

Positive Expression of Programmed Death Ligand 1 in Peritumoral Liver Tissue is Associated with Poor Survival after Curative Resection of Hepatocellular Carcinoma¹



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Abstract

BACKGROUND: Recurrence or metastasis of hepatocellular carcinoma (HCC) is mainly intrahepatic after curative resection, demonstrating that the peritumoral environment is important but often neglected. Programmed death ligand 1 (PD-L1) in intratumoral liver tissues is a poor prognosis factor whose impact is removed after curative resection. However, PD-L1 expression remains in the peritumoral liver tissues and its distribution and prognostic value are still not clear. **METHODS:** We assessed the expression of PD-L1 by immunohistochemistry in peritumoral liver tissues from 90 HCC patients who underwent curative hepatectomy. The results were validated in an independent cohort of additional 90 HCC patients. **RESULTS:** We found PD-L1 positive expression in 31.11% (28/90) of peritumoral tissues. Peritumoral PD-L1 expression was associated with a significantly worse overall survival (OS) ($P = .000$) and disease-free survival (DFS) ($P = .001$) compared to the negative expression group. Additionally, peritumoral PD-L1 positivity significantly correlated with vascular invasion and a lower albumin level (≤ 35 g/L). Univariate and multivariate Cox regression models both revealed peritumoral PD-L1 as an independent prognostic factor for OS (HR = 2.853, $P = .002$) and DFS (HR = 2.362, $P = .003$). The prognostic value of PD-L1 positivity was validated in the independent data set. **CONCLUSIONS:** Our data suggest PD-L1 expression in peritumoral hepatocytes is an independent prognostic factor for OS and DFS. This implies that future anti-cancer therapy should target not only residual tumor cells but also the “soil” for promoting tumor growth. Peritumoral PD-L1 could be a good target for adjuvant therapy after hepatectomy.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common and aggressive malignant tumors, and the leading cause of cancer-related death worldwide [1]. Chronic hepatitis B and C virus (HBV and HCV, respectively) infection are two leading HCC etiologies [2]. Despite considerable advances in surgical techniques and targeted therapies over the past decade, the long-term survival of patients with HCC remains dismal due to metastasis or recurrence [3]. Previous studies highlighted that tumor-infiltrating cytotoxic and regulatory T cells were independent prognosticators for both survival and recurrence in HCC patients [4,5], which imply that HCC tumors may be immunogenic. Moreover, long-term chronic hepatitis B and C virus infection also promoted the development of inflammation, leading to the formation of liver cirrhosis and even tumor occurrence [2]. This evidence indicates the development of HCC could be closely associated with the host immune system. Accordingly, immunomodulatory treatments may be an interesting alternative strategy.

Recently, the advent of monoclonal antibodies (nivolumab and pembrolizumab) targeting the programmed death ligand 1 (PD-L1) and programmed death receptor 1 (PD-1) has provided a major break-through in oncology by opening a new avenue for studying tumor immunotherapy. PD-1 is an immune checkpoint transmembrane receptor involved in the negative regulation of immune responses and peripheral tolerance and expressed on activated T, B, natural killer (NK), and antigen-presenting cells [6,7]. PD-L1, the major PD-1 ligand, is widely expressed by both hematopoietic and nonhematopoietic cells. In the context of tumor microenvironment, the interaction of PD-L1 with PD-1 is pro-tumorigenic. Ligated PD-1 inhibited tumor-related T cell activation and induced T cell apoptosis, thereby destroying local immune activity and allowing tumor cells to escape immune surveillance, a mechanism supporting tumor growth and metastasis [8,9]. A series of studies demonstrated that PD-L1 expression in tumor tissues correlated with tumor aggressiveness, poor clinicopathologic features, and recurrence and survival in multiple types of cancers [4,10–13]. In agreement with this data, the PD-1 inhibitors nivolumab and pembrolizumab have demonstrated impressive antitumor efficacy in various types of cancers [14–17]. In most clinical trials to date, the response rate after treatment with PD-1/PD-L1 inhibitors was associated with the immunohistochemical expression of PD-L1 in tumor cells and tumor-infiltrating immune cells of multiple cancers [18–21]. Therefore, PD-L1 expression in tumor cells and tumor-infiltrating immune cells may serve as a predictive marker of sensitivity to immunotherapy. However, given that impact of PD-L1 was removed after curative hepatectomy, whether PD-L1 protein expression only in tumor tissues for HCC patients, can precisely and adequately

predict prognosis and efficacy of anti-PD-1/PD-L1 therapy remains controversial.

Commonly known, recurrence or metastasis of HCC is mainly intrahepatic after curative resection. This supports the notion that the peritumoral environment may provide “fertile soil” for subclinical metastatic tumor cells [22]. The association of intratumoral PD-L1 in HCC with tumor aggressiveness and a poor prognosis factor has been previously described [4,10,23]; however, the impact of intratumoral PD-L1 is removed after curative resection. Moreover, the peritumoral microenvironment favorable for HCC growth and metastasis persists after curative hepatectomy; and it is unknown whether PD-L1 expressed in peritumoral tissues after curative hepatectomy indicate a poor prognosis. Therefore, in the present study, we examined PD-L1 expression by immunohistochemistry in peritumoral tissues for 90 HCC patients who had received curative hepatectomy and investigated the correlation of PD-L1 expression with clinicopathological features, overall survival (OS), and disease free survival (DFS). Furthermore, we

Table 1. Main Demographic, Biochemical, and Clinical Characteristics of the 90 HCC Patients

Variable	Unit	Value
Age	years	54 (13–81)
Gender	male	84 (93.3)
Albumin	g/L	41 (29–50)
ALT	U/L	49 (14–283)
Total bilirubin	μmol/L	10 (4–83)
HCC diameter	cm	4.2 (1.3–15)
AFP	ng/mL	40 (2–699,800)
Risk factor	HBV/HCV	81 (91.0)

Data are presented as median (range) or absolute frequency (%). ALT, alanine aminotransferase; HCC, hepatocellular carcinoma; HBV/HCV, hepatitis B/C virus.

Table 2. Correlations of PD-L1 Protein Expression in Peritumoral Tissues with Clinicopathological Characters

Parameters	PD-L1 (Peritumor)		P
	Positive	Negative	
Age			.621
<50y	7	20	
≥50y	21	42	
Gender			.661
Male	27	57	
Female	1	5	
Cirrhosis			.067
Absence	7	28	
Presence	21	33	
Unknown	0	1	
Tumor size			.359
<5 cm	13	36	
≥5 cm	15	25	
Unknown	0	1	
AFP			.798
<400 μg/L	21	39	
≥400 μg/L	7	17	
Unknown	0	6	
Histological grade			.534
Well and moderate	25	48	
Poor	3	11	
Unknown	0	3	
Vascular invasion			.031 *
Absence	14	46	
Presence	14	16	
Number of tumor lesions			.134
Single	20	53	
Multiple	8	8	
Unknown	0	1	
HBV/HCV infection			.703
Yes	25	56	
No	3	5	
Unknown	0	1	
ALT			.107
>80 U/L	10	10	
≤80 U/L	18	44	
Unknown	0	8	
Albumin			.018 *
>35 g/L	20	50	
≤35 g/L	8	4	
Unknown	0	8	
Bilirubin			1.000
>20 μmol/L	1	2	
≤20 μmol/L	27	52	
Unknown	0	8	

AFP, alpha-fetoprotein; ALT, alanine aminotransferase; HBV/HCV, hepatitis B/C virus. * indicates P < .05.

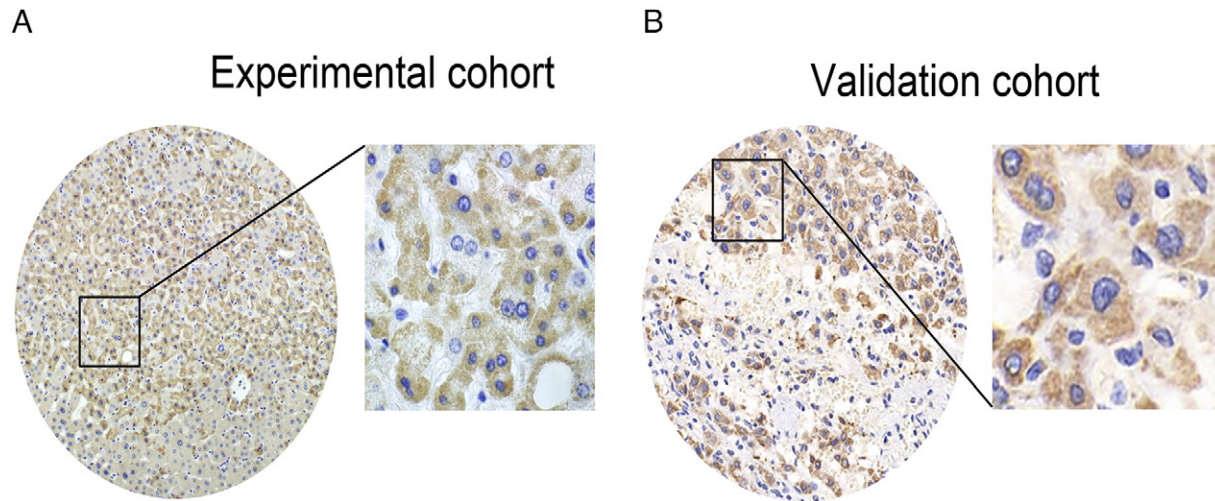


Figure 1. Peritumoral positive hepatocellular cytoplasmic/membranous staining for PD-L1 in experimental group (A), and validation cohort (B).

validated our results in an external cohort of an additional series of 90 HCC tissue arrays from BioChip (Shanghai, China).

Methods

Patients and Clinicopathology Information

The study was designed and performed strictly according to the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) [24] and the Transparent Reporting of a Multivariable Prediction Model for Individual Prognosis or Diagnosis (TRIPOD) Statement [25]. Written consent was obtained from all patients or their legal representative. The study was approved by the Joint Chinese University of Hong Kong–New Territories East Cluster Clinical Research Ethics Committee. We examined 90 HCC patients who underwent curative hepatectomy between November 1996 and January 2010 at the Prince of Wales Hospital, Hong Kong. Curative resection was defined as the complete removal of cancer tissues without residual tumor cells in resection margins (R0 resection). The criteria for patient inclusion and exclusion were as follows: (1) distinctive pathologic diagnosis of HCC for the first time without distant metastasis and no anticancer therapy before surgery; (2) curative hepatectomy with tumor-negative resection margins; (3) detailed clinicopathologic and follow-up information, and (4) other malignant tumors, autoimmune disease, or serious heart, lung, kidney, or blood diseases were excluded. A total of 90 eligible HCC patients were identified. Two independent, experienced pathologists, blinded to all patient related information, carefully reviewed surgical specimens to definitively diagnose HCC by histology.

Conventional clinicopathologic and biological variables, including gender, HBV or HCV infection, liver cirrhosis, tumor number, size, vascular invasion, histological differentiation, α -Fetoprotein (AFP), albumin, alanine aminotransferase (ALT) and bilirubin were recorded in Tables 1 and 2.

Tissue Microarray and Immunohistochemistry

Peritumoral liver tissue microarrays (TMAs) were constructed as previously described [22]. TMAs were performed on peritumoral liver tissue adjacent to the tumor within a distance of 2 cm. The primary

antibody was an anti-human PD-L1 mouse monoclonal antibody (clone 2B11D11, dilution 1:500; Proteintech, Chicago, USA). Immunostaining was performed by standard protocol as described elsewhere [22]. PD-L1 expression was assessed in peritumoral hepatocytes. PD-L1 positive was evaluated according to the intensity of the hepatocellular cytoplasmic/membranous staining (0, negative; 1, very weak; 2, moderate; and 3, strong expression). Staining scores of 0 and 1 were defined as negative staining, whereas 2 and 3 were regarded as positive staining [23,26].

Statistical Analysis

All statistical analyses were performed using SPSS® version 21.0 (IBM, Armonk, NY, USA). Continuous data were displayed as the median (range). The correlations between immunostaining parameters and clinicopathological features were analyzed using Pearson χ^2 test or Fisher's exact test as appropriate. Disease-free survival (DFS) was defined as the length of time from the date of curative hepatectomy to the first recurrence of disease (locoregional recurrence or distant metastases) or to death. Overall survival (OS) was defined as the interval between the dates of curative hepatectomy and death. Data was censored at the last follow-up (November 11, 2014) for patients without recurrence, metastases, or death. The overall and disease-free survival were calculated by the Kaplan–Meier method and compared by the log-rank test. Univariate Cox-regression model was performed against all the clinicopathologic features as covariates. Multivariate Cox proportional hazards analysis was performed on the significant factors determined by univariate analysis. $P < .05$ (two-tailed) was considered statistically significant for each analysis.

Independent Validation Cohort

To further validate our data, we examined the peritumoral hepatocellular PD-L1 expression in an independent cohort of 90 HCC tissue arrays purchased from BioChip (Shanghai, China). The tissue arrays were also performed on peritumoral liver tissue adjacent to the tumor within a distance of 2 cm. The detailed clinicopathologic characteristics of the validation cohort patients are displayed in Tables S1 and S2. Immunohistochemical and statistical analysis were performed in the same manner as the experimental cohort in this study.

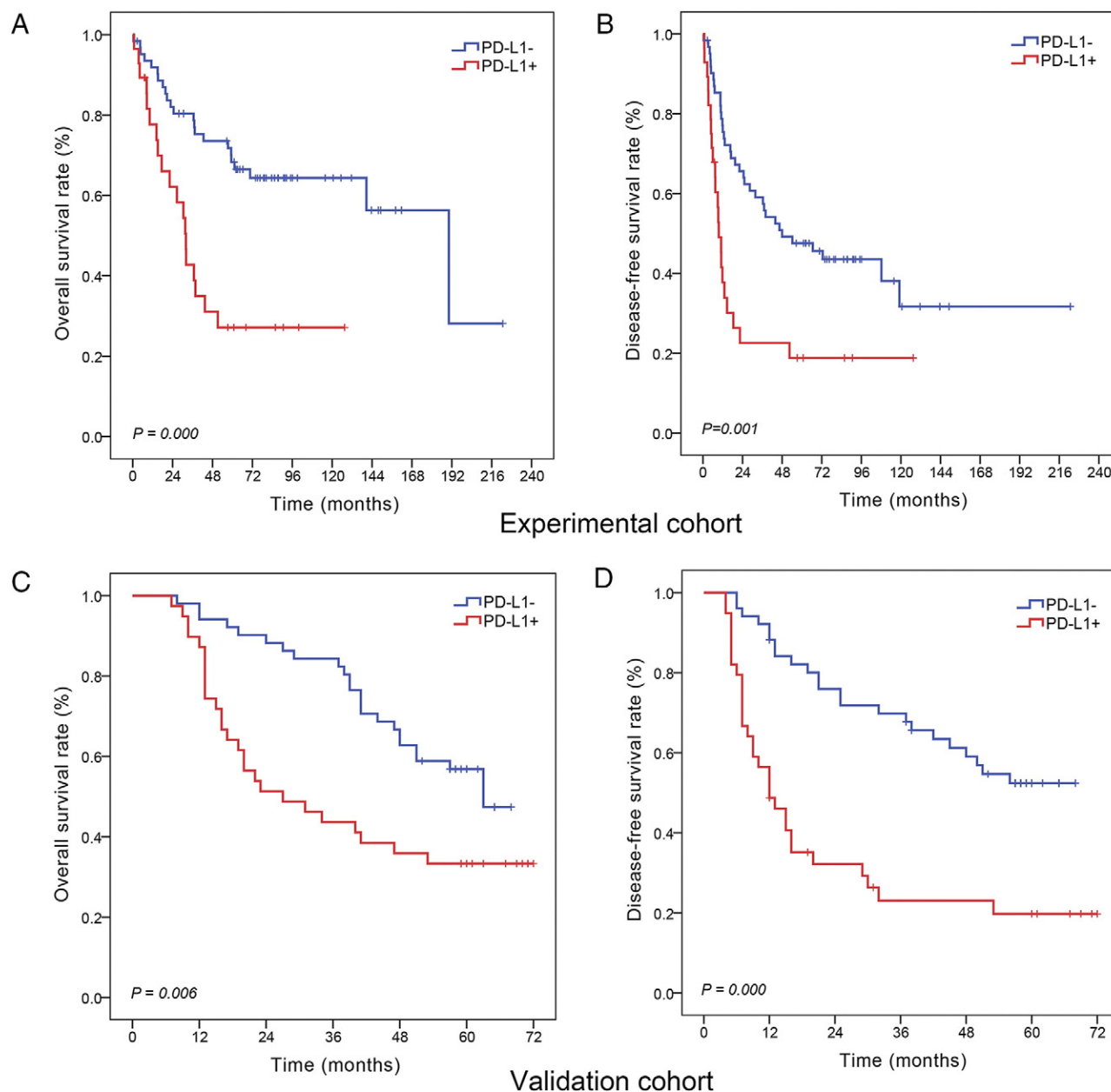


Figure 2. Prognostic values of peritumoral PD-L1 expression. Kaplan–Meier overall survival (OS) and disease-free survival (DFS) analysis in experimental cohort (A and B), and validation cohort (C and D), respectively, according to peritumoral PD-L1 expression.

Results

Patient Characteristics and Clinicopathologic Profiles

A total of 90 eligible HCC patients who underwent curative resection at the Prince of Wales Hospital, Hong Kong, from November 1996 to January 2010, were recruited in the present study. Patients were majority male (84/90, 93.3%) with a median age of 54 (range, 13–81 years). HBV/HCV infection rate was 91.0% (81/90). The main clinical and biological features of the experimental cohort are summarized in Tables 1 and 2. All patients were followed until November 11, 2014, with a median time of 60.8 months (range, 0.4–222.7 months).

Immunohistochemical staining for PD-L1 was performed in peritumoral liver tissue microarrays (TMAs) of all 90 HCC patients. We identified PD-L1 positive staining at the hepatocellular membrane or

in the cytoplasm (or both) of peritumoral TMAs (Figure 1A). Twenty-eight (31.1%) patients were PD-L1 positive. As shown in Table 2, peritumoral PD-L1 protein expression was significantly associated with more frequent vascular invasion ($P = .031$), and tended to correlate with lower albumin levels (≤ 35 g/L) ($P = .018$). Additionally, peritumoral PD-L1 staining almost significantly correlated with presence of liver cirrhosis ($P = .067$). However, no statistically significant correlation was observed between PD-L1 and other clinicopathological features.

Survival Analysis

Kaplan–Meier survival and disease analysis demonstrated that peritumoral PD-L1 positive patients had worse overall survival (OS) ($P = .000$) (Figure 2A) and disease-free survival (DFS) ($P = .001$) (Figure 2B) compared to the PD-L1 negative patients. The mean OS and

Table 3. Cox Proportional Hazard Regression Analysis of Patients' Overall Survival

Variables	Univariable			Multivariable		
	Hazard Ratio	95% CI	P	Hazard Ratio	95% CI	P
Gender (male vs female)	0.301	0.060–1.495	.142	-	-	-
Age (≥50 vs <50)	1.402	0.587–3.352	.447	-	-	-
AFP (≥400 ng/ml vs <400 ng/ml)	1.578	0.688–3.619	.282	-	-	-
ALT (>80 U/L vs ≤80 U/L)	1.653	0.758–3.065	.206	-	-	-
Albumin (>35 g/L vs ≤35 g/L)	0.784	0.292–2.109	.630	-	-	-
Bilirubin (>20 μmol/L vs ≤20 μmol/L)	0.602	0.070–5.157	.643	-	-	-
Number of tumor lesions (single vs multiple)	1.272	0.438–3.695	.658	-	-	-
Vascular invasion (absent vs present)	2.471	1.060–5.762	.036*	2.516	1.302–4.861	0.006*
Cirrhosis (absent vs present)	1.226	0.533–2.819	.632	-	-	-
Histological differentiation						
Well/moderately vs poorly	1.268	0.427–3.764	.669	-	-	-
HBV/HCV (positive vs negative)	0.648	0.179–2.345	.509	-	-	-
Tumor diameter						
(≥5 cm vs <5 cm)	2.861	1.253–6.533	.013*	2.341	1.225–4.476	0.010*
PD-L1 (positive vs negative)	3.167	1.427–7.030	.005*	2.853	1.482–5.493	0.002*

CI, confidence interval; AFP, alpha-fetoprotein; ALT, alanine aminotransferase; HBV/HCV, hepatitis B/C virus. * indicates $P < .05$.

DFS of patients with PD-L1 negative expression in peritumoral tissues was 138.5 ± 13.4 months and 97.1 ± 13.5 months, respectively, while those with PD-L1 positive expression was only 51.2 ± 9.4 and 32.6 ± 9.0 months, respectively. In univariate analysis, the clinical and pathological features associated with shorter OS were vascular invasion ($P = .036$), tumor diameter ($P = .013$), and PD-L1 positive expression ($P = .005$) (Table 3). Alanine aminotransferase (ALT) >80 U/L ($P = .021$), vascular invasion ($P = .024$), and PD-L1 positive expression ($P = .014$) were significantly associated with poor DFS in HCC patients (Table 4).

The multivariate Cox proportional hazards analysis was performed for factors determined to be significant in the above univariate analysis. In the multivariable model, vascular invasion (hazard ratio [HR] = 2.516, 1.302–4.861; $P = .006$), tumor size ≥ 5 cm (HR = 2.341, 1.225–4.476; $P = .010$), and PD-L1 positive expression (HR = 2.853, 1.482–5.493; $P = .002$) were independent indicators of poor OS. Moreover, tumor size ≥ 5 cm (HR = 2.163, 1.238–3.778; $P = .007$) and PD-L1 positive expression (HR = 2.362, 1.347–4.141; $P = .003$) can also independently predict poor DFS in HCC patients, whereas elevated ALT level did not independently predict poor DFS.

Independent Validation Cohort

To validate our results, we examined peritumoral hepatocellular PD-L1 expression, using the same approach, in an independent cohort, consisting of 90 additional HCC patients. PD-L1 positive expression in peritumoral hepatocytes was observed in 43.3% (39/90) of patients. This PD-L1 expression pattern in experimental cohort and validation cohort was similar to that reported previously [23]. PD-L1 immunohistochemical staining profile in peritumoral tissues are shown in Figure 1B. Patients' detailed clinical and biological features are presented in Tables S1 and S2. The median follow-up time was 51 (range, 7–72 months). Although there were no statistically significant correlations between PD-L1 expression and any clinicopathological features in the validation cohort (Table S2), PD-L1 positive staining indicated a poor prognosis for OS ($P = .006$) and DFS ($P = .000$) in the additional 90 HCC patients (Figure 2, C and D). Furthermore, univariate and multivariate survival analyses also revealed that patients with peritumoral PD-L1 positivity had poorer prognoses than those without PD-L1 expression (OS, [HR] = 2.165, 1.137–4.125, $P = .019$; DFS, HR = 4.549, 2.489–8.312, $P = .000$) (Tables S3, S4).

Table 4. Cox Proportional Hazard Regression Analysis of Disease-Free Survival

Variables	Univariable			Multivariable		
	Hazard Ratio	95% CI	P	Hazard Ratio	95% CI	P
Gender (male vs female)	0.374	0.099–1.410	.146	-	-	-
Age (≥50 vs <50)	1.165	0.603–2.252	.649	-	-	-
AFP (≥400 ng/ml vs <400 ng/ml)	1.701	0.877–3.297	.116	-	-	-
ALT (>80 U/L vs ≤80 U/L)	2.193	1.126–4.271	.021	1.801	0.983–3.298	.057
Albumin (>35 g/L vs ≤35 g/L)	0.723	0.324–1.615	.429	-	-	-
Bilirubin (>20 μmol/L vs ≤20 μmol/L)	0.291	0.037–2.270	.239	-	-	-
Number of tumor lesions (single vs multiple)	2.185	0.952–5.016	.065	-	-	-
Vascular invasion (absent vs present)	1.349	0.690–2.637	.381	-	-	-
Cirrhosis (absent vs present)	1.529	0.777–3.009	.218	-	-	-
Histological differentiation						
Well/moderately vs Poorly	1.626	0.683–3.871	.272	-	-	-
HBV/HCV (positive vs negative)	1.801	0.546–5.945	.334	-	-	-
Tumor diameter						
(≥5 cm vs <5 cm)	2.216	1.111–4.419	.024*	2.163	1.238–3.778	.007*
PD-L1 (positive vs negative)	2.221	1.173–4.207	.014*	2.362	1.347–4.141	.003*

CI, confidence interval; AFP, alpha-fetoprotein; ALT, alanine aminotransferase; HBV/HCV, hepatitis B/C virus. * indicates $P < .05$.

Discussion

In the present study, we first demonstrated that HCC patients with PD-L1 expression in peritumoral hepatocytes had a significantly higher risk of cancer recurrence or metastasis and cancer-related death. Additionally, peritumoral PD-L1 positivity significantly correlated with vascular invasion, a lower albumin level (≤ 35 g/L), and liver cirrhosis (with a borderline significant trend). Univariate and multivariate survival analyses further supported that peritumoral PD-L1 positive expression was an independent prognostic factor for OS and DFS. The prognostic value was validated in an independent cohort, containing an additional 90 HCC patients.

Previous studies have indicated PD-L1 ligation of PD-1, inhibited and destroyed T-cell-mediated antitumor immunity [27], potentially the basis for the association between peritumoral PD-L1 expression and poor prognosis. As we know, PD-1/PD-L1 immune checkpoint is a critical negative regulatory pathway for tumor-associated immune responses. Some studies suggested PD-L1 is a key regulator for the accumulation and deletion of CD8⁺ T cells [28–30]. Moreover, a study recently indicated that a high level of peritumoral neutrophil infiltration and neutrophil-to-lymphocyte T cell ratio (pNLR) correlated with poor patient survival after curative resection. This study also showed that peritumoral neutrophils negatively regulated adaptive immunity via the PD-L1/PD-1 signaling pathway in HCC patients [31]. Accordingly, we speculated that PD-L1 expression in peritumoral infiltrating immune cells may also be a poor prognostic marker partly resulting from peritumoral infiltration of PD-L1 positive neutrophil interacting with PD-1 positive CD8⁺ T cells to inhibit and destroy T-cell-mediated antitumor immunity. However, our peritumoral samples were hardly PD-L1 positive infiltrating immune cells, hence, we only assessed PD-L1 expression in peritumoral hepatocytes. We considered the main reason was that the sample size was relatively small. Relevant studies with larger sample size are needed to test this hypothesis in future.

Relapse and metastasis are the major contributors to HCC patients' dismal prognosis after curative resection [3]. HCC relapse included two types: (1) a true metastasis by subclinical metastatic HCC cells' dissemination, and (2) a new neoplasm of residual liver tissue after hepatectomy caused by chronic virus infection and inflammation [4]. HBV and HCV, two leading etiologies for HCC, affects approximately 250 million people worldwide [32]. The HBV/HCV infection rate of HCC patients in either our experimental cohort (91.0%) or our validation cohort (84.4%) are more than 80%. As previously described, PD-L1 can induce T-cell exhaustion to inhibit anti-tumor immune responses. PD-L1 can also inhibit T-cell responses necessary for the clearance of HBV or HCV [33]. Given HCC patients' high HBV/HCV infection rate in the present study, we speculate that peritumoral PD-L1 expression's association with a poor prognosis may be caused, in part, by de novo neoplasms due to the failure of HBV/HCV clearance. Moreover, the formation of liver cirrhosis can result from chronic inflammation and our data show that peritumoral PD-L1 expression was associated with the presence of liver cirrhosis with a borderline significant trend ($P = .067$). Therefore, the association between PD-L1 and liver cirrhosis further supports our hypothesis.

Recurrence or metastasis of hepatocellular carcinoma (HCC) is mainly intrahepatic after curative resection, demonstrating that the peritumoral environment is important but often neglected [22]. We therefore evaluated the role of peritumoral PD-L1 expression as a prognostic indicator of OS and DFS in HCC patients. In conclusion, we first demonstrated that PD-L1 expression in peritumoral liver

hepatocytes was an independent prognostic factor for OS and DFS, which was validated in an independent cohort, containing an additional 90 HCC patients. Therefore, future anti-cancer therapy should target not only residual tumor cells but also the “soil” for promoting tumor growth after hepatectomy. Our studies indicate that peritumoral PD-L1 could be a good target for adjuvant therapy after hepatectomy.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.tranon.2017.03.009>.

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