Association of PD-L1 and HIF-1α Coexpression with Poor Prognosis in Hepatocellular Carcinoma ⓇCrossMark

Xiaomeng Dai[*,1], Guoliang Pi[†,1], Sheng-li Yang[*], George G. Chen[‡], Li-ping Liu[§] and Han-Hua Dong[¶]

[*]Cancer Center, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China; [†]Department of Radiation Oncology, Hubei Cancer Hospital, Wuhan 430079, China; [‡]Department of Surgery, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, NT, Hong Kong, China; [§]Department of Hepatobiliary and Pancreas Surgery, the Second Clinical Medical College of Jinan University (Shenzhen People's Hospital), Shenzhen, Guangdong Province, China; [¶]Hepatic Surgery Centre, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430030, China

Abstract

OBJECTIVE: To investigate the correlation between the expression of PD-L1 and HIF-1α in hepatocellular carcinoma (HCC) tissue and further analyze the association with clinical parameters and the prognostic value of coexpression in HCC patients. *METHODS:* We assessed the expression of PD-L1 and HIF-1α by immunohistochemistry in tumor tissue from 90 HCC patients who underwent curative hepatectomy. The results were validated in an independent cohort of additional 90 HCC patients. *RESULTS:* PD-L1 and HIF-1α exhibited in tumor tissue high expression rates of 41.11% (37/90) and 43.33% (43/90), respectively, and their expressions were positively correlated ($r = 0.563$, $P < .01$). High expression of PD-L1 was significantly associated with low albumin levels ($P < .05$); high expression of HIF-1α was significantly correlated with high alpha-fetoprotein (AFP) levels and low albumin levels ($P < .05$); high expression of both PD-L1 and HIF-1α was also significantly associated with high AFP levels and low albumin levels ($P < .05$). High expression of PD-L1, HIF-1α, as well as both PD-L1 and HIF-1 α was respectively significantly associated with worse overall survival (OS) and disease-free survival (DFS) ($P < .05$). Patients with co-overexpression of PD-L1 and HIF-1α had the worst prognosis compared with other groups. Additionally, multivariate Cox regression models suggested that high expression of PD-L1, HIF-1α, as well as both PD-L1 and HIF-1α was an independent prognostic factor for OS and DFS ($P < .05$). Furthermore, the positive correlation and prognostic values of PD-L1 and HIF-1α were validated in an independent data set. *CONCLUSION:* We demonstrated that HCC patients with co-overexpression of PD-L1 and HIF-1α in tumor tissue had a significantly higher risk of recurrence or metastasis and death compared with others. Therefore, more frequent follow-up is needed for patients with co-overexpression of PD-L1 and HIF-1α. At the same time, a combinational therapy with HIF-1α inhibitors in conjunction with PD-L1 blockade may be beneficial for HCC patients with co-overexpression in the future.

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Introduction

Hepatocellular carcinoma (HCC) remains one of the most frequently occurring and aggressive human malignancies worldwide [1]. Chronic hepatitis B virus and C virus infections, metabolic syndrome, and chronic alcohol consumption are major leading HCC etiologies [2]. Curative therapy for HCC, including surgical resection, ablation, and liver transplantation, is only suitable for 10% to 30% of all HCC patients [3]. Although considerable progress has been made in surgical

techniques and molecular targeted treatment (e.g., with sorafenib), long-time outcome remains to be dismal. Frequent drug resistance, recurrence, and metastasis are the main obstacles to the current clinical management of HCC [4]. Accordingly, novel systemic therapies are required to improve the patients' prognosis.

A series of clinical investigations indicated that immune cell infiltration in peritumoral and intratumoral liver tissue correlated with poor prognosis [5,6], suggesting that HCC may be immunogenic. The advent of immune checkpoint inhibitors represents a breakthrough in cancer treatment. Indeed, the representative immune checkpoint inhibitors ipilimumab (against T-lymphocyte-associated protein-4 (CTLA-4)), nivolumab (against programmed death-1 (PD-1)), and pembrolizumab (against programmed death-ligand 1 (PD-L1)) have been approved by the US Food and Drug Administration for the treatment of non–small cell lung cancer and metastatic melanoma [7,8], opening a new avenue for tumor immunotherapy. PD-1, a cell surface glycoprotein receptor, is normally expressed in activated T cells, B cells, and natural killer cells. PD-L1, as the major ligand of PD-1, binds to PD-1 to suppress anticancer immunity by inducing T-cell apoptosis and exhaustion [9,10]. Recently, several clinical investigations suggested that PD-L1 is overexpressed in various tumors, including melanoma, non–small cell lung cancer, breast cancer, as well as HCC, and correlated with poor clinicopathological features and poor prognosis [11–15]. Currently, the immunohistochemical expression of PD-L1 in tumor cells or tumor-associated stromal cells is the best predictive biomarker of response to PD-1/PD-L1 targeted therapy [16]. Although PD-1/PD-L1 antibodies showed promising outcomes for cancer treatment, only a proportion of patients responded to the treatments [17]. Therefore, the response to anti–PD-1/PD-L1 antibodies cannot be predicted only based on the expression of PD-L1.

Hypoxia is a common feature of HCC, especially in patients with liver cirrhosis, and plays an important role in the development of HCC [18]. Intrahepatic hypoxia stimulates cancer development, invasion and metastasis, and resistance to chemotherapy and radiation [19]. Hypoxia-inducible factor-1α (HIF-1α) is a major transcription factor involved in the hypoxic response of cancer cells and activates hundreds of genes that play vital roles in angiogenesis, proliferation, glucose metabolism, invasion and metastasis, and resistance to radiation and chemotherapy in HCC [18]. Several studies demonstrated that high HIF-1α expression in tumor tissue is associated with poor outcomes in multiple types of cancer, including HCC [20–22]. Recently, relevant studies exhibited that HIF-1α upregulates PD-L1 expression on myeloid-derived suppressor cells and tumor cells, contributing to cancer immune evasion [23–26]. However, to the best of our knowledge, the association between the expression of PD-L1 and HIF-1α in HCC remains obscure. Therefore, we examined the expression of PD-L1 and HIF-1α by immunohistochemistry in tumor tissue of 90 HCC patients and investigated the correlation of the expression of PD-L1 and HIF-1α with clinicopathological features and clinical outcomes. The obtained results were further validated in an external cohort of an additional series of 90 HCC tissue arrays.

Methods

Patients and Clinicopathological Information

The study was designed and carried out strictly according to the Reporting Recommendations for Tumor Marker Prognostic Studies and the Transparent Reporting of a Multivariable Prediction Model for Individual Prognosis or Diagnosis statement [27,28]. Included were 90 HCC patients who underwent curative hepatectomy between November 1995 and January 2013 in the Prince of Wales Hospital (Hong Kong, China). Detailed eligibility criteria were as follows: 1) patients with diagnosed with HCC for the first time and no distant metastasis; 2) no history of any anticancer therapy before surgery and underwent radical hepatectomy; 3) follow-up information was available; and 4) patients with autoimmune disease, other malignant tumors, or serious heart, lung, kidney, or blood diseases were excluded. Written consent was obtained from all patients or their legal representatives. The study was approved by the Joint Chinese University of Hong Kong–New Territories East Cluster Clinical Research Ethics Committee. All available surgical specimens were reviewed by two independent, experienced pathologists blinded to all patient-related information. Conventional clinicopathological variables have been described and detailed in previous reports [12].

Tissue Microarray and Immunohistochemistry

Construction of liver cancer tissue microarrays as well as immunostaining was performed according to standard protocols described elsewhere [6]. The following primary antibodies were used: anti–PD-L1 (mouse monoclonal antibody, clone 2B11D11, Proteintech, Chicago, IL) and anti–HIF-1α antibody (mouse monoclonal antibody, clone H1alpha67, Santa Cruz Biotechnology, CA). PD-L1 and HIF-1α expressions were assessed in neoplastic tumor cells. The semiquantitative analytical criteria of the PD-L1 and HIF-1α expression levels have been previously described in detail [21,29]: cytoplasmic/membranous PD-L1 expression according to the intensity of the staining (0, negative; 1, very weak; 2, moderate; and 3, strong expression) [30] and cytoplasmic and nuclear HIF-1α expression according to intensity and extensity of the staining (0, no staining; 1, nuclear staining in less than 10% of cells and/or with weak cytoplasmic staining; 2, nuclear staining in 10%-50% of cells and/or with distinct cytoplasmic staining; and 3, nuclear staining in more than 50% of cells and/or with strong cytoplasmic staining) [31]; 0 and 1 were defined as low-expression group, whereas 2 and 3 were defined as high-expression group.

Statistical Analysis

Statistical analyses were conducted using the SPSS software (version 23.0; IBM, Armonk, NY). Either χ^2 test or Fisher's exact test was used to explore the association between immunostaining markers and clinicopathological variables. The correlation was analyzed by Pearson's contingency analysis. Overall survival (OS) was defined as the length of time from the date of curative hepatectomy to death. Disease-free survival (DFS) was defined as the interval between the date of curative hepatectomy and recurrence (locoregional recurrence or distant metastases) or death. Data were censored at the last follow-up (November 11, 2014) for patients without recurrence, metastases, or death. Kaplan-Meier analysis was used to determine the survival, and the two-sided log-rank test was used to compare differences. The univariate Cox regression model was conducted including all clinicopathological features as covariates. Multivariate Cox proportional-hazards analysis was performed on the significant covariates

A

	Low	High	
			PD-L1
			HIF-1a

B

	HIF-1a		Pearson's Contingency Coefficient	P
PD-L1	High	Low		
High	31	6		
Low	8	45	0.563	0.000

Experimental cohort

C

	Low	High	
			PD-L1
			HIF-1a

D

	HIF-1a		Pearson's Contingency Coefficient	P
PD-L1	High	Low		
High	22	22		
Low	9	37	0.305	0.002
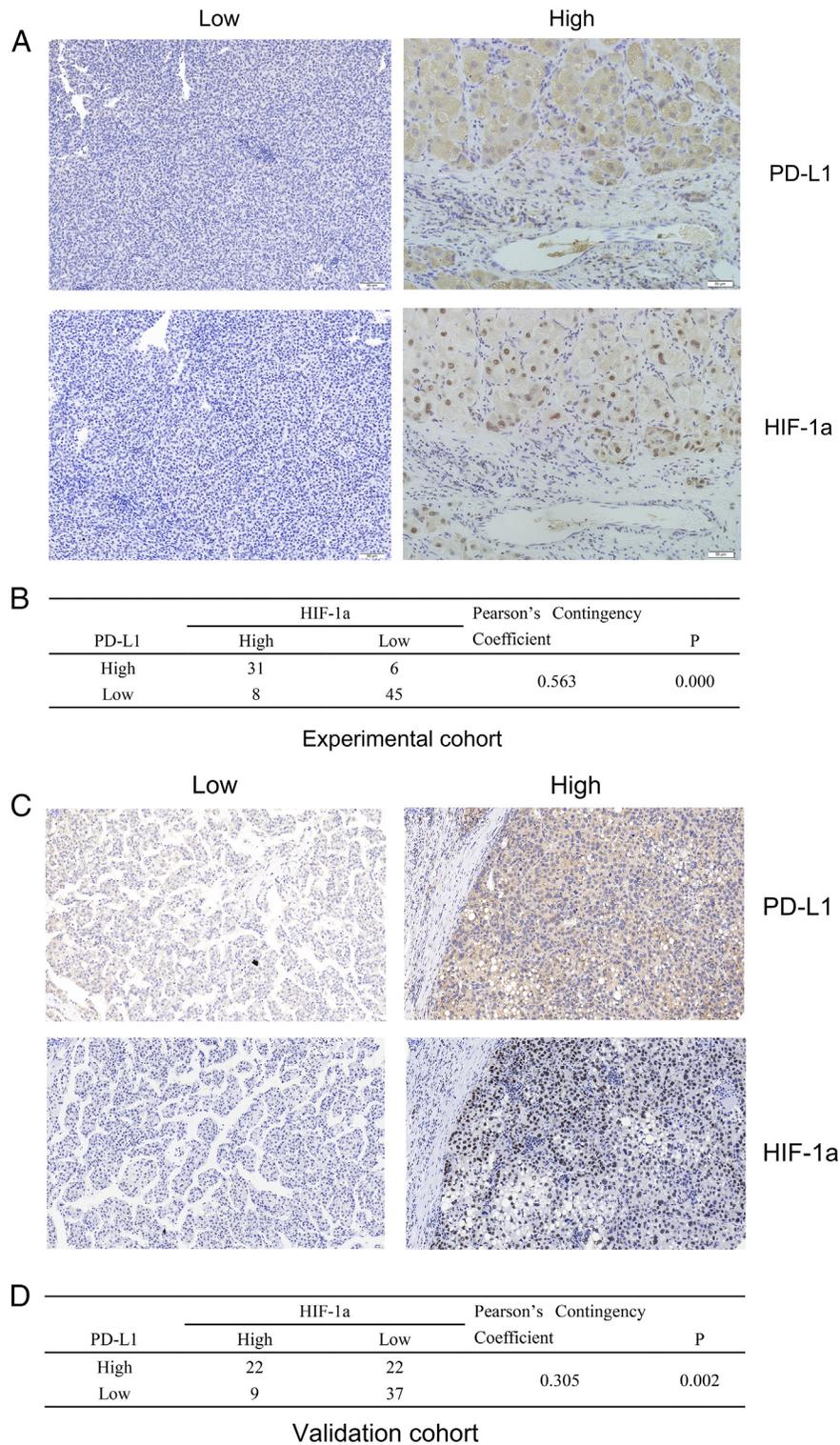
Validation cohort

Figure 1. Representative immunohistochemistry images of HCC tissues for detection of PD-L1 and HIF-1α in experimental (A) and validation cohort (C); positive correlation between the expression of PD-L1 and HIF-1α in experimental (B) and validation cohort (D).

determined by the univariate analysis. Two-sided P values < .05 were considered as statistically significant in all cases.

Independent Validation Cohort

To further validate our results, we examined the correlation and prognostic performance of the expression of PD-L1 and HIF-1α in an independent cohort containing an additional tissue array of 90 HCC patients. The tissue array was purchased from BioChip (Shanghai, China). The detailed clinicopathological features of this

Table 1. Correlations of PD-L1 and HIF-1α Protein Expression with Clinicopathological Characters

Parameters	PD-L1			HIF-1α			PD-L1 and HIF-1α		
	High	Low	P	High	Low	P	Both high	Others	P
Age			.101			.063			.092
<50 y	15	12		16	11		13	14	
≥50 y	22	41		23	40		18	45	
Gender			.687			.397			1.000
Male	34	50		35	49		29	55	
Female	3	3		4	2		2	4	
Cirrhosis			.080			.383			.176
Absence	10	25		13	22		9	26	
Presence	26	28		26	28		22	32	
Unknown	1	0		0	1		0	1	
Tumor size			.516			.519			.509
<5 cm	18	31		19	30		15	34	
≥5 cm	18	22		19	21		15	25	
Unknown	1	0		1	0		1	0	
AFP			.226			.003			.042
<400 μg/l	23	37		20	40		17	43	
≥400 μg/l	13	11		17	7		13	11	
Unknown	1	5		2	4		1	5	
Histological grade			.241			.145			.238
Well and moderate	28	45		30	43		24	49	
Poor	8	6		9	5		7	7	
Unknown	1	2		0	3		0	3	
Vascular invasion			.115			.114			.103
Absence	21	39		22	38		17	43	
Presence	16	14		17	13		14	16	
Number of tumor lesions			.785			1.000			.744
single	29	44		31	42		24	49	
multiple	7	9		7	9		6	10	
Unknown	1	0		1	0		1	0	
HBV/HCV infection			.261			.279			1.000
Yes	31	50		33	48		27	54	
No	5	3		5	3		3	5	
Unknown	1	0		1	0		1	0	
ALT			.604			.798			.589
>80 U/l	10	10		8	12		8	11	
≤80 U/l	25	37		28	34		22	43	
Unknown	2	6		3	5		1	5	
Albumin			.024			.027			.026
>35 g/l	26	44		27	43		22	48	
≤35 g/l	9	3		9	3		8	4	
Unknown	2	6		3	5		1	7	
Bilirubin			1.000			1.000			1.000
>20 μmol/l	1	2		1	2		1	2	
≤20 μmol/l	34	45		35	44		29	50	
Unknown	2	6		3	5		1	7	

HBV/HCV, hepatitis B/C virus.
  P < .05.

cohort of patients have been described in detail in our previous article [12]. Immunohistochemistry, semiquantitative analysis of markers, and statistics were performed in the same manner as the experimental cohort in this study.

Results

PD-L1 and HIF-1α Expression and Clinicopathological Features

In the HCC tissues, PD-L1 protein was distributed in a diffuse manner and was mainly found in the cellular membrane and/or cytoplasm of tumor cells; HIF-1α protein was mainly expressed in the cytoplasm and/or nucleus of tumor cells (Figure 1A). PD-L1 and HIF-1α exhibited high expression rates in the HCC tissue with values of 41.11% (37/90) and 43.33% (43/90), respectively. The high PD-L1 expression in tumor cells was significantly associated with low albumin levels (P = .024), whereas high HIF-1α expression significantly correlated with high alpha-fetoprotein (AFP) levels (P = .003) and low albumin levels (P = .027). Furthermore, high expression of both PD-L1 and HIF-1α was also significantly associated with higher AFP levels (P = .042) and lower albumin levels (P = .026) compared with others (Table 1). However, no statistically significant association was observed between the expression of PD-L1 and HIF-1α and other clinicopathological features. Pearson's contingency analysis showed a positive correlation between the expression of PD-L1 and HIF-1α (r = 0.563, P < .01; Figure 1B).

Survival Analysis

Kaplan-Meier survival and disease analysis of 90 HCC patients (Figure 2) showed significantly worse OS (P = .001) and DFS (P = .004) for the PD-L1 high-expression group compared with the low-expression group. Furthermore, the HIF-1α high-expression group had both shorter OS (P = .000) and DFS (P = .001) than the
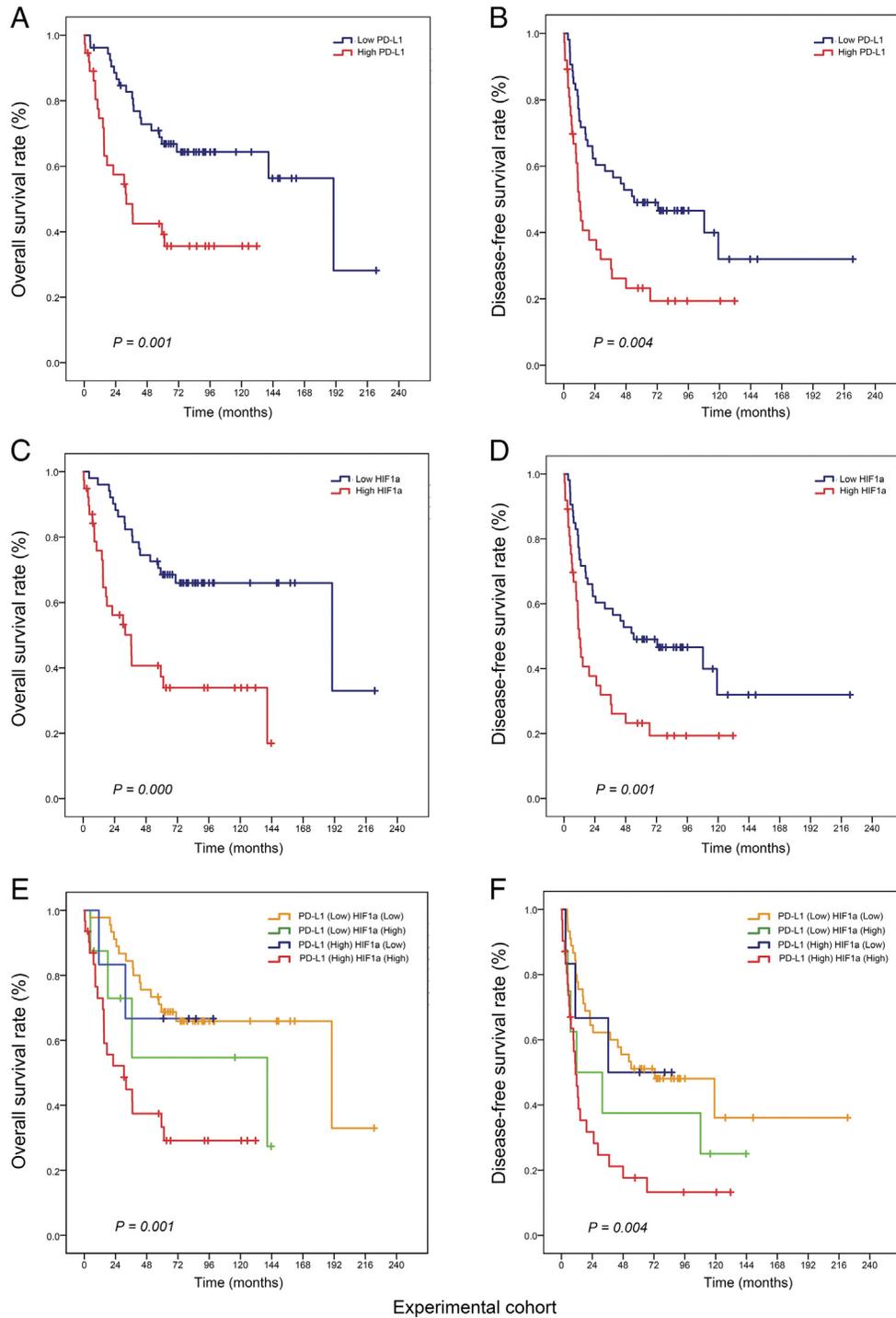
Figure 2. Prognostic values of PD-L1 and HIF-1α expression in HCC tissues. Kaplan-Meier OS and DFS analysis of different types stratified by PD-L1 (A, B), HIF-1α (C, D), and PD-L1 and HIF-1α (E, F) in experimental cohort.

corresponding low-expression group. Based on these results, we divided the 90 HCC patients into four groups for further Kaplan-Meier analyses. Patients in both PD-L1 and HIF-1α high-expression group had worst OS ($P < .01$) and DFS ($P < .01$) than other groups (Figure 2).

Given the significant correlation between the expression of PD-L1 and HIF-1α, we performed multiple univariate and multivariate Cox proportional-hazards analyses, including analyses of the PD-L1, HIF-1α, and both PD-L1 and HIF-1α high-expression groups,

respectively. Multivariate Cox regression model of the PD-L1 expression groups revealed that vascular invasion ($P < .01$), tumor diameters ≥5 cm ($P < .01$), and PD-L1 high expression ($P < .01$) were independent indicators for poor OS (Table 2). Multivariate Cox proportional-hazards analysis showed that alanine aminotransferase (ALT) > 80 U/l ($P < .05$), multiple tumor lesions ($P < .01$), tumor diameters ≥5 cm ($P < .01$), and high expression of PD-L1 ($P < .01$) can independently predict poor DFS (Table 3). Moreover, multivariate analysis of HIF-1α expression groups identified that

Table 2. Multivariate Analysis of OS in Hepatocellular Carcinoma Patients (Cox Regression Model)

	HR (95% CI)	P
Including PD-L1		
Vascular invasion (present vs absent)	2.936 (1.547-5.572)	0.001
Tumor diameter (≥5 cm vs <5 cm)	2.555 (1.344-4.857)	0.004
PD-L1 (high vs low)	2.643 (1.387-5.035)	0.003
Including HIF-1α		
ALT (> 80 U/l vs ≤ 80 U/l)	2.240 (1.117-4.495)	0.023
Vascular invasion (present vs absent)	3.020 (1.519-6.007)	0.002
Tumor diameter (≥5 cm vs < 5 cm)	2.670 (1.327-5.372)	0.006
HIF-1α (high vs low)	3.109 (1.576-6.131)	0.001
Including PD-L1 and HIF-1α		
Vascular invasion (present vs absent)	2.822 (1.494-5.329)	0.001
Tumor diameter (≥5 cm vs < 5 cm)	2.691 (1.415-5.116)	0.003
PD-L1 and HIF-1α (both vs others)	3.047 (1.599-5.804)	0.001

CI, confidence interval.

ALT > 80 U/l ($P < .05$), vascular invasion ($P < .01$), tumor diameters ≥5 cm ($P < .01$), and high expression of HIF-1α ($P < .01$) were independent predictive factors for OS (Table 2). Moreover, ALT >80 U/l ($P < .01$), multiple tumor lesions ($P < .01$), tumor diameters ≥5 cm ($P < .01$), and high expression of HIF-1α ($P < .01$) were independent predictive factors for DFS (Table 3). In addition, multivariate analysis, including the covariates of PD-L1 and HIF-1α (both vs others), exhibited that vascular invasion ($P < .01$), tumor diameters ≥5 cm ($P < .01$), and co-overexpression of both HIF-1α and PD-L1 were independent predictive factors for OS and DFS. Furthermore, ALT >80 U/l ($P < .05$) also independently indicated poor DFS (Tables 2-3).

Independent Validation Cohort

In the validation group, the distribution of PD-L1 and HIF-1α in tumor cells was the same as in the experimental group. Representative PD-L1 and HIF-1α immunohistochemical staining profiles were shown in Figure 1C. High expression of PD-L1 and HIF-1α in the HCC tissue was observed in 48.89% (44/90) and 34.44% (31/90) of patients, respectively. The expression of PD-L1 was found to be significantly higher in male HCC patients ($P < .05$) and HCC patients with higher AFP levels ($P < .05$) and cancer embolus ($P < .01$). The expression of HIF-1α was found to be significantly higher in HCC patients with higher AFP levels ($P < .05$). No statistically significant correlations were found between high expression of both PD-L1 and HIF-1α and any clinicopathological features in the

Table 3. Multivariate analysis of DFS in HCC Patients (Cox Regression Model)

	HR (95% CI)	P
Including PD-L1		
ALT (>80 U/l vs ≤80 U/l)	1.994 (1.091-3.647)	.025
Number of tumor lesions (multiple vs single)	3.314 (1.584-6.932)	.001
Tumor diameter (≥5 cm vs < 5 cm)	2.415 (1.377-4.234)	.002
PD-L1 (high vs low)	2.488 (1.425-4.345)	.001
Including HIF-1α		
ALT (>80 U/l vs ≤80 U/l)	2.302 (1.251-4.237)	.007
Number of tumor lesions (multiple vs single)	3.385 (1.612-7.106)	.001
Tumor diameter (≥5 cm vs <5 cm)	2.199 (1.249-3.873)	.006
HIF-1α (high vs low)	2.728 (1.543-4.820)	.001
Including PD-L1 and HIF-1α		
ALT (>80 U/l vs ≤80 U/l)	2.101 (1.147-3.847)	.016
Number of tumor lesions (multiple vs single)	3.209 (1.539-6.693)	.002
Tumor diameter (≥5 cm vs <5 cm)	2.267 (1.291-3.981)	.004
PD-L1 and HIF-1α (both vs others)	2.541 (1.448-4.458)	.001

validation cohort (Table S1). Pearson's contingency analysis exhibited that PD-L1 expression was significantly associated with HIF-1α expression ($r = 0.371$, $P < .01$) (Figure 1D). The prognostic values of high expression of PD-L1, HIF-1α, as well as both HIF-1α and PD-L1 were validated in the additional 90 HCC patients by Kaplan-Meier survival analysis for OS and DFS ($P < .05$; Figure 3). Furthermore, univariate Cox proportional-hazards analysis revealed that high expressions of PD-L1, HIF-1α as well as both HIF-1α and PD-L1 were independent predictive factors for OS respectively ($P < .05$; Table S2, S3, S6). In the multivariable model, PD-L1 high expression and AFP ≥ 400 ng/ml were identified as independent predictive factors for DFS ($P < .01$; Table S4). However, the Cox proportional-hazard ratios for high expression of HIF-1α and co-overexpression of both HIF-1α and PD-L1 were not statistically significant for DFS ($P > .05$; Table S5, S7).

Discussion

In the present study, we demonstrated that HCC patients with high expression of PD-L1 or HIF-1α in tumor tissue had a significantly higher risk of recurrence or metastasis and death, which was in line with previous studies [11,21]. We first found a positive correlation between the expression of PD-L1 and HIF-1α in HCC tumor tissue. Both high PD-L1 and HIF-1α expressions were significantly correlated with higher AFP levels and lower albumin levels compared with others. Additionally, HCC patients with high expression of both HIF-1α and PD-L1 had the worst prognosis compared with others. Multivariate survival analyses further supported that the co-overexpression of HIF-1α and PD-L1 in HCC tissue was an independent prognostic factor for OS and DFS. The prognostic value was further validated in an independent cohort containing an additional 90 HCC patients.

Hypoxia is a common characteristic of many solid cancers as well as HCC. Recently, a series of studies demonstrated that hypoxia contributes to immune escape in cancer via a multifaceted mechanism [23]. Strikingly, hypoxia can significantly increase the PD-L1 expression on myeloid-derived suppressor cells, macrophages, dendritic cells, and tumor cells, and further acquired resistance to CTL-mediated lysis is in a certain manner dependent on HIF-1α [24,25]. Although our study found a positive correlation between PD-L1 and HIF-1α in HCC tissue, it remains obscure whether HIF-1α can selectively upregulate the expression of PD-L1 to favor anticancer immune escape in HCC cells and animal models. This hypothesis needs to be verified in future studies.

Although a series of clinical trials has shown promising outcomes for cancer treatment with PD-1/PD-L1 antibodies, only a proportion of patients responded to the treatments. The main reason is that the PD-L1 expression is not sufficient to predict the response to Abs against PD-1/PD-L1. Our results exhibited that patients with PD-L1 and HIF-1α co-overexpression had the worst prognosis, and increasing evidence shows that the expression of PD-L1 in cancer cells is mediated by HIF-1α [24,25]. Accordingly, we speculated that HCC patients with high expression of both PD-L1 and HIF-1α may be more suitable for anti–PD-1/PD-L1 therapy. It is well known that HIF-1 is a major oncogenic factor for HCC, and a relevant study showed that the nitric oxide mimetics GTN or 8-bromo-cGMP, which can prevent the accumulation of HIF-1α protein, can block the hypoxia-induced upregulation of PD-L1 and further inhibit hypoxia-induced resistance to CTL-mediated lysis in B16-OVA cells
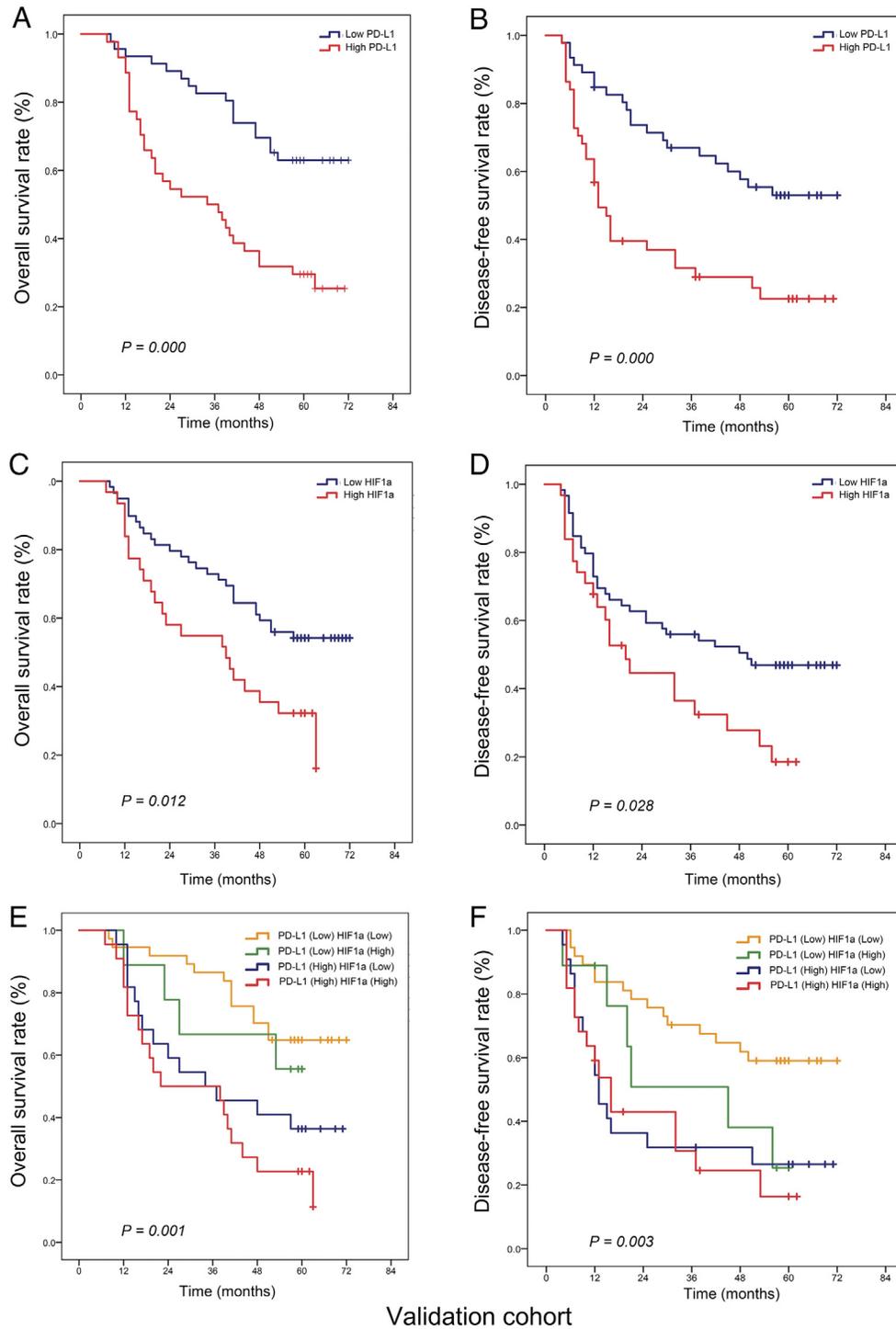
Figure 3. Prognostic values of PD-L1 and HIF-1α expression in HCC tissues. Kaplan-Meier OS and DFS analysis of different types stratified by PD-L1 (A, B), HIF-1α (C, D), and PD-L1 and HIF-1α (E, F) in validation cohort.

[32]. Moreover, inhibition of HIF-1 has been applied for the treatment of cancer [18,33,34]. Therefore, a novel combinational therapy targeting tumor hypoxia by using HIF-1α inhibitors in conjunction with PD-L1 blockade may boost the immune system in cancer patients. As our results suggested that co-overexpression of PD-L1 and HIF-1α is an independent prognostic factor for OS and DFS, and the co-overexpression group had the worst prognosis, we speculated that patients with PD-L1 and HIF-1α co-overexpression may be more suitable for the combined PD-L1/HIF-1α inhibition therapy. Relevant clinical trials need to be designed to verify our speculation for HCC patients in the future.

In conclusion, we first reported the positive correlation between the expression of PD-L1 and HIF-1α in HCC tissue. Co-overexpression of PD-L1 and HIF-1α was found to be an independent prognostic factor for OS and DFS, and patients with high expression of both PD-L1 and HIF-1α had the worst prognosis compared with others. Therefore,

more frequent follow-up is needed for patients with co-overexpression of PD-L1 and HIF-1α. At the same time, a combinational therapy with HIF-1α inhibitors in conjunction with PD-L1 blockade may be beneficial for HCC patients with co-overexpression and needs to be examined in future studies.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2018.02.014.

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