# Overexpression of PD-L1 Significantly Associates with Tumor Aggressiveness and Postoperative Recurrence in Human Hepatocellular Carcinoma

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**Abstract**

**Purpose:** The aberrant expression of programmed cell death 1 ligands 1 and 2 (PD-Ls) on tumor cells dampens antitumor immunity, resulting in tumor immune evasion. In this study, we investigated the expression of PD-Ls in human hepatocellular carcinoma (HCC) to define their prognostic significance after curative surgery.

**Experimental Design:** Immunohistochemistry was used to investigate PD-Ls expression as well as granzyme $B^+$ cytotoxic and $FoxP3^+$ regulatory T cell infiltration on tissue microarrays containing 240 randomly selected HCC patients who underwent surgery. The results were further verified in an independent cohort of 125 HCC patients. PD-Ls expression on HCC cell lines was detected by Western blot assay.

**Results:** Patients with higher expression of PD-L1 had a significantly poorer prognosis than patients with lower expression. Although patients with higher expression of PD-L2 also had a poorer survival, the difference in recurrence was not statistically significant. Multivariate analysis identified tumor expression of PD-L1 as an independent predictor for postoperative recurrence. No correlation was found between PD-Ls expression and granzyme $B^+$ lymphocyte infiltration, whereas a significant positive correlation was detected between PD-Ls expression and $FoxP3^+$ lymphocyte infiltration. In addition, tumor-infiltrating cytotoxic and regulatory T cells were also independent prognosticators for both survival and recurrence. The prognostic value of PD-L1 expression was validated in the independent data set.

**Conclusion:** Our data suggest for the first time that PD-L1 status may be a new predictor of recurrence for HCC patients and provide the rationale for developing a novel therapy of targeting the PD-L1/PD-1 pathway against this fatal malignancy.

Hepatocellular carcinoma (HCC), epidemic to Asia and Africa with an increasing incidence in western countries, is one of the most common and aggressive cancers worldwide (1). Surgery is potentially curative and holds as priority; however, only 10% to 30% of patients are eligible for curative surgery. To make it worse, particular high rate of postsurgical recurrence and metastasis (50-70% at 5 years) produces a major challenge as this disease is highly refractory to conventional chemotherapy and radiation (2). Clinically significant intratumoral immune infiltration (3), naturally acquired tumor antigen-specific T-cell responses (4), as well as natural killer cell-mediated association between depressive symptoms and survival (5) in HCC patients altogether implicate HCC as an immunogenic tumor and provide the rationale for the development of immunomodulatory approaches as alternative treatment strategies. The limited clinical experience validates that HCC tumor growth and recurrences could be controlled in selected patients with immunotherapies (e.g., IFN-α, autologous tumor-pulsed dendritic cells, or adoptively transferred lymphocytes; ref. 6).

Effective antitumor immunity depends on the concordant activity of CTLs, whose fate and activity are the results of a balance between positive and negative signals conferred through interactions between various T-cell coregulatory receptors and ligands (7). It has been clearly known that inadequate, inappropriate, or inhibitory T-cell costimulatory pathway signaling all play key roles in a host's inability to generate productive immune responses against cancer. Programmed cell death 1 (PD-1), an immunoinhibitory receptor belonging to the CD28 family, has been shown as a frequently used physiologic immunosuppressive mechanism by tumors

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## Translational Relevance

Hepatocellular carcinoma (HCC) is highly refractory to conventional chemotherapy and radiation, whereas less than one-third of patients are eligible for curative surgery. As an immunogenic tumor, immunomodulatory approaches in HCC emerge as promising alternative treatment strategies. We showed for the first time that elevated programmed cell death 1 ligand 1 (PD-L1) expression in HCC is significantly associated with tumor aggressiveness and enhanced risk for postoperative recurrence. As such, PD-L1 may represent a target for HCC immunotherapy and a potential biomarker to facilitate patient assignment to treatment as well as aid in the determination of prognosis both before and after therapy. Moreover, together with previous findings that targeted blockade of the PD-L1/PD-1 pathway could facilitate hepatitis virus clearance, PD-L1 may represent a single bullet with the power to simultaneously target HCC recurrence and *de novo* cancer, the two major components of HCC relapse.

to invade host immunity (8). PD-1, being involved in the negative regulation of immune responses and peripheral tolerance, is expressed on activated T, B, and myeloid cells, and the ligation of PD-1 inhibits T-cell activation and the production of cytokines such as IFN-γ and interleukin-2 (9, 10). Two ligands for PD-1, PD-1 ligand 1 (PD-L1; B7-H1) and PD-1 ligand 2 (PD-L2; B7-DC), have been identified based on the similarity to other B7 superfamily (11, 12). In contrast to the limited expression of PD-L2 on activated dendritic cells and macrophages, PD-L1 is broadly expressed on nonimmune cells as well as T cells, B cells, macrophages, and dendritic cells and is upregulated after their activation. PD-Ls expressed on antigen-presenting cells have been shown to induce T-cell anergy or apoptosis via PD-1 on T cells, whereas PD-L1 expressed on peripheral tissues (e.g., the liver) directly determines accumulation or deletion of intrahepatic $CD8^+$ T lymphocytes (13, 14).

An association between tumor-associated PD-L1 expression and tumor aggressiveness, poor clinicopathologic features, as well as reduced survival has been recently reported in a group of human malignancies (15–17). Forced or constitutive PD-L1 expression on tumor cells has been shown to enhance apoptosis of activated tumor-specific T cells *in vitro*. Also, in murine tumor models, PD-L1 blockade using anti-PD-L1 monoclonal antibody potentiated antitumor immunity and inhibited tumor growth (8, 18). Interfering into the PD-L1/PD-1 pathway has been even in considerations in clinical settings (19).

However, conflicting data, indicating PD-Ls' augmentation of antitumor immunity, still exists (20, 21) and PD-Ls' clinical significance has not yet been explored in HCC. Specifically, dysfunction of tumor suppressor PTEN (22) and overexpression of apoptosis inhibitor survivin (23) have newly been suggested to promote tumor PD-L1 protein expression. As an immunogenic tumor that is amenable to immune-based therapy, HCC initiation and progression are also characterized by high prevalence of the oncologic events pertaining to PTEN and survivin (24, 25). Additionally, hepatitis B and C virus infection, two leading HCC etiologies, has been shown to damage protective antiviral immunity via the PD-L1/PD-1 pathway (26, 27). Hence, it is logical to speculate that PD-L1 may have a crucial role in HCC immune escape and were of value in precisely formulating a prognosis.

To this end, we investigated the extent of PD-Ls expression in HCC cell lines and tumor samples from a large, random HCC cohort. Herein, for the first time, we showed that elevated PD-L1 expression in HCC is significantly associated with tumor aggressiveness and enhanced risk for postoperative recurrence. The results were further validated in an independent data set. As such, PD-L1 may represent a target for HCC immunotherapy and a potential biomarker to facilitate patient assignment to such treatment as well as aid in the determination of prognosis both before and after therapy.

## Materials and Methods

***Cell lines.*** Seven HCC cell lines HCCLM6, HCCLM3, MHCC97H, and MHCC97L (established from the same parental cell line at our institute; refs. 28, 29), PLC (Japanese Cancer Research Bank), and HepG2 and Hep3B (American Type Culture Collection) were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C with 5% $CO_2$.

***Patient selection.*** After institutional review board approval, under the following the inclusion and exclusion criteria: (*a*) distinctive pathologic diagnosis of HCC, (*b*) without anticancer treatment and distant metastases before surgery, (*c*) underwent primary and curative resection for HCC between 2002 and 2006, defined as macroscopically complete removal of the tumor, as described previously (3, 30, 31), and (*d*) with complete clinicopathologic and follow-up data, a total of 2,523 patients were finally identified representing a continuous, unselected cohort of patients in a single liver cancer center (Liver Cancer Institute and Zhong Shan Hospital, Fudan University). Then, 240 HCC patients were randomly selected from this cohort as the study population and reviewed retrospectively.

Conventional clinicopathologic variables, including age, gender, hepatitis history, liver cirrhosis, α-fetoprotein, γ-glutamyl transferase, tumor number, size, encapsulation, differentiation, vascular invasion, stage, therapy, and status, were recorded and detailed in Table 1. Tumor stage was determined according to the 2002 American Joint Committee on Cancer/International Union Against Cancer tumor-node-metastasis (TNM) classification system (32). Tumor differentiation was graded by the Edmondson grading system. If patients had multiple lesions in the liver, we selected the main nodule for our study. In case of tumor with different histologic grades, the grade of the tumor was regarded as the most advanced one among them. Postoperative treatments and surveillance according to a uniform guideline were described in our previous study (3, 30, 31, 33). Data were censored at last follow-up for patients without relapse or death. Disease-free survival (DFS) time was defined as the period from the date of surgery to confirmed tumor relapse date for relapsed patients or from the date of surgery to the date of last follow-up for nonrecurrent patients.

***Western blot analysis.*** The cells lysing, protein extraction, concentration detection, and Western analysis were done as we described previously (31, 34) with the following primary antibodies: goat anti-human PD-L1 (1:1,000) and PD-L2 (1:1,000) monoclonal antibodies (R&D Systems) with β-actin (1:500; Chemicon) as an internal loading control. Results were representative of three experiments.

***Tissue microarray and immunohistochemistry.*** Tissue microarrays were produced as described previously (3, 33). All HCC cases were histologically reviewed by H&E staining and representative areas were premarked in the paraffin blocks, away from necrotic and hemorrhagic materials. Triplicate of 1 mm diameter cylinders were included in each case, along with different controls (spleen, lymph node, artery, and glioma), to ensure reproducibility and homogenous staining of the slides (Shanghai Biochip). Thus, three different tissue microarray blocks

were constructed, each containing a total of 246 cores. Sections of 4 μm thickness were taken on 3-aminopropyltriethoxysilane-coated slides.

Mouse anti-human granzyme B (Novocastra), FoxP3 (AbD Serotec), PD-L1 (eBioscience), and goat anti-human PD-L2 (R&D Systems) monoclonal antibodies were purchased. Immunohistochemistry of serial tissue microarrays was carried out as described previously (3, 19, 31, 33). Briefly, sections were dewaxed, hydrated, and washed. After neutralization of endogenous peroxidase and microwave antigen retrieval, slides were preincubated with blocking serum and then incubated overnight with each monoclonal antibody. Subsequently, the sections were serially rinsed, incubated with second antibodies, and treated with horseradish peroxidase-conjugated streptavidin. Reaction products were visualized with 3,3′-diaminobenzidine tetrahydrochloride and counterstained with hematoxylin. For each antibody, including negative staining controls, all tissue microarray stainings were done in a single experiment.

***Quantification of PD-Ls expression and immune-cell infiltration.*** For PD-Ls, a digital image system was used to evaluate the signals as described previously (33). Briefly, three images of representative fields were captured under a Leica CCD camera DFC420 connected to a Leica DM IRE2 microscope (Leica Microsystems Imaging Solutions) at a magnification of ×200 and saved as TIFF files using the Leica QWin Plus version 3 software. Images were analyzed with Image-Pro Plus version 6.2 software (Media Cybernetics) using a special function called measurement of integrated absorbance, which evaluate both the area and the intensity of the positive staining (35). With this function, integrated absorbance of all the positive staining of PD-Ls in each photograph was measured and its ratio to total area of each photograph was calculated as PD-Ls density. The average integrated absorbance value (integrated absorbance/total area) on each slide (three images) was used to represent a particular sample.

As for granzyme $B^+$ and $FoxP3^+$ staining, the entire 1-mm-diameter core was counted manually under high-power field and the average count per 1-mm disk was calculated. All samples were anonymized and independently scored by two investigators. In case of disagreement, the slides were reexamined and a consensus was reached by the observers.

***Statistical analysis.*** All statistical analyses were conducted using SPSS 15.0 statistical software. The association between immunoreactive markers and clinicopathologic variables was analyzed using the $\chi^2$ test or Fisher's exact test or *t* test as appropriate. The correlation between the density of PD-Ls and tumor-infiltrating lymphocytes (TIL) was analyzed using Spearman's rank correlation. The survival curves were estimated by the Kaplan-Meier method and compared by the log-rank test. The Cox-regression model was used to perform univariate and multivariate analyses, including all the clinicopathologic features as covariates. $P < 0.05$ (two-tailed) was considered to indicate statistical significance.

The 75th percentile was selected as cutoff for high or low PD-Ls density based on previous reports, suggesting that PD-Ls-positive or high expression patients accounted for ~25% in other malignancies (19, 36). For TIL counts, the distinguishing factor for subgroups was the median values as our previous report (3).

***Independent validation.*** For further validation in an independent data set, we examined the prognostic performance of PD-L1 expression and $FoxP3^+$ TIL density in tissue microarrays containing an additional series of 125 HCC patients. These tissue microarrays were kind gift from Prof. Huichuan Sun (Liver Cancer Institute, Fudan University). Clinicopathologic features of this cohort of patients have been described elsewhere, with a median follow-up of 30.0 months (range, 1.0-105.0; SD, 27.6; ref. 37). Immunohistochemistry, quantification of immunomarkers, and statistics were conducted in the same manner.

## Results

***Patient clinicopathologic profiles.*** DFS and overall survival (OS; in brackets) rates at 1, 3, and 5 years posthepatectomy were 67% (81%), 50% (54%), and 47% (53%) for the whole study population. The cancer-specific survival was also calculated, with the 1-, 3-and 5-year rates 92%, 78%, and 62%. At last follow-up (December 31, 2007), 102 (42.5%) patients were confirmed as relapse, including 75 intrahepatic recurrence, 10 extrahepatic metastasis, and 17 cases with both events. The median follow-up period was 16.0 months (range, 1.5-68.0; SD, 14.6). Postrecurrent treatments including reoperation ($n$ = 13), chemoembolization ($n$ = 54), and regional therapy ($n$ = 4) were given as appropriate. However, 31 recurrent patients with severe liver dysfunction or week general performance cannot sustain any anticancer treatments (Table 1).

***PD-Ls expression pattern in HCC cell lines and tissue samples.*** Western blot revealed constitutive PD-Ls expression

**Table 1.** Clinicopathologic features of the HCC patients

| Variables | Results |
|---|---|
| Median (range) age, y | 52 (18-81) |
| Gender (male/female) | 204/36 |
| Virus infection [hepatitis B virus/hepatitis C virus/hepatitis B + C virus/ (-)] | 219/1/4/16 |
| Liver cirrhosis (no/yes) | 28/212 |
| Median (range) preoperative α-fetoprotein, ng/mL | 127.0 (0.9-60,500.0) |
| Preoperative γ-glutamyl transferase (units/L) | 64.0 (8.0-1,111.0) |
| Tumor multiplicity (single/multiple) | 184/56 |
| Median (range) tumor size, cm | 5.5 (0.9-23.0) |
| Child-Pugh classification (A/B) | 239/1 |
| Vascular invasion (absence/presence) | 131/109 |
| Tumor encapsulation (complete/non) | 119/121 |
| Tumor differentiation (well/poor) | 135/105 |
| American Joint Committee on Cancer/International Union Against Cancer TNM stage (I/II/III) | 106/76/58 |
| Adjuvant therapy (none/TACE/immunotherapy)* | 82/128/30 |
| Postrecurrent therapy (none/TACE/regional † /resection) | 31/54/4/13 |
| Alive with recurrence (without recurrence)/died of tumor (non-tumor) | 30 (117)/42 (51) |

Abbreviation: TACE, transcatheter arterial chemoembolization.
*Cytokine-based immunotherapy, such as IFN-α and interleukin-2.
†Radiofrequency ablation and percutaneous ethanol injection.

in all 7 HCC cell lines examined (Fig. 1A). Among all HCC specimens, the expression of PD-L1 and PD-L2 was shown in the cell membrane, cytoplasm, or both in a focal or scattered pattern (Fig. 1B and C; Supplementary Fig. S1). For vast majority of HCC cases, PD-Ls-positive cells were evenly scattered throughout the specimens, similar to their expression fashion in glioma (38) and ovarian cancer (17). In addition, PD-Ls expression was also detected in some infiltrating lymphocytes and endothelial cells in HCC tissues (Supplementary Fig. S2).

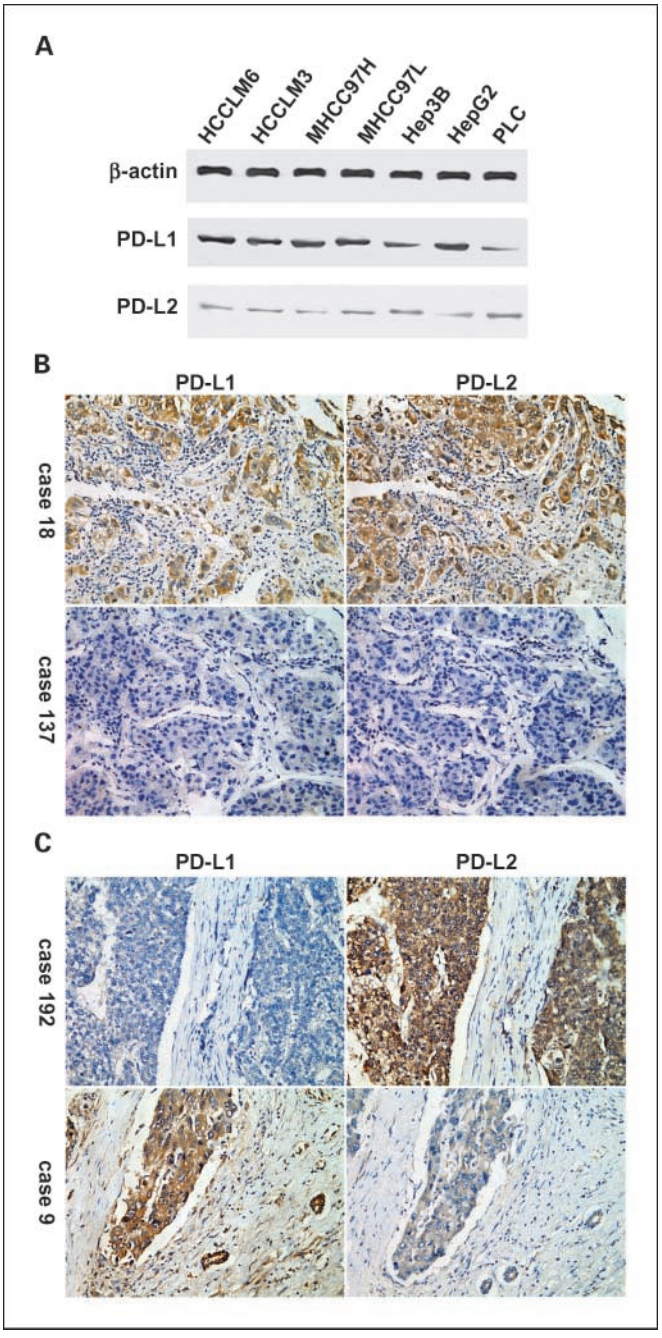


**Fig. 1.** PD-Ls expression in HCC cell lines and tissue samples. Western blots detected constitutive PD-L1 and PD-L2 expressions on 7 HCC cell lines (*A*). Total protein (50 μg) was loaded per lane. Consecutive sections were used for immunohistochemical study on expression of PD-L1 and PD-L2 on HCC tumor tissues. Representative cases of concurrent high or low (*B*) as well as either high (*C*) expression of PD-L1 and PD-L2 on HCC tumor tissues were shown. Positive cells were stained brown. Magnification, ×200.

***PD-Ls expression and patient prognosis.*** PD-L1-positive (high expression) patients had significantly poorer DFS and OS than PD-L1-negative (low expression) patients (Table 2; Fig. 2A). The median DFS and OS were 14.9 and 29.6 months for PD-L1-positive patients compared with not reached and 59.4 months for PD-L1-negative patients, respectively. However, there was significant difference in OS but only broadline significance in DFS between PD-L2-positive and PD-L2-negative patients (Table 2; Fig. 2B). On multivariate analysis, tumor PD-L1 status was defined to be an independent prognostic factor for DFS. PD-L1-positive patients were nearly two times more likely to suffer from relapse than PD-L1-negative patients [hazard ratio (HR), 1.71; 95% confidence interval (95% CI), 1.11-2.65; Table 3; Supplementary Table S1]. The difference in DFS remained significant when the density of PD-L1 expression was evaluated as a continuous variable (Table 3; Supplementary Table S2). However, for PD-Ls, no significant differences in cancer-specific survival were uncovered and the significance in OS disappeared on multivariate analysis (Table 3; Supplementary Table S2) partly due to these two types of events are suboptimal endpoints in HCC clinical settings (39).

Furthermore, subgroup analysis also indicated that significant differences in recurrence were found between PD-L1-positve and PD-L1-negative patients after categorization by the following variables: small tumor ($P$ = 0.024), single tumor ($P$ = 0.0045), tumor with vascular invasion ($P$ = 0.020), tumor with encapsulation ($P$ = 0.021), poor differentiation tumor ($P$ = 0.028), and tumor stage II ($P$ = 0.028, log-rank test; Supplementary Fig. S3).

We also investigated combined PD-Ls expression on patient outcome. Patients were divided into three groups: (*a*) both PD-L1 and PD-L2 were positive ($n$ = 13), (*b*) either PD-L1 or PD-L2 was positive ($n$ = 94), and (*c*) both PD-L1 and PD-L2 were negative ($n$ = 133). Significant differences in recurrence and survival were found between groups I and III as well as between groups II and III. The median DFS and OS were 6.0 and 11.0 months for group III, 35.5 and 35.5 months for group II, and not reached and 50.0 months for group I, respectively (Fig. 2C). Multivariate analysis revealed that the differences in OS and DFS between groups I and III had a tendency toward statistical significance ($P$ = 0.09 and 0.09, respectively; Table 3; Supplementary Table S2).

***Relationship of immunomarkers with clinicopathologic features.*** To evaluate the association of PD-Ls with tumor biology, comparisons of the clinicopathologic features with PD-Ls expression were made. Patients with high PD-Ls expression were more likely to exhibit aggressive clinicopathologic features: PD-L1-positive patients harbored more tumors with the presence of vascular invasion, whereas PD-L2-positive patients harbored more tumor vascular invasion and advanced TNM stage (Table 4). Adjuvant therapy and postrecurrent treatment did not differ significantly between PD-Ls-positive and PD-Ls-negative groups. Meanwhile, high-dense FoxP3$^+$ and low-dense granzyme B$^+$ TILs were significantly associated with aggressive phenotypes (Table 4).

***Correlation between PD-Ls expression and TILs.*** Granzyme B$^+$ and FoxP3$^+$ TILs were also found to be independent prognosticators for DFS, OS, and even cancer-specific survival (Table 3; Supplementary Table S2; Supplementary Fig. S4), conferring a

**Table 2.** Univariate analyses of prognosis factors associated with survival

| Variables | DFS | | OS | | Cancer-specific survival | |
|---|---|---|---|---|---|---|
| | HR (95% CI) | P | HR (95% CI) | P | HR (95% CI) | P |
| Age, y (≤52 vs >52) | 0.78 (0.53-1.15) | 0.21 | 0.80 (0.53-1.21) | 0.29 | 0.98 (0.54-1.80) | 0.95 |
| Gender (female vs male) | 0.69 (0.38-1.26) | 0.22 | 0.70 (0.37-1.31) | 0.27 | 0.57 (0.20-1.59) | 0.28 |
| Hepatitis history (no vs yes) | 0.76 (0.40-1.46) | 0.42 | 1.26 (0.55-2.90) | 0.58 | 0.88 (0.31-2.48) | 0.80 |
| α-Fetoprotein, ng/mL (≤20 vs >20) | 1.70 (1.14-2.53) | 0.0095 | 2.08 (1.37-3.15) | 0.0005 | 2.27 (1.11-4.66) | 0.025 |
| γ-Glutamyl transferase, units/L (≤54 vs >54) | 1.63 (1.08-2.44) | 0.019 | 2.04 (1.32-3.17) | 0.0015 | 2.31 (1.18-4.52) | 0.015 |
| Liver cirrhosis (no vs yes) | 1.35 (0.72-2.53) | 0.34 | 2.13 (0.99-4.62) | 0.055 | 1.71 (0.61-4.81) | 0.31 |
| Tumor differentiation (well vs poor) | 1.50 (1.02-2.21) | 0.041 | 1.85 (1.22-2.78) | 0.0035 | 2.05 (1.11-3.80) | 0.023 |
| Tumor size, cm (≤5 vs >5) | 2.46 (1.63-3.71) | <0.0001 | 2.60 (1.68-4.02) | <0.0001 | 1.97 (1.06-3.69) | 0.033 |
| Tumor multiplicity (single vs multiple) | 2.37 (1.57-3.58) | <0.0001 | 1.82 (1.17-2.83) | 0.008 | 2.62 (1.39-4.94) | 0.003 |
| Tumor encapsulation (complete vs none) | 2.51 (1.66-3.80) | <0.0001 | 1.67 (1.10-2.53) | 0.016 | 1.73 (0.93-3.23) | 0.08 |
| Vascular invasion (no vs yes) | 4.60 (3.01-7.03) | <0.0001 | 4.78 (3.03-7.55) | <0.0001 | 4.10 (2.12-7.92) | <0.0001 |
| TNM stage (I vs II vs III) | 2.96 (2.29-3.83) | <0.0001 | 2.90 (2.21-3.80) | <0.0001 | 3.38 (2.22-5.14) | <0.0001 |
| FoxP3$^+$ TILs (low vs high) | 2.09 (1.41-3.12) | 0.0003 | 2.79 (1.81-4.30) | <0.0001 | 3.33 (1.72-6.44) | 0.0004 |
| Granzyme B$^+$ TILs (low vs high) | 0.49 (0.33-0.74) | 0.0006 | 0.49 (0.32-0.74) | 0.0008 | 0.43 (0.23-0.80) | 0.008 |
| PD-L1 (low vs high) | 1.79 (1.18-2.70) | 0.0058 | 1.61 (1.04-2.50) | 0.032 | 1.24 (0.62-2.48) | 0.54 |
| PD-L2 (low vs high) | 1.45 (0.93-2.24) | 0.099 | 1.60 (1.01-2.52) | 0.044 | 1.09 (0.52-2.28) | 0.82 |
| Combined PD-Ls* | | | | | | |
| Overall | NA | 0.0007 | NA | 0.0019 | NA | 0.64 |
| I vs II | 1.42 (0.94-2.14) | 0.096 | 1.41 (0.92-2.18) | 0.12 | 1.33 (0.72-2.47) | 0.37 |
| I vs III | 3.72 (1.87-7.39) | 0.0002 | 3.61 (1.75-7.41) | 0.0005 | 0.83 (0.11-6.15) | 0.85 |

NOTE: Cox proportional hazards regression model.
Abbreviation: NA, not applicable.
*Patients were divided into three groups: (*a*) both PD-L1 and PD-L2 were positive ($n$ = 13), (*b*) either PD-L1 or PD-L2 was positive ($n$ = 94), and (*c*) both PD-L1 and PD-L2 were negative ($n$ = 133).

validation of our previous reports (3). Representative images of TILs were shown in Supplementary Fig. S5. There was no significant correlation neither between PD-L1/PD-L2 expression and granzyme B$^+$ activated CTLs ($r$ = 0.06, $P$ = 0.35; $r$ = -0.10, $P$ = 0.87) nor between PD-L1 and PD-L2 expression ($r$ = -0.06, $P$ = 0.37). Intriguingly, significant positive correlation between PD-L1 expression and FoxP3$^+$ regulatory T (Treg) cell infiltration ($r$ = 0.17, $P$ = 0.009) as well as between PD-L2 expression and Treg infiltration ($r$ = 0.20, $P$ = 0.002) were found.

***Association of immunomarkers with true tumor recurrence and* de novo *hepatocarcinogenesis.*** Using 12 months as the cutoff value, all of the intrahepatic recurrences were divided into early recurrence ($n$ = 59), which is mainly from intrahepatic metastasis, and late recurrence ($n$ = 16), which is usually a result of a multicentric new tumor (40). In addition, extrahepatic recurrence ($n$ = 27) was more likely to be a true metastasis from primary tumor. This may provide simple basis for distinguishing a true HCC relapse from *de novo* hepatocarcinogenesis, that is, extrahepatic or early intrahepatic recurrence representing the true versus late intrahepatic recurrence representing the false. The correlation of immunostaining and recurrence status was summarized in Supplementary Table S3. More patients with high PD-L1 or low granzyme B$^+$ TILs or high FoxP3$^+$ TILs, compared with patients with low PD-L1 or high granzyme B$^+$ TILs or low FoxP3$^+$ TILs, had extrahepatic or early intrahepatic recurrence ($P$ = 0.007, 0.001, and 0.002, respectively) rather than late intrahepatic recurrence ($P$ = 0.80, 0.09, and 0.54, respectively).

Although PD-Ls expression, CTL and Treg counts were all significantly higher in patients with extrahepatic or early intrahepatic recurrence compared with patients without recurrence, PD-L1 was the unique immunomarker significantly higher in patients with late intrahepatic recurrence than nonrecurrent patients ($P$ = 0.007; Supplementary Fig. S6).

***Independent validation.*** The prognostic ability of tumor PD-L1 expression and FoxP3$^+$ TIL density was validated in independent data set on multivariate analysis (Table 3; Supplementary Table S3). The 1-and 4-year DFS rates were 75% and 45% for patients with lower PD-L1 expression, compared with 59% and 12% for patients with higher expression, respectively (Fig. 2D). Of note, tumor PD-L1 expression also showed the ability to subclassify stage I patients, with the median DFS 55.0 versus 15.0 months for patients with lower versus higher expression levels, respectively ($P$ = 0.022, log-rank test). A statistically significant positive correlation between tumor PD-L1 expression and FoxP3$^+$ TIL density was also revealed ($r$ = 0.22, $P$ = 0.016).

## Discussion

We here describe that PD-L1, a costimulatory molecule of the B7 family, is constitutively expressed in HCC cells *in vitro* as well as in tumor specimens *in vivo*. More importantly, we present the first large-scale study using high-throughput tissue microarray analyses to examine the prognostic effect of tumor PD-Ls expression in a random population of surgically resected HCC patients and also in an independent validation data set.

We found that HCC patients with tumor PD-L1 expression are at significant high risk of cancer recurrence. Multivariate analysis further strengthened that PD-L1 expression was an independent prognostic factor with the smallest $P$ value along with the well-established factors including tumor vascular invasion, encapsulation, and TNM stage. The results were then

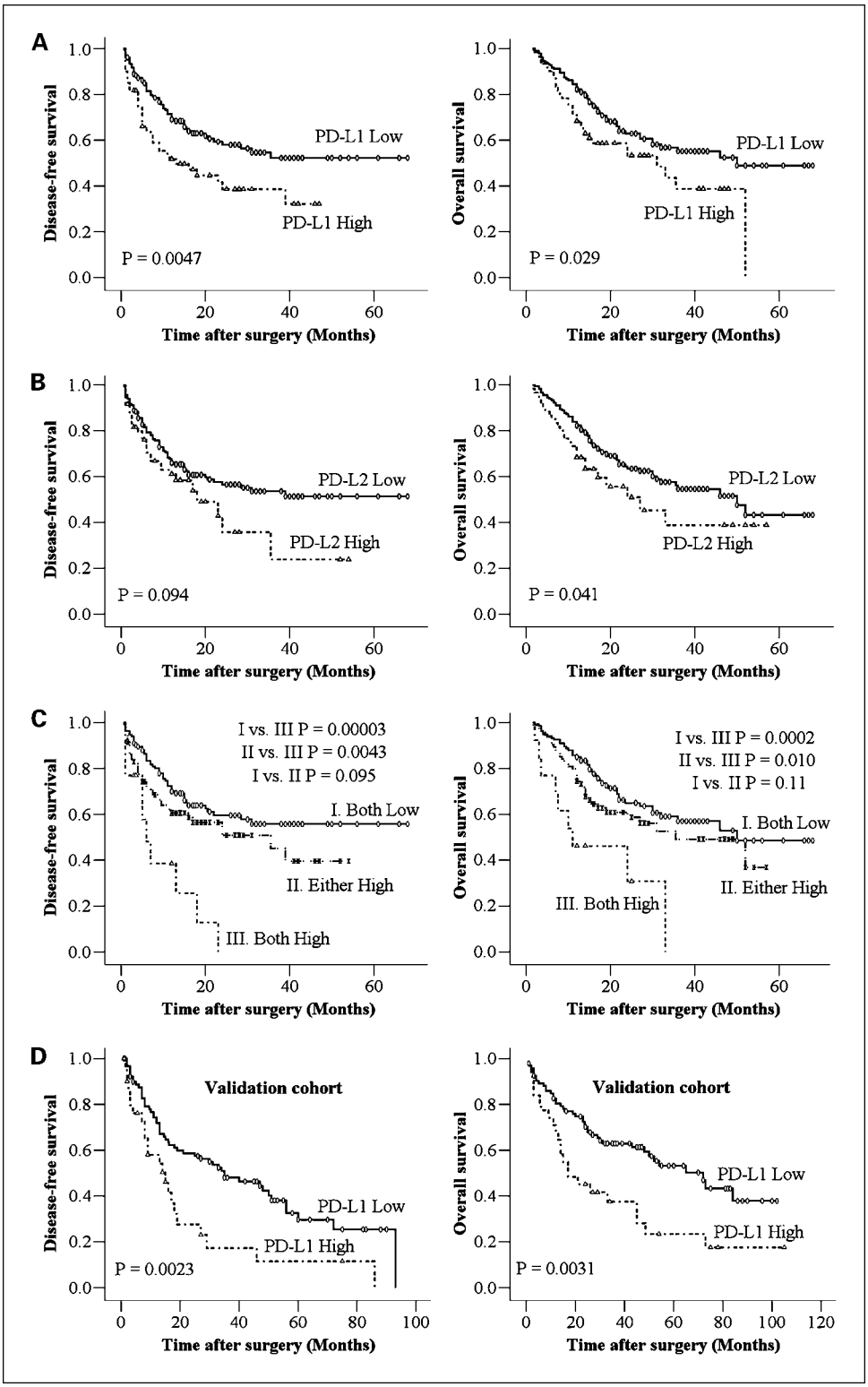


**Fig. 2.** Kaplan-Meier curves of survival differences among HCC patients. DFS and OS for expression of PD-L1 (*A*), PD-L2 (*B*), and their combination (*C*) were found to be statistically significant. Significant differences in DFS and OS were validated in an independent cohort based on PD-L1 expression (*D*). *P* values were determined by the log-rank test.

validated in an independent cohort of patients with nearly two times median follow-up period than that of the original cohort (30.0 versus 16.0 months). The basis for this association may relate to the recognized ability of PD-L1 to inhibit T-cell-mediated antitumor immunity as well as its newly identified function as a ubiquitous antiapoptotic receptor on cancer cells (41). As shown in coculture experiments, PD-L1$^+$ HCC cells induced T-cell apoptosis, which was further augmented by IFN-mediated up-regulation of PD-L1 on HCC cells, while in the presence of blocking antibodies to PD-L1, apoptosis in T cells was significantly reduced (42). Also, a study using PD-L1-deficient mice model suggested that PD-L1 was a key

regulator in determining accumulation and deletion of intrahepatic CD8$^+$ T cells (13). Therefore, the assessment of tumor PD-L1 offers additional information for patient prognosis and represents an attractive target for immune manipulation in the multimodal treatment of HCC. Furthermore, given that the antitumor effect of interleukin-12-based gene therapy on HCC-bearing mice was partly counteracted by constitutive or induced PD-L1 expression and significant differences in tumor PD-L1 expression detected between responder and nonresponder mice (43), we speculate that intratumoral PD-L1 may function as a critical determinant of treatment responses in patients who receive immunotherapy.

HCC relapse is characterized by two types: a true metastasis resulted from HCC dissemination before resection and multicentric occurrence in the liver remnant caused by continuous virus infection and inflammation (accounting for 20-60%; ref. 44). Treatments that are effective against metastasis may not prevent *de novo* cancer and vice versa. Because PD-L1 can facilitate failure of T-cell responses to terminate hepatitis C or B virus infections, targeted blockade of the PD-L1/PD-1 pathway may have additional implications for virus clearance (45) and hence prevent *de novo* hepatocarcinogenesis on the background of infected liver. Thus, PD-L1 may represent a single bullet with the power to simultaneously target HCC recurrence and *de novo* cancer. Supporting this hypothesis, we found that only PD-L1 was expressed significantly higher in patients with late intrahepatic recurrence, a probable condition of *de novo* hepatocarcinogenesis, compared with nonrecurrent patients. However, down-regulation or dysfunction of PD-Ls/PD-1 systems in autoimmune hepatitis and primary biliary cirrhosis, especially in autoimmune hepatitis, are suggested to play important roles in the development of these diseases where immune responses are aberrantly enhanced (46). It will be important to determine how effectively they can be therapeutically manipulated to enhance antitumor immunity and pathogen control while maintaining effective regulation of immunopathology.

In the current study, although PD-L2 expression correlated with patients' survival and to a less extent tumor recurrence, it was not significant anymore on multivariate analysis. Similar results have also been reported in ovarian (17) and pancreatic (19, 47) cancers, whereas conflicting conclusion suggesting PD-L2 as an independent prognosticator in esophageal cancer (48) also exists. Our results sustained the hypothesis that the involvement of PD-L1 and PD-L2 in the tumor immune escape differs depending on the organs or tumor types (17). In addition, patients with concurrent low expression of PD-L1 and PD-L2 had a far better prognosis than those with high expression of either or both of them, showing a tendency toward significance on multivariate analysis.

In line with no correlation revealed between PD-L1 and CD8$^+$ TILs in other solid tumors (48), we found no significant correlation between PD-Ls and activated CTLs either. The reduction of CTLs may not be the only mechanism by which PD-L1 promotes tumor immune escape, and it may also be possible that PD-L1 on tumor cells induces functional impairment of tumor-specific T cells without reducing their number. Notably, a significant positive correlation between PD-Ls expression and Treg infiltration was found and further validated. Mechanistically, it has shown that naive T cells cultured with *Helicobacter pylori*-infected gastric epithelial cells can develop into cells with the Treg phenotype, which was completely dependent on induced PD-L1 expressed on gastric

**Table 3.** Multivariate analyses of prognosis factors associated with survival

| Variables | DFS | | OS | | Cancer-specific survival | |
|---|---|---|---|---|---|---|
| | HR (95% CI) | *P* | HR (95% CI) | *P* | HR (95% CI) | *P* |
| A* | | | | | | |
| FoxP3$^+$ TILs (low vs high) | 1.93 (1.25-2.96) | 0.003 | 2.45 (1.55-3.87) | 0.0001 | 2.68 (1.32-5.43) | 0.006 |
| Granzyme B$^+$ TILs (low vs high) | 0.59 (0.38-0.90) | 0.014 | 0.66 (0.42-1.03) | 0.065 | 0.57 (0.29-1.12) | 0.10 |
| PD-L1 (low vs high) | 1.71 (1.11-2.65) | 0.015 | 1.31 (0.84-2.07) | 0.24 | 1.06 (0.51-2.20) | 0.88 |
| PD-L2 (low vs high) | 0.96 (0.60-1.53) | 0.86 | 1.10 (0.68-1.79) | 0.71 | 0.77 (0.35-1.69) | 0.51 |
| Combined PD-Ls † | | | | | | |
| Overall | NA | 0.22 | NA | 0.22 | NA | 0.71 |
| I vs II | 1.19 (0.78-1.82) | 0.42 | 1.05 (0.67-1.64) | 0.84 | 1.03 (0.54-1.99) | 0.92 |
| I vs III | 1.95 (0.90-4.23) | 0.09 | 2.03 (0.90-4.59) | 0.09 | 0.43 (0.05-3.49) | 0.43 |
| B* | | | | | | |
| FoxP3$^+$ TILs | 1.01 (1.01-1.02) | 0.0008 | 2.45 (1.55-3.87) | 0.0001 | 1.02 (1.01-1.03) | 0.0049 |
| Granzyme B$^+$ TILs | 0.99 (0.98-1.00) | 0.012 | 0.99 (0.98-1.00) | 0.0047 | 0.98 (0.97-1.00) | 0.010 |
| PD-L1 | 1.01 (1.00-1.02) | 0.0097 | 1.00 (1.00-1.01) | 0.27 | 1.00 (0.99-1.01) | 0.76 |
| PD-L2 | 1.00 (0.99-1.00) | 0.36 | 1.00 (0.99-1.01) | 0.62 | 0.99 (0.98-1.01) | 0.30 |
| C* | | | | | | |
| FoxP3$^+$ TILs (low vs high) | 1.74 (1.04-2.91) | 0.034 | 1.63 (0.92-2.90) | 0.094 | | ND |
| PD-L1 (low vs high) | 2.06 (1.12-3.81) | 0.020 | 1.84 (1.00-3.40) | 0.050 | | ND |

NOTE: Cox proportional hazards regression model, including all the clinicopathologic features as covariates (for details, see Supplementary Tables S1-S3).

Abbreviations: NA, not applicable; ND, not done.

*(A) Patients were dichotomized according to the 75th percentile of PD-Ls density and median of TIL counts. (B) PD-Ls and TIL density were modeled as a continuous variable. The HR is for each increase of 5% in expression intensity. (C) Validation of the prognostic significance of PD-L1 and FoxP3$^+$ TIL density in an independent data set.

†Patients were divided into three groups: (*a*) both PD-L1 and PD-L2 were positive ($n$ = 13), (*b*) either PD-L1 or PD-L2 was positive ($n$ = 94), and (*c*) both PD-L1 and PD-L2 were negative ($n$ = 133).

**Table 4.** Correlation of clinicopathologic findings with tumor PD-Ls expression and TIL counts

| Characteristics | PD-L1 | | | PD-L2 | | | CTLs | | | Treg | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Low | High | *P* | Low | High | *P* | Low | High | *P* | Low | High | *P* |
| Age, y | | | | | | | | | | | | |
| ≤52 | 92 | 32 | 0.77 | 92 | 32 | 0.77 | 70 | 54 | 0.053 | 58 | 66 | 0.24 |
| >52 | 88 | 28 | | 88 | 28 | | 51 | 65 | | 63 | 53 | |
| Gender | | | | | | | | | | | | |
| Male | 155 | 49 | 0.40 | 152 | 52 | 0.68 | 104 | 100 | 0.68 | 107 | 97 | 0.13 |
| Female | 25 | 11 | | 28 | 8 | | 17 | 19 | | 14 | 22 | |
| Hepatitis history | | | | | | | | | | | | |
| Yes | 168 | 56 | 1.00 | 169 | 55 | 0.55 | 115 | 109 | 0.29 | 111 | 113 | 0.32 |
| No | 12 | 4 | | 11 | 5 | | 6 | 10 | | 10 | 6 | |
| α-Fetoprotein (ng/mL) | | | | | | | | | | | | |
| ≤20 | 67 | 15 | 0.084 | 62 | 20 | 0.88 | 43 | 39 | 0.65 | 54 | 28 | 0.001 |
| >20 | 113 | 45 | | 118 | 40 | | 78 | 80 | | 67 | 91 | |
| γ-Glutamyl transferase (units/L) | | | | | | | | | | | | |
| ≤54 | 77 | 27 | 0.76 | 82 | 22 | 0.23 | 45 | 59 | 0.053 | 58 | 46 | 0.15 |
| >54 | 103 | 33 | | 98 | 38 | | 76 | 60 | | 63 | 73 | |
| Liver cirrhosis | | | | | | | | | | | | |
| Yes | 158 | 54 | 0.64 | 158 | 54 | 0.64 | 101 | 111 | 0.018 | 101 | 111 | 0.018 |
| No | 22 | 6 | | 22 | 6 | | 20 | 8 | | 20 | 8 | |
| Tumor size (cm) | | | | | | | | | | | | |
| ≤5 | 91 | 26 | 0.33 | 89 | 28 | 0.71 | 52 | 65 | 0.071 | 58 | 59 | 0.80 |
| >5 | 89 | 34 | | 91 | 32 | | 69 | 54 | | 63 | 60 | |
| Tumor encapsulation | | | | | | | | | | | | |
| None | 87 | 34 | 0.26 | 88 | 31 | 0.71 | 49 | 70 | 0.005 | 56 | 65 | 0.20 |
| Complete | 93 | 26 | | 92 | 29 | | 72 | 49 | | 65 | 54 | |
| Tumor multiplicity | | | | | | | | | | | | |
| Single | 138 | 46 | 1.00 | 140 | 44 | 0.48 | 88 | 96 | 0.15 | 102 | 82 | 0.005 |
| Multiple | 42 | 14 | | 40 | 16 | | 33 | 23 | | 19 | 37 | |
| Tumor differentiation | | | | | | | | | | | | |
| I-II | 106 | 29 | 0.15 | 100 | 35 | 0.71 | 62 | 73 | 0.12 | 77 | 58 | 0.020 |
| III-IV | 74 | 31 | | 80 | 25 | | 59 | 46 | | 44 | 61 | |
| Vascular invasion | | | | | | | | | | | | |
| Yes | 75 | 34 | 0.043 | 71 | 38 | 0.001 | 55 | 76 | 0.004 | 45 | 64 | 0.010 |
| No | 105 | 26 | | 109 | 22 | | 66 | 43 | | 76 | 55 | |
| TNM stage | | | | | | | | | | | | |
| I | 84 | 22 | 0.40 | 88 | 18 | 0.038 | 45 | 61 | 0.009 | 68 | 38 | 0.001 |
| II | 54 | 22 | | 52 | 24 | | 37 | 39 | | 28 | 48 | |
| III | 42 | 16 | | 40 | 18 | | 39 | 19 | | 25 | 33 | |
| Adjuvant therapy | | | | | | | | | | | | |
| None | 63 | 19 | 0.84 | 61 | 22 | 0.12 | 41 | 41 | 0.94 | 42 | 40 | 0.017 |
| TACE | 94 | 34 | | 92 | 36 | | 64 | 64 | | 57 | 71 | |
| Immunotherapy * | 23 | 7 | | 27 | 3 | | 16 | 14 | | 22 | 8 | |
| Postrecurrent therapy | | | | | | | | | | | | |
| None | 20 | 11 | 0.83 | 27 | 4 | 0.12 | 18 | 13 | 0.34 | 15 | 16 | 0.013 |
| TACE | 35 | 19 | | 34 | 20 | | 31 | 23 | | 15 | 39 | |
| Regional † | 3 | 1 | | 3 | 1 | | 4 | 0 | | 3 | 1 | |
| Resection | 10 | 3 | | 10 | 3 | | 7 | 6 | | 9 | 4 | |

NOTE: Pearson's $\chi^2$ test.
*Cytokine-based immunotherapy, such as IFN-α and interleukin-2.
†Radiofrequency ablation and percutaneous ethanol injection.



epithelial cells (49). Additionally, Treg was suggested to be highly resistant to PD-L1-induced apoptosis compared with antitumor effector T cells (50). Similar results have also been just reported in pancreatic cancer (47). This suggests that PD-L1-induced tumor immune evasion may operate through the arm of Treg, although their causal link and precise nature warrant further investigation.

In conclusion, we report that elevated PD-L1 expression in HCC is significantly associated with tumor aggressiveness and enhanced risk for postoperative recurrence. As such, PD-L1 may represent a target for HCC immunotherapy and a potential biomarker to facilitate patient assignment to treatment as well as aid in the determination of prognosis both before and after therapy.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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