis were included in a multivariate analysis. Clinically relevant variables that may have impacted outcomes, such as age, gender, TNM stage, lymph node metastasis, histological type and so on. Finally, multivariate Cox regression analysis was performed to identify predictive factors for OS. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Patients characteristics

The clinical characteristics of the 338 patients are shown in Table 1. The median age of the patients at diagnosis was 67 years (range, 33–91 years). Two hundred and fourteen (61%) of the patients were male. By anatomic site, 27% tumors were in the rectum, 33% in the left colon, and 40% in the right colon. Lymph node metastasis was observed in 41% of patients, and 46% of patients were at late-stage disease. According to AJCC Staging Manual (seventh edition), there were very few highly differentiated cases in the follow-up data. Seventy-six percent cases were well/moderate differentiated, and 24% were poorly differentiated. Vessel invasion was observed in about 36% patients, but only 1.2% patients was lymph nodes positive. Intriguingly, schistosoma infection was observed in 38% (128 out of 338) CRC patients(Supplementary Fig. 1).

Table 1
Clinicopathological characteristics of the CRC cohort

Characteristics	**All patients (N = 338)**	
	N	*%*
$CD8^{low}$	104	*69*
$tPD\text{-}L1^{pos}$	138	*41*
$sPD\text{-}L1^{pos}$	200	*64*
Age (≥60ys)	83	*24*
Gender (Male)	214	*61*
Tumor location		
Rectum	91	*27*
Left colon	112	*33*
Right colon	135	*40*
Tumor diameter (≥5cm)	166	*49*
Tumor differentiation		
Well/moderate diff.	256	*76*
Poor diff.	82	*24*
Vessel invasion (present)	120	*36*
Nervous invasion (present)	31	*0.9*
Lymph nodes positive for CRC (≥2 nodes)	42	*1.2*
Bowel perforation (present)	13	*0.4*
Tumor budding (≥ 5 buds)	215	*64*
Ulceration (Yes)	145	*43*
Histological type		
Adenocarcinoma	297	*88*
Mucinous/SRCC	41	*12*
Pathological T stage		
T1-2	80	24
T3-4	258	76

Characteristics	All patients (N = 338)	
Lymph node metastasis (Yes)	140	*41*
TNM stage		
I + II	184	*54*
III + IV	154	*46*
Schistosomiasis (Positive)	128	*38*

Note: CD8low = density ≤ 279 cell /mm2; Abbreviation: CRC = Colorectal Cancer; N = Number; SRCC = Signet ring cell carcinoma.

Staining Results of Each Marker

It has been reported that PD-L1 on either tumor cells or host immune cells contributes to tumor escape, and the relative contributions of PD-L1 on these cells seem to be context-dependent[30]. Thus, the immunostaining for PD-L1 was observed in the membrane of the tumor cells and stromal lymphocytes. Figure 1 shows representative PD-L1-stained images on both tumor cells and tumor-infiltrating mononuclear cells. Among 338 cases analyzed, 41% of cases showed tumoral PD-L1 expression (tPD-L1$^+$: defined as ≥ 1%), and 64% showed PD-L1 expression within the immune stroma (sPD-L1$^+$: defined as ≥ 1%) (Table 1 and Fig. 1A-B). The median of CD8$^+$ density was 405 cell/mm^2 (range, 0-2466 cell/mm^2) (Table 1 and Fig. 1C).

Relationship between Schistosomiasis and CD8$^+$ TIL Density and PD-L1 Expression

Previous studies indicated that schistosomasis was a risk factor for CRC[26, 31, 32]. And chronic schistosomal infection was related with inflammation. Thus, we further made subgroups according to whether the patient has schistosomiasis: patients with schistosomiasis (CRC-S) and without schistosomiasis (CRC-NS). As shown in Fig. 2A, there were no significant correlation between CD8 + TILs density and schistosomiasis (p 0.05).

We next compared the density of CD8$^+$ T cells in CRC with or without expression of PD-L1 on the tumour cells or in the immune stroma, respectively. As shown in Fig. 2B and Table 2, CD8$^+$ T cell density was significantly higher within sPD-L1$^+$ group than sPD-L1$^-$ group (sPD-L1$^-$ group versus sPD-L1$^+$ group, median 347 versus 460 cell/mm^2, $p < 0.0001$), and it was also obviously higher within the tPD-L1$^+$ group than in the tPD-L1$^-$ group (tPD-L1$^+$ group versus tPD-L1$^-$ group, median 371 versus 454 cell/mm^2, $p = 0.0102$).

Table 2
The association between clinicopathological characteristics and PD-L1.

Variables	No.	sPD-L1 expression		P		iPD-L1 expression		P
		Negative (N = 196)	Positive (N = 142)		No.	Negative (N = 200)	Positive (N = 138)	
Age				0.898				0.698
≤60	80	47(24%)	33(23%)		80	49(25%)	31(22%)	
≥ 60	258	149(76%)	109(77%)		258	151(75%)	107(78%)	
Gender				0.599				0.429
Male	205	120(61%)	85(60%)		133	125(62%)	80(58%)	
Female	133	76(39%)	57(40%)		205	75(38%)	58(42%)	
Tumor site				0.518				0.216
Rectum	91	49(25%)	42(30%)		91	52(26%)	39(28%)	
Left colon	112	68(35%)	44(31%)		112	74(37%)	38(28%)	
Right colon	135	79(40%)	56(39%)		135	74(37%)	61(44%)	
Tumor diameter				0.123				0.077
≤5cm	166	89(45%)	77(53%)		166	90(45%)	76(55%)	
≥ 5cm	172	107(55%)	65(47%)		172	110(55%)	62(45%)	
Tumor differentiation				0.797				0.521
Moderate	256	147(75%)	109(77%)		256	154(76%)	102(75%)	
Poor	82	49(25%)	33(23%)		82	46(24%)	36(25%)	
Pathological T stage				≤0.001*				0.177
I-II	77	28(14%)	49(35%)		65	39(20%)	26(19%)	
III	261	168(86%)	93(65%)		231	161(80%)	70(81%)	
Lymph node metastasis				0.034*				0.370
No	198	105(54%)	93(65%)		198	113(57%)	85(62%)	
Yes	140	91(46%)	49(35%)		140	87(43%)	53(38%)	
Tumor budding				0.039*				0.563

Variables	No.	sPD-L1 expression		P		iPD-L1 expression		P
		Negative (N = 196)	Positive (N = 142)		No.	Negative (N = 200)	Positive (N = 138)	
Negative (5 buds)	123	62(32%)	61(43%)		123	67(34%)	56(41%)	
Positive (≥ 5 buds)	215	134(68%)	81(57%)		215	133(66%)	82(59%)	
Vessel invasion				0.250				0.425
No	218	121(62%)	97(68%)		272	126(63%)	91(66%)	
Yes	120	75(38%)	45(32%)		67	74(37%)	47(34%)	
Nerve invasion				0.849				0.614
No	307	177(90%)	130(92%)		307	181(91%)	126(91%)	
Yes	31	19(10%)	12(8%)		31	19(9%)	12(9%)	
Lymph nodes positive for CRC				0.018*				0.866
No	297	165(84%)	132(93%)		297	174(87%)	123(89%)	
Yes	41	31(16%)	10(7%)		41	26(13%)	15(11%)	
Histological type				0.314				0.471
Adenocarcinoma	297	169(86%)	128(90%)		297	175(88%)	122(88%)	
Mucinous/SRCC	41	27(14%)	14(10%)		41	25(12%)	16(12%)	
Bowel perforation				0.001*				0.859
No	325	185(94%)	140(99%)		332	192(96%)	133(96%)	
Yes	13	11(6%)	2(9%)		13	8(4%)	5(4%)	
Ulceration				0.825				0.738
No	193	113(58%)	80(56%)		193	116(58%)	77(56%)	
Yes	145	83(42%)	62(44%)		145	84(42%)	61(44%)	
TNM Stage				0.037*				0.020*
I	185	97(49%)	88(61%)		184	104(52%)	80(58%)	
II								
III	155	99(51%)	56(39%)		138	96(48%)	42(42%)	
IV								

Variables	No.	sPD-L1 expression		P		iPD-L1 expression		P
		Negative (N = 196)	Positive (N = 142)		No.	Negative (N = 200)	Positive (N = 138)	
Schistosomiasis				0.650				0.210
Negative	210	124(63%)	86(61%)		210	130(65%)	80(58%)	
Positive	128	72(37%)	56(39%)		128	70(35%)	58(42%)	
$CD8^+$ T cell density				0.001*				0.023*
Low group	1044	74(38%)	30(21%)		104	71(36%)	33(24%)	
High group	234	122(62%)	112(79%)		234	129(64%)	105(76%)	

—:Data is not applicable; Abbreviation: sTILs = stromal tumour-infiltrating lymphocytes; CRC-NS = patients without schistosomiasis; CRC-S = patients with schistosomiasis; N = Number; LN = Lymph node. The association between schistosomiasis and clinicopathological characteristics was evaluated by using the Chi square and Fisher's exact tests.

Correlation between PD-L1 Expression and Patient Characteristics

The relationships between tPD-L1 and sPD-L1 expression with clinicopathologic features are detailed in Table 3. A ROC analysis showed that the optimal cut-off value of tPD-L1 and sPD-L1 were both 2% for CRC OS. One hundred and thirty-eight patients (41%) and 142 (42%) were placed in the $tPD\text{-}L1^{high}$ (expression level ≥ 2%) and $sPD\text{-}L1^{high}$ group (expression level ≥ 2%) based on the optimum cutoff point, respectively. Stromal PD-L1 positivity were significantly associated with less aggressive tumor features, including early pathological T stage (p□0.001), absence of lymph node metastasis ($p = 0.031$), absence of lymph nodes positive for CRC ($p = 0.012$), early TNM Stage ($p = 0.034$), less tumor budding($p = 0.039$) and less bowel perforation (p□0.001). Meanwhile, tumoral PD-L1 positivity were significantly associated with early TNM Stage ($p = 0.020$), but there were no correlation between tPD-L1 expression and other clinicopathological factors (Table 2). It was suggested that higher PD-L1 expression lever both in tumoral cells and stromal cells were along with less aggressive features of tumors.

Table 3
Univariate and multivariate Cox regression of clinicopathological for overall survival

Variables	Univariate analysis		Multivariate analysis	
	P	HR(95%CI)	P	HR(95%CI)
Age (≥60ys)	0.012	1.754(1.129–2.726)		
Gender (male)	0.011	1.590(1.112–2.272)	0.005	1.626(1.133–2.335)
Tumor diameter(5cm)	0.881	0.975(0.669–1.360)		
Tumor site				
Rectum		Refer		
Left colon	0.906	1.026(0.673–1.562)		
Right colon	0.438	0.849 (0.561–1.284)		
Pathological T stage	＜0.001	2.453 (1.477–4.074)		
Lymph node metastasis	＜0.001	2.891(2.058–4.060)		
TNM stage	＜0.001	3.273(2.305–4.649)	＜0.001	2.755(1.887–4.022)
Tumor differentiation	0.002	1.775(1.242–2.537)		
Vessel invasion	＜0.001	1.925(1.376–2.692)		
Nerve invasion	0.133	1.509(0.882–2.584)		
Lymph nodes positive for CRC	＜0.001	4.095(2.724–6.156)	＜0.001	2.102(1.351–3.270)
Bowel perforation	0.815	0.888(0.328–2.401)		
Tumor budding	＜0.001	1.856(1.274–2.705)		
Schistosomiasis	0.048	1.388(0.994–1.940)	0.042	1.424(1.016–1.996)
Ulceration	0.554	0.903(0.644–1.266)		
Histological type	0.521	1.168(0.727–1.875)		
CD8 density	＜0.001	0.424(0.294–0.611)	0.010	0.635(0.449–0.897)
sPD-L1	0.046	0.702(0.496–0.993)		
iPD-L1	0.540	0.637(0.326–1.266)		

—:Data is non-significant ; Abbreviation: CRC-NS = patients without schistosomiasis; CRC-S = patients with schistosomiasis; CI = confidence interval; HR = Hazard ratio; LN = Lymph node; $P < 0.05$ was defined as the criterion for variable deletion when performing backward stepwise selection.

Prognostic Significance of PD-L1 Expression and $CD8^+$ T cells Density

Mean and median time to OS was 62.54 and 62.85(1.25–134.4) months, respectively. During the follow-up, there were 42% (141 out of 338) patients who died. PD-L1 expression on both tumour cells (expression level ≥ 2%, $tPD\text{-}L1^{high}$) and in the immune stroma (expression level ≥ 2%, $sPD\text{-}L1^{high}$) was associated with better OS in CRC patients, but only the association with immune stromal expression of PD-L1 reached the level of statistical significance ($p = 0.0023$, Fig. 3A for sPD-L1; $p = 0.3693$, Fig. 3B for tPD-L1).

With regard to $CD8^+$T cell, the optimum cutoff value of $CD8^+$T cell density were determined by X-tile program, which were 279 cell/mm^2 (Supplementary Fig. 2). Patients were divided into 2 groups for further analysis ($CD8^{low}$ < 279 and $CD8^{high}$ ≥ 279 cell/mm^2). Tumours with higher $CD8^+$ T cell density had better OS compared with tumours with lower $CD8^+$ T cell densities ($p < 0.0001$, respectively, Fig. 3C).

The univariate Cox regression model indicated that age, gender, pathological T stage, lymph node metastasis, TNM stages, tumor differentiation, vessel invasion, lymph nodes positive for CRC, tumor budding, *Schistosomiasis*, $CD8^+$ T cells and sPD-L1 were significantly associated with CRC OS ($p < 0.05$, Table 3), whereas none of the other factors examined were significantly associated with OS. Multivariate analysis after adjustment indicated that gender, TNM stage, lymph nodes positive for CRC, *Schistosomiasis*, $CD8^+$ T cells were independent prognostic factors for OS of CRC patients ($p < 0.05$,Table 3), but sPD-L1 was not an independent predictor.

Survival analysis based on subgroups

Kaplan-Meier analysis demonstrated that merely sPD-L1 expression level was associated with favorable OS in the CRC-NS group ($p = 0.0040$) (Fig. 4A), sPD-L1 expression level in the CRC-S group and tPD-L1 in the both groups were not correlated with OS ($p > 0.05$) (Fig. 4B, C and D). In the CRC-NS set, the univariate Cox regression model revealed that gender, TNM stage, pathological T stage, lymph node metastasis, tumor differentiation, tumor budding, vessel invasion, lymph nodes positive for CRC, sPD-L1 expression level and $CD8^+$ T cells density were associated with OS ($p < 0.05$) (Supplementary Table 1), and the multivariate Cox regression analysis showed that gender, lymph node metastasis, vessel invasion, lymph nodes positive for CRC, sPD-L1 expression level and $CD8^+$ T cells density were independent prognosis factors ($p < 0.05$) (Supplementary Table 1). In the CRC-S set, the univariate analysis demonstrated that lymph node metastasis, TNM stage, tumor differentiation, lymph nodes positive for CRC and CD8 + T cell density were associated with OS ($p < 0.05$), and multivariate analysis results showed that only TNM stage, lymph nodes positive for CRC and CD8 + T cell density were independent factors for OS ($p < 0.05$).

Discussion

Various tumor entities with elevated immune response, including MSI-H CRC, have dense CD8 pos T-cell infiltrates in common, which are responsible for a local production of interferon gamma (IFNγ) [33, 34]. IFNγ, in turn, provokes the adaptive upregulation of PD-L1 on nearby tumor cells via NFκB[35], thereby mediating a

negative feedback mechanism that ultimately leads to T-cell exhaustion in tumor-infiltrating lymphocytes. Emerging data in other tumor types suggest that negative immune checkpoint proteins are usually upregulated in tumor tissues with a "T cell inflamed phenotype" and that infiltration of tumours by effector T cells is necessary to drive upregulation of immune checkpoints[36]. Our results showed that PD-L1 expression in tumoral cells and stromal cells were positively correlated with CD8 + TILs density.

In this study, the expression of PD-L1 in tumour cells and immune stroma were associated with less aggressive tumor features and translated into favorable OS in patients with CRC cancer. however, only the association with immune stroma cell expression was statistically significant. Consistent with our findings, *J Wyss et al* showed that merely stromal PD-L1 were associated with less aggressive features of colon cancer and with better OS in colon cancer, although they had excluded rectal cancer patients given disease`s different tumor biology, treatment, and prognosis[8]. The association of PD-L1 expression with beneficial clinical outcome has been reported in a diverse set of tumour types, such as NSCLC[37], melanoma[38], breast cancer[39, 40] and including CRC[41]. This might seem inconsistent with the immunosuppressive function of PD-L1. However, this might be explained that PD-L1 expression within tumor microenvironment is not only as a immunosuppression factor, but rather acts as a reflection of adaptive antitumor immunity, where tumor-infiltrating lymphocytes are activated in response to tumor antigens. Contrary to our findings, *Thompson et al*[42] showed that in patients with locally advanced gastric cancer, both tumoral and stromal PD-L1 expression and CD8 + TILs were associated with unfavorable outcome. These opposite results might because the interaction between tumor and tumor-associated stroma and TILs might be different among different tumor types.

Our results showed that CD8 density was also associated with a good OS and it was also an independent predictor for CRC patients. CD8, which is predominantly expressed on cytotoxic T cells, is a crucial component of the cellular immune system and pivotal for cell-mediated anti-tumor immune response[43, 44]. Previous studies of association between CD8 and prognosis have reported that patients whose tumors contained infiltrating CD8 + TIL showed better survival in non-small cell lung cancer (NSCLC)[45–52]. These results further suggest that PD-L1 expression may reflects an association with a TIL-mediated antitumour inflammatory response, rather than always being associated with tumour immune evasion[53]. Anti-PD-L1 antibody MPDL3280A elicited a response in patients with tumours expressing high levels of PD-L1 and tumor-infiltrating immune cells[54], suggesting that patients who have PD-L1-positive tumors with CD8 + TILs might achieve a better outcome through blocking of PD-1/PD-L1 pathway. These results further confirmed that CD8 plays crucial role in the immune microenvironment, and the association of CD8 + TIL density with PD-L1 expression may be more important than PD-L1 expression alone predicting survival. Unexpectedly, there were no correlation between CD8 + TILs and PD-L1 and schistosomiasis. It was possible that the patients in the cohort with schistosomiasis are obviously older than patients without schistosomiasis. And the vigour of immunity of older people is weak[55]. In order to confirm this speculation, we excluded patients younger than 60 years old, then to analysis the relationship between schistosomiasis and CD8 + TILs. However, the small percentage of CRC-S patients did not allow us to perform further analysis stratified by age. Thus, further work in larger cohort are still needed to investigate the impact of *s. japonicum* on CD8 + TILs density and PD-L1 expression.

Our retrospective study had several limitations. First, we do recognized the limitation of utilizing a TMA approach to assess expression of a biomarker that may only be locally present in samples, raising the possibility of false negatives, which could possibly change the significance of PD-L1 expression in CRC. Second, we speculated that IFNγ which secreted by CD8 + T cells upregulated the expression of PD-L1. However, further studies needed to clarify the association between PD-L1 expression and CD8 + TILs, and to determine whether this combination has predictive relevance as a biomarker for selecting individual patients for treatment involving PD-1/PD-L1 blockade or for selection of certain tumour types for development. Third, determination of PD-L1 expression in tumour samples was generally performed by immunohistochemistry using various antibodies. Fifth, the threshold for positivity was not formally assessed.

In conclusion, results in present study demonstrated that stomal PD-L1 expression, but not tumoral PD-L1 expression in the whole cohort and in the CRC-NS set were associated with less aggressive tumor feature and translated into better OS. And the expression of PD-L1 was positively associated with CD8 + TILs density.

Abbreviations

programmed cell death-ligand 1=PD-L1; tumour-infiltrating lymphocytes=TILs; colorectal cancer=CRC; expression of PD-L1 within tumor cells =tPD-L1; expression of PD-L1 in stromal cells=sPD-L1; overall survival=OS; American Joint Committee on Cancer =AJCC; TMA= Tissue microarray; Immunohistochemical =IHC; formalin fixed paraffin-embedded =FFPE; colorectal cancer patients with schistosomiasis =CRC-S; patients without schistosomiasis=CRC-NS; non-small cell lung cancer=NSCLC

Declarations

Ethics approval and consent to participate:

This study was approved by the medical ethics committee of Fudan University, in accordance with the Helsinki Declaration of 1975. Prior written informed consent was obtained from all patients.

Consent for publication Written informed consent was obtained from each participant.

Availability of data and materials:

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Conflict of interest: The authors declare that they have no competing interests.

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Authors' contributions

Weixia Wang contributed data analysis, manuscript editing, article revision. Jican Liu and Limei Wang assessed all the dyeing slices. Hongyan Jing, Xi Yu and Yeying Gu contributed to the research design, data analysis, and manuscript writing. Yingyi Zhang, Kui Lu, Ting Zhu, Yanchao Xu, Dacheng Bu, Meihong Cheng, Jing Liu, Weidong Shen, Yingyi Zhang and Junxia Yao contributed to the data collection and perform experiments. Sinian Huang and Limei Wang contributed to the data analysis and manuscript editing. All authors read and approved the final manuscript.

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Figures

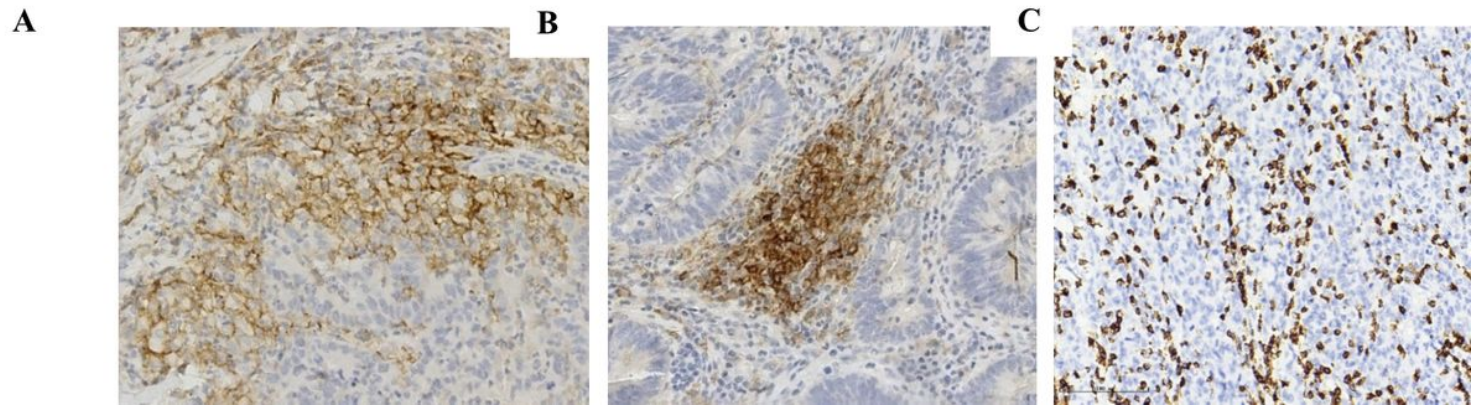

Figure 1

Immunohistochemical staining of representative programmed death-ligand 1 (PD-L1) expression (×200) and CD8 (×200) positivity. A. PD-L1 expression positivity on tumor cells. B. PD-L1 expression positivity on tumor-infiltrating mononuclear cells. C. Immunohistochemical staining of representative CD8 positivity (×200).

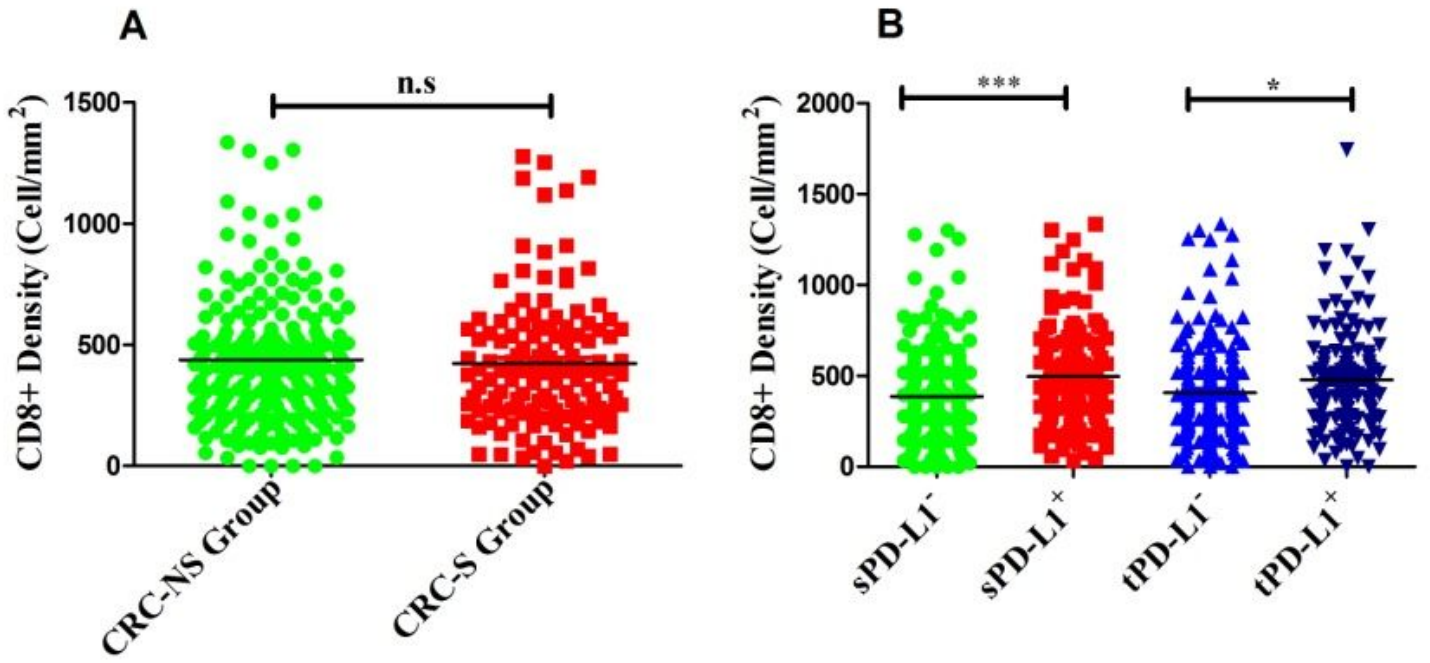

Figure 2

A. The relationship between schistosomial infection and CD8+ TILs density. B. PD-L1 expression by both immune stroma (p= 0.0001) and tumour cells (p= 0.0102) increased with increasing CD8 density in each location; Correlation between CD8 density and PD-L1 expression by location were examine using the unpaired t test. The optimum cutoff value of CD8+T cell density were determined by X-tile program, which were 279 cell/mm2. CD8low group was defined as CD8+ T cells density 279, and CD8high group was defined as CD8+ T cells density≥ 279 cell/mm2.

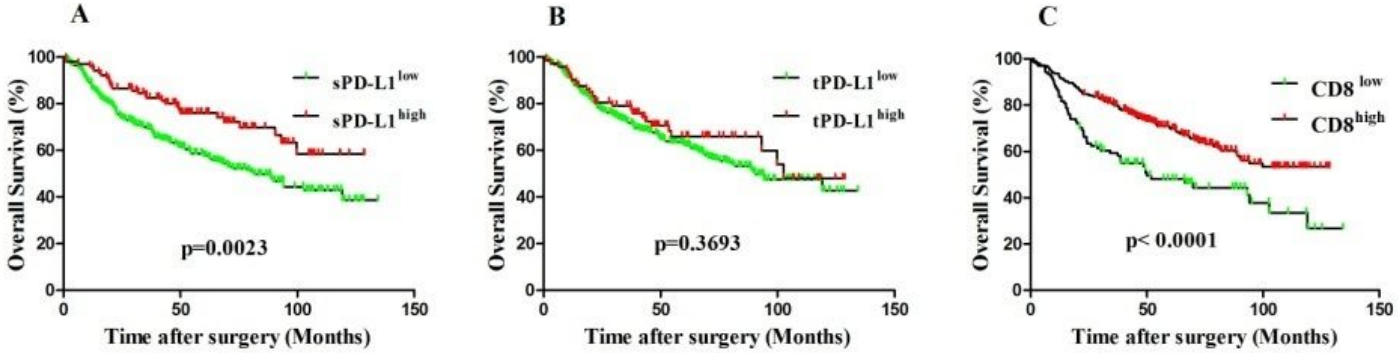

Figure 3

Kaplan-Meier curves for overall survival (OS) of CRC patients, OS was calculated using the Kaplan–Meier method and analyzed using the log-rank test. A. OS of CRC patients with expression of PD-L1 on the immune stroma cells (sPD-L1 positive expressing ≥2%) (p=0.0023); B. OS of CRC patients with expression of PD-L1 on tumoral cells (tPD-L1 positive expressing≥2%) (p=0.3693); C. OS of CRC patients with CD8+ cells density (p < 0.0001). The optimum cutoff value of CD8+T cell density were determined by X-tile program, which were

279 cell/mm2. CD8low group was defined as CD8+ T cells density 279, and CD8high group was defined as CD8+ T cells density≥ 279 cell/mm2.

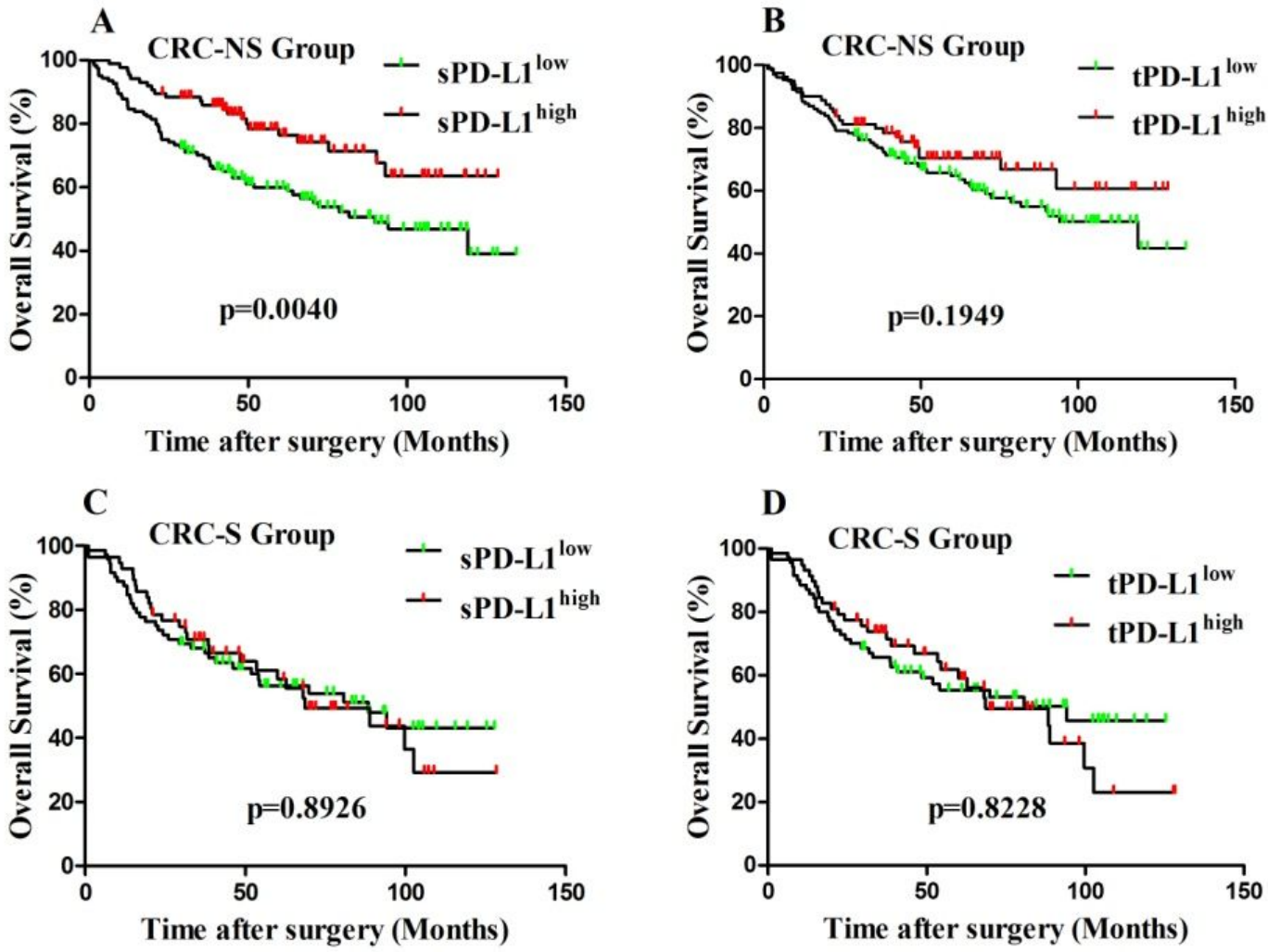

Figure 4

Kaplan-Meier curves for overall survival OS of CRC patients, OS was calculated using the Kaplan–Meier method and analyzed using the log-rank test. A. PD-L1 expressing on the immune stroma cells (sPD-L1 positive expressing ≥2%) for OS of schistosomiasis-associated CRC patients (CRC-S) (p=0.0040); or for OS of CRC patients without schistosomiasis (CRC-NS) (B) (p=0.1949); C. PD-L1 expressing on the tumoral cells (tPD-L1 positive expressing ≥2%) for OS of CRC-S patients (p=0.8926), or for OS of CRC-NS patients (D) (p=0.8228).

Supplementary Files

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