Original Article

PD-L1 combined with HDAC9 is a useful prognostic predictor in hepatocellular carcinoma

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Background: The expression of programmed death-ligand 1 (PD-L1) is associated with the response of patients to PD-1/PD-L1 blockade immunotherapy. It has been demonstrated that histone deacetylase (HDAC) inhibitors may alter the expression of PD-L1/PD-L2 and enhance the antitumor immune responses. However, the profile of PD-L1 expression and its association with HDACs in hepatocellular carcinoma (HCC) has not been accurately investigated.

Methods: The expression of PD-L1 and HDACs were examined by immunohistochemical (IHC) staining using tissue microarray (TMA) of 109 HCC specimens. Expression data from TCGA database and IHC staining of TMA were used for correlation analysis. Survival rates were analyzed based on data from 109 HCC patients.

Results: We found that PD-L1 was upregulated in the majority of HCC samples. Furthermore, correlation analysis revealed that PD-L1 expression was positively correlated with HDAC9 and HDAC2 expression in HCC. Survival analysis showed that high levels of PD-L1 combined with increased expression of HDAC9 decreased overall survival (OS) in patients with HCC. In addition, univariate and multivariate analysis further suggested that the increased PD-L1/HDAC9 expression was an independent prognostic biomarker in HCC. HDAC9 overexpression promoted HCC growth and PD-L1 expression.

Conclusions: The results of the current study highlighted the strong association between HDACs with immunotherapy, thus providing a rational basis for combining HDAC9 specific inhibitors and PD-1 blockade in a future clinical approach for HCC.

Keywords: Programmed death-ligand 1 (PD-L1); hepatocellular carcinoma (HCC); histone deacetylase 9; histone deacetylase 2; predictor

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignancy and the third leading cause of cancer-related death worldwide (1,2). Currently, surgical resection and liver transplantation are considered as the only available approaches to treat HCC. However, large percentage of patients are diagnosed with HCC at an advanced stage, thereby missing the local treatment opportunity (3,4). Several multi-targeted tyrosine kinase inhibitors (TKIs), including sorafenib, lenvatinib, regorafenib and cabozantinib, are considered to be the most efficient targeted drugs for the systemic treatment of patients with advanced HCC (5-7). However, a study demonstrated that sorafenib could extend the median overall survival (OS) by

only 3 months (8). Therefore, novel therapeutic modalities are urgently needed to treat patients with advanced HCC.

Programmed cell death protein 1 (PD-1), an inhibitory receptor expressed on activated T cells, negatively regulates T cell responses. When PD-1 binds to the co-inhibitory receptors on tumor cells, such as programmed death ligand 1 or 2 (PD-L1 or PD-L2), s T cell the proliferation and cytokine secretion of T cells were attenuated, ultimately leading to T cell dysfunction or apoptosis (9-11). Immunotherapy, especially the immune checkpoint blockade with anti-cytotoxic T lymphocyte associated antigen 4 (CTLA-4) and anti-PD-1/PD-L1 antibodies, has been successfully utilized in treating several types of advanced tumors, including non-small cell lung cancer (NSCLC) (12), melanoma, bladder carcinoma (13,14), Hodgkin's lymphoma (15,16), and Merkel cell carcinoma (17). Clinical studies on antibodies against PD-1 in advanced HCC, showed safety and durable antitumor activity, suggesting that immunotherapy with immune checkpoint inhibitors could bring new hope for advanced HCC treatment. Nivolumab, a PD-1 monoclonal antibody, has been approved by the Food and Drug Administration (FDA) for the systemic treatment of HCC, in patients who have previously received sorafenib treatment (18). However, the response rate of patients underwent anti-PD-1 monotherapy was <20% (19). Importantly, reliable predictive biomarkers to assess the response of patients with HCC to immunotherapy are still lacking. Accumulating evidence has suggested that the expression profile of PD-L1 on tumor cells is significantly associated with the therapeutic effect of PD-1 blocking antibodies (20,21). In addition, a study revealed that anti-PD-1 therapy exerted an increased objective response rate in patients with PD-L1 positive disease (22). Since the expression of PD-L1 is very dynamic, revealing the regulatory mechanism underlying PD-L1 expression remains a main challenge for tumor immunotherapy.

It has been shown that histone deacetylases (HDACs) are overexpressed and associated with tumor progression in several cancer types such as lung, gastric, colorectal, breast, prostate and pancreatic carcinomas (23-28). Clinical trials are currently exploring the potential of HDAC inhibitors as anticancer agents. Several studies have shown that treatment with epigenetic modulators, including HDAC inhibitors, upregulates the expression of checkpoint inhibitors in tumor cells and enhances the responses to blockade therapy (29,30). These findings prompted any investigation into whether there was any association between HDACs and PD-Ls. Therefore, the present study evaluated the protein expression levels and clinical significance of PD-L1 in HCC and evaluated the correlation between HDACs and PD-L1.

We present the following article in accordance with the ARRIVE reporting checklist (available at http://dx.doi.org/10.21037/tcr-20-3415).

Methods

Patients and sample collection

One hundred nine patients were randomly collected from HCC patients who underwent liver resection in Eastern Hepatobiliary Surgery Hospital (Shanghai, China) from September 2012 to October 2014. The patients did not receive any preoperative anticancer treatment. All patients were observed until November 2019. OS referred to the interval between the dates of surgery and death. Disease-free survival (DFS) referred to the interval between the surgery and recurrence. If recurrence was not diagnosed, patients were censored on the date of death or the last follow-up. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethic Committee of Eastern Hepatobiliary Surgery Hospital (No.: SHDC12017122) and informed consent was taken from all the patients.

Real time RT-PCR

Total RNA of HCC tumor tissues was extracted using Trizol reagent (Gibco, Carlsbad, CA, USA). Real-time PCR was performed using an ABI 7300 Fast Real-time PCR System (Applied Biosystems) and SYBR Green PCR kit (Applied TaKaRa, Japan). The primer sequences are shown as following (to detect all isoforms of HDAC9, the primers used for HDAC9 qPCR were designed to amplify a fragment from exon 21 to exon 23 of the HDAC9 mRNA):

Gene	Forward primer	Reverse primer
PD-L1	5'-GTTGAAGGACCAGCTCTCCC-3'	5'-CTTGTAGTCGGCACCACCAT-3'
HDAC2	5'-TGAAGTCTTGTTTCAGTGGCT-3'	5'-AAGCTATAGAGGGCAAGGT-3'
HDAC9	5'-TTCAGGACCATCGTGAAGCC-3'	5'-TAGAGCCAACACCACACGTC-3'
β-actin	5'-CGCGAGAAGATGCCCAGATC-3'	5'-TCACCGGAGTCCATCACGA-3'

Immunohistochemical staining and quantification

Paraffin embedded tissue samples were cut into 5 μm sections. Immunohistochemical staining were performed following the routine protocol. The primary antibodies were the following: anti-PD-L1 (ab205921, Abcam, Cambridge, UK), anti-HDAC9 (ab109446, Abcam, Cambridge, UK) and anti-HDAC2 (ab32117, Abcam, Cambridge, UK). The density of IHC staining was quantified by Image-Pro Plus v6.2 software (Media Cybernetics Inc., Bethesda, MD).

Cell line and lentivirus

HCC cell line MHCC-97L were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Full length human HDAC9 or green fluorescent protein (GFP) CDS sequence was inserted into pLenti-CMV-3FLAG lentiviral vector (OBiO Technology, Shanghai). The lentivirus, LV-HDAC9 or LV-GFP were infected into MHCC-97L cells with a multiplicity of infection (MOI) 20 for 4 hours. After 12 hours, the original medium was replaced with fresh medium.

Western blot analysis

Whole cell extracts were prepared in lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM PMSF, 10 mM NaF, 5 mg/mL aprotinin, 20 mM leupeptin, and 1 mM sodium orthovanadate) and centrifuged at 12,000 rpm for 15 min. Protein extracts were subjected to SDS-PAGE. The primary antibodies used were anti-HDAC9 (ab109446, Abcam, Cambridge, UK) and anti-GAPDH (60004, Proteintech, Wuhan, China).

Nude mice xenograft model

Experiments were performed under a project license (No. 2018ZX10723204) granted by institutional ethics committee of Second Military Medical University (Shanghai, China), in compliance with the institutional guidelines for the care and use of animals. Five-week-old male athymic BALB/c nude mice (weight ~19–22 g) were obtained from Shanghai Model Organisms Center (Shanghai, China) and allowed to acclimate to their new surroundings for one week. All mice were kept in home cages in a specific pathogen-free (SPF) environment with free access to sterilized food and water, and animal experiments were conducted at the Second Military Medical University Animal Experiment Center (Shanghai, China). The home cages were cleaned, and sterilized food and water were exchanged weekly during the experiments. In the animal xenograft assays, 5×10^6 HDAC9-transfected MHCC-97L cells or control cells were injected subcutaneously in the right flank of mice (n=6). Tumor size was measured every three days, and tumor volume was calculated by the formula: $(\text{width})^2 \times \text{length}/2$. Mice were sacrificed 3 weeks after injection and tumors were excised, fixed in 10% buffered formalin solution and used for further experiments.

Statistical analysis

The SPSS version 21 and R version 3.2.3 softwares were used in data analysis. The experiment results were shown as mean ± SEM. Data analysis was carried out by Student's *t* test, χ^2 test, Fisher's exact test. In survival analysis, the median value for PD-L1 expression was used to divide the patients into high-PD-L1 (scored as 1) and low-PD-L1 (scored as 0) groups. In the same way, the patients were divided into high-HDAC9 (scored as 1) and low-HDAC9 (scored as 0) groups. We define the patient cases that have two "0" scores as "combined low PD-L1 and HDAC9", and those have two "1" scores as "combined high PD-L1 and HDAC9". Kaplan-Meier was used to calculate survival curves. Multivariate Cox proportional hazards regression model was used to determine prognostic factors. The significant difference was determined by $P<0.05$.

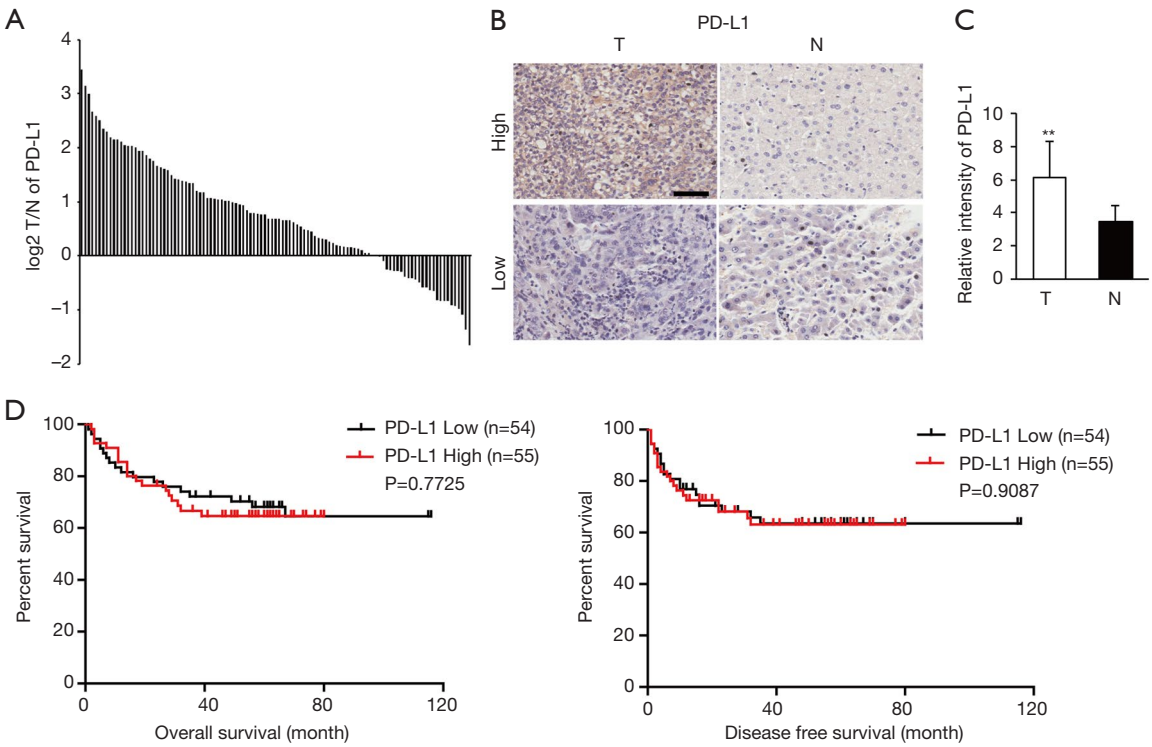

Figure 1 PD-L1 is upregulated in HCC samples. (A) IHC staining of PD-L1 in HCC TMA. The results from all 109 specimens were quantified with the Image-Pro Plus v6.2 software. (B) Representative micrographs showing high and low expression levels of PD-L1 in HCC and normal peritumoral tissues (scale bar, 50 μm). (C) The mean expression levels of PD-L1 were compared between HCC (T) and normal peritumoral (N) tissues. Data are shown as the mean ± standard deviation (SD). **, P<0.01. (D) Kaplan-Meier survival analysis of OS and DFS rates of the high and low PD-L1 expression groups. PD-L1, programmed death-ligand 1; HCC, hepatocellular carcinoma; TMA, tissue microarray; IHC, immunohistochemistry; OS, overall survival; DFS, disease-free survival.

Results

PD-L1 is upregulated in HCC samples

To determine the expression status of PD-L1 in HCC, 109 patients with HCC, who underwent liver resection (Table S1), from the tissue microarrays (TMA) were used to detect the protein levels of PD-L1 by immunohistochemical (IHC) staining. The results showed that PD-L1 was increased in 83/109 (76.1%) HCC tumor samples compared with the peritumoral normal tissues (*Figure 1A*). Representative PD-L1 immunostaining results showing the different levels of PD-L1 in HCC and peritumoral tissues are illustrated in *Figure 1B*. In addition, the average PD-L1 protein exhibited remarkably higher levels in HCC tissues in comparison with normal peritumoral tissues (*Figure 1C*). Based on the IHC results of PD-L1 expression in tumor tissues, we divided 109 HCC patients into two groups, namely the high-expression (n=55) and low-expression (n=54) groups. Although PD-L1 expression was positively associated with recurrence (Table S2), no obvious difference in DFS or OS was observed between the high- and low-expression groups (*Figure 1D*). These findings suggested that the upregulated PD-L1 expression could not serve as a prognostic predictor for HCC.

mRNA expression of PD-L1 in HCC is associated with HDACs

To investigate whether PD-L1 expression was associated with that of HDACs in HCC, a gene correlation analysis was carried out using Gene Expression Profiling Interactive Analysis (GEPIA) using RNA expression data from The Cancer Genome Atlas (TCGA) database. The results indicated that the mRNA expression levels of seven HDACs in 369 HCC tissue samples, including HDAC1, HDAC2, HDAC3, HDAC4, HDAC7, HDAC8 and

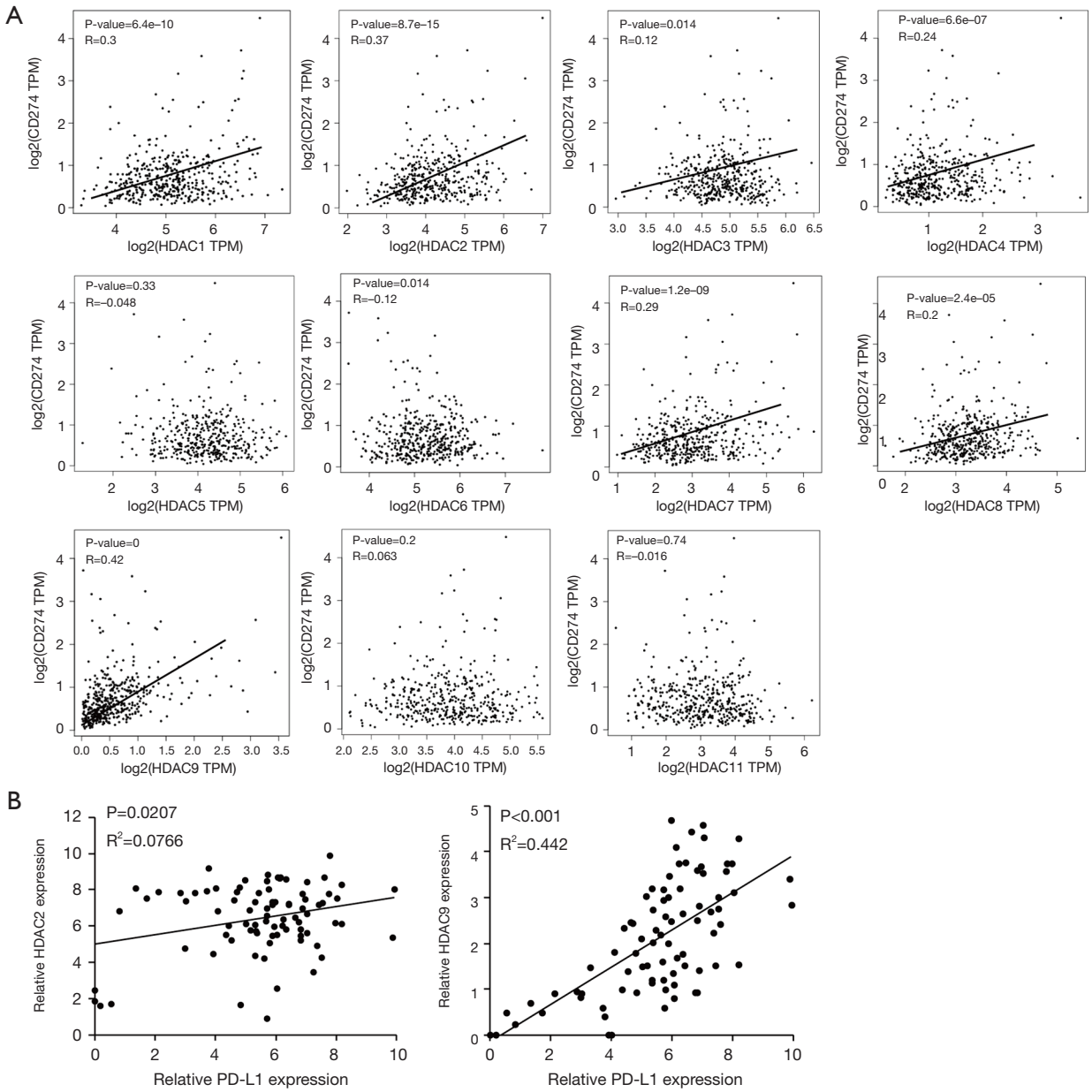

Figure 2 PD-L1 mRNA expression in HCC is associated with HDAC mRNA expression. (A) Correlation analysis of PD-L1 with HDAC9 and HDAC2 in the GEPIA database (http://gepia.cancer-pku.cn) using mRNA expression data of HCC specimens from TCGA database (n=369). (B) Correlation analysis of PD-L1 with HDAC2 and HDAC9 using the corresponding mRNA expression levels from HCC tissues. PD-L1, programmed death-ligand 1; HCC, hepatocellular carcinoma; HDAC, histone deacetylase; GEPIA, Gene Expression Profiling Interactive Analysis; TCGA, The Cancer Genome Atlas.

HDAC9, showed a positive correlation with the mRNA levels of PD-L1 (CD274; P<0.01, *Figure 2A*). Notably, among the seven HDACs tested, HDAC9 (R=0.42) and HDAC2 (R=0.37) were the most significantly correlated with PD-L1 expression. The mRNA expression levels of PD-1, HDAC2 and HDAC9 were also evaluated in 84 clinically and pathologically characterized HCC tissues from our hospital. RT-qPCR analysis showed that the expression of HDAC2 (R^2=0.0766) and HDAC9 (R^2=0.442) was positively correlated with PD-L1 expression in HCC

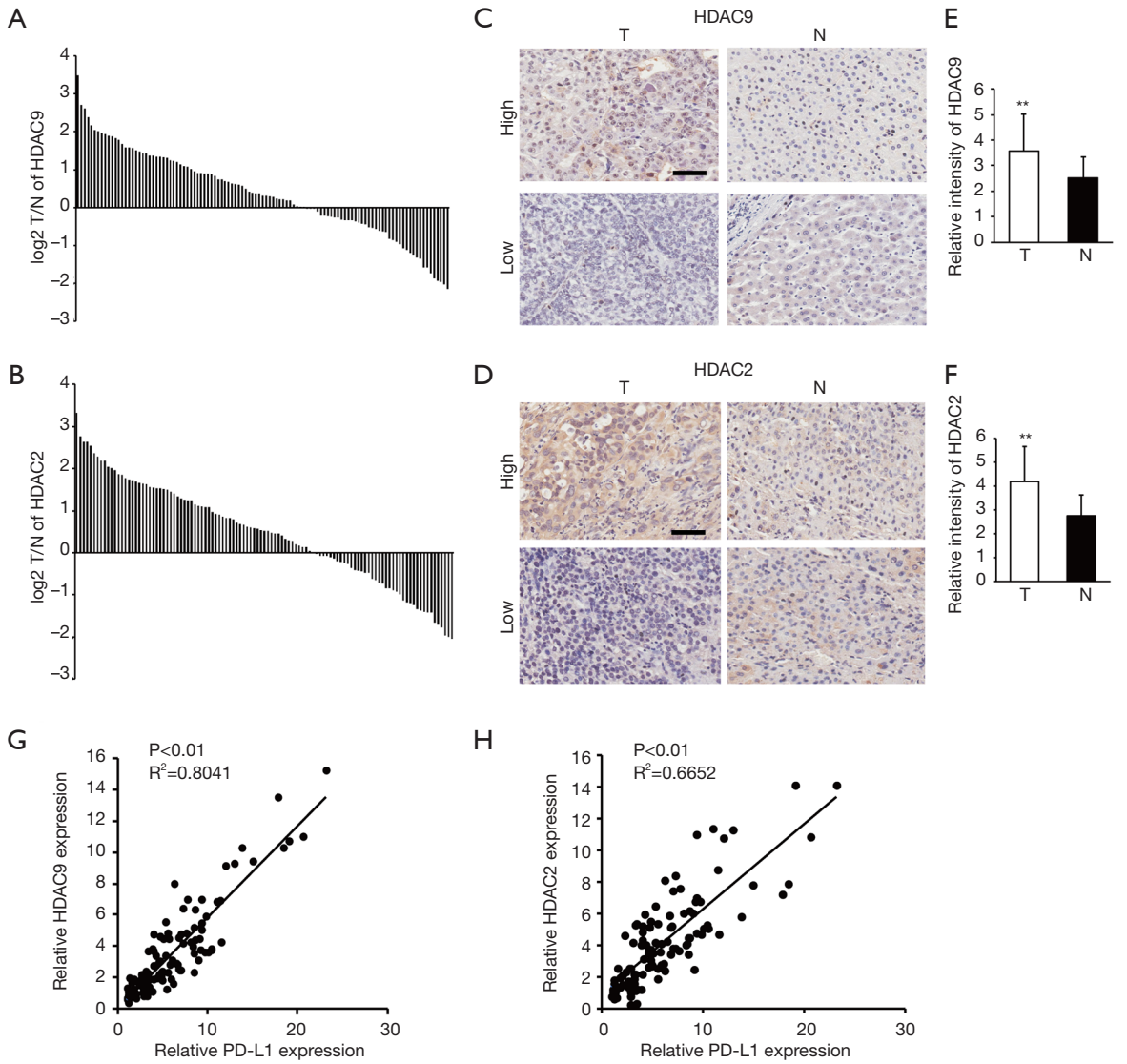

Figure 3 HDAC9 and HDAC2 are overexpressed in HCC. The expression of (A) HDAC9 and (B) HDAC2 in HCC TMAs were determined by IHC. The results of all 109 specimens were quantified with the Image-Pro Plus v6.2 software. Representative micrographs showing the different expression levels of (C) HDAC9 and (D) HDAC2 in HCC and normal peritumoral tissues (scale bar, 50 μm). The mean expression levels of (E) HDAC9 and (F) HDAC2 were compared between HCC (T) and peritumoral (N) tissues. Data are shown as the mean ± standard deviation (SD). **, P<0.01. Correlation analysis of PD-L1 with (G) HDAC9 and (H) HDAC2 using the corresponding IHC expression levels in HCC TMA. HDAC, histone deacetylase; HCC, hepatocellular carcinoma; TMA, tissue microarray; IHC, immunohistochemistry; PD-L1, programmed death-ligand 1.

samples (*Figure 2B*).

Protein expression levels of HDAC9 and HDAC2 are associated with PD-L1 in HCC TMA

To further verify the correlation between PD-1 and HDAC expression, the protein expression levels of HDAC9 and HDAC2 in HCC TMAs were determined by IHC staining. As illustrated in *Figure 3A*, the HDAC9 and HDAC2 protein levels were elevated in 67 (67/109; 61.5%) and 69 (69/109; 63.3%) HCC samples, respectively, compared with those in the matched peritumoral tissues (*Figure 3B*).

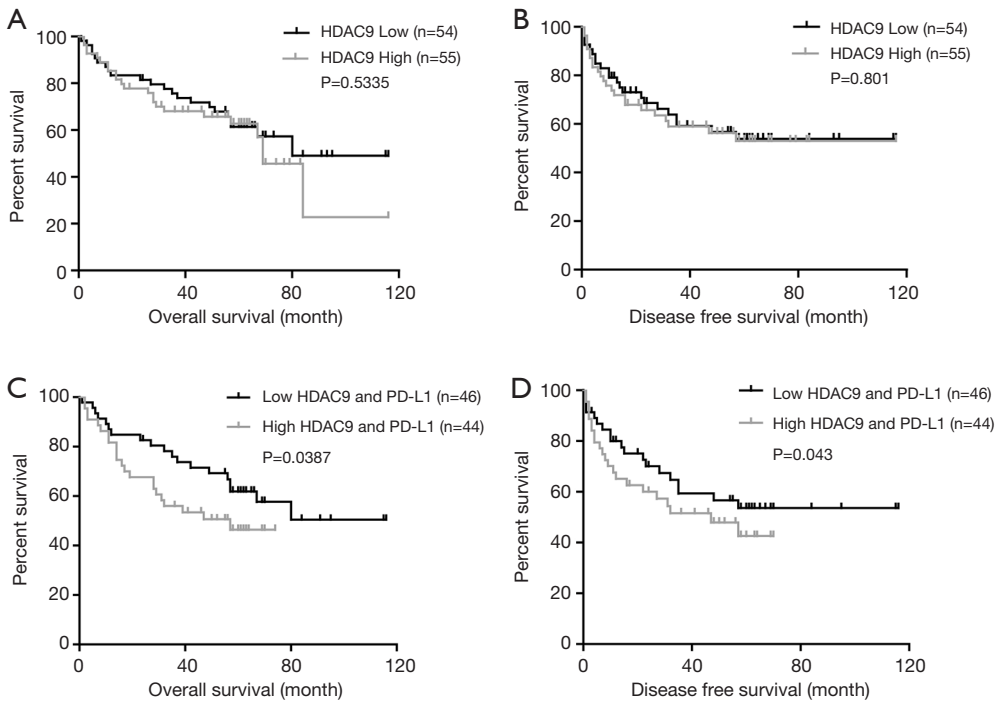

Figure 4 Combined increased HDAC9/PD-L1 expression in HCC can predict a poor prognosis. Kaplan-Meier survival analysis of (A) OS and (B) DFS of the high and low HDAC9 expression groups. Kaplan-Meier survival analysis of (C) OS and (D) DFS for the combined high and low HDAC9/PD-L1 expression groups. HDAC, histone deacetylase; PD-L1, programmed death-ligand 1; HCC, hepatocellular carcinoma; OS, overall survival; DFS, disease-free survival.

Representative IHC results in HCC tissues with high or low HDAC9 and HDAC2 protein levels are shown in *Figure 3C* and *D*. Furthermore, the average protein expression levels of HDAC9 and HDAC2 were dramatically higher in HCC tissues in comparison with normal peritumoral tissues (*Figure 3E*,*F*).

Subsequently, the association of HDAC9 and HDAC2 with PD-L1 protein expression was analyzed. The expression of both HDAC9 and HDAC2 was positively correlated with PD-L1 protein expression levels, while the association was stronger for HDAC9 than HDAC2. This finding was consistent with the data obtained on the mRNA level from HCC TCGA (*Figure 3G*,*H*).

Combined high expression of HDAC9 and PD-L1 in HCC tissues predicts poor prognosis

To determine the clinical significance of HDAC9 expression in human HCC, HCC patients were divided into high- (> median) and low-expression (< median) groups according to the median values for HDAC9 and PD-L1 expression. The survival rates were compared between the low (n=54) and high (n=55) HDAC9 expression groups. No significant differences were observed in OS and DFS between the two groups (*Figure 4A*,*B*). However, patients with high levels of both HDAC9 and PD-L1 (n=44) exhibited increased distant metastasis (P=0.036) and recurrence (P=0.006) in comparison with those with low levels of HDAC9 and PD-L1 (n=46; *Table 1*). Kaplan-Meier survival analyses showed that patients with high HDAC9 and PD-L1 expression had markedly reduced DFS and OS (*Figure 4C*,*D*). In addition, the clinical significance of HDAC2 expression in human HCC samples was also investigated; however, no differences were observed in then survival rate when the HDAC2 and PD-L1 expression levels were compared (Table S3; Figure S1). Moreover, univariate and multivariate analyses also suggested that the combined high HDAC9 and PD-L1 expression was an independent factor for worse OS (HR =2.164; 95% CI =1.1–4.257; P=0.025; *Table 2*). These results indicated that the combination of PD-L1 and HDAC9 expression could be a powerful predictor for poor prognosis in patients with HCC.

Table 1 Relationship between intratumoral HDAC9 + PD-L1 expression and clinicopathologic features

Variable	All cases	HDAC9 + PD-L1 density		P value
		Low (n=46)	High (n=44)	
Age (years)				0.506
<50	42	21	21	
>50	48	25	23	
Gender				0.618
Female	8	4	4	
Male	82	42	40	
HBsAg				0.459
Positive	73	38	35	
Negative	17	8	9	
AFP (ng/mL)				0.143
<20	24	15	9	
>20	66	31	35	
Cirrhosis				0.148
Yes	47	27	20	
No	43	19	24	
Tumor size (cm)				
<5	34	18	16	0.479
>5	56	28	28	
TNM stage				0.165
I–II	73	35	38	
III–IV	17	11	6	
Distant metastasis				*0.036*
Yes	11	2	9	
No	79	44	35	
Microvascular invasion				0.498
Yes	46	23	23	
No	44	23	21	
Involucrum				0.275
Complete	31	14	17	
Incomplete or absent	59	32	27	
Recurrence				*0.006*
Yes	38	12	26	
No	52	34	18	

Patients with HCC were divided into Low HDAC9 + PD-L1 expression group (whose final density was lower than the median) and High expression group (whose final density was higher than the median). The patient and disease profiles in each group were compared. Italic P values indicate statistical difference.

Table 2 Univariable and multivariable analysis of risk factors of OS after hepatectomy

Variable	Univariable analysis			Multivariable analysis		
	HR	95% CI	P	HR	95% CI	P
PDL1 expression	1.1	0.576–2.101	0.774	–	–	–
HDAC9 expression	1.389	0.724–2.664	0.322	–	–	–
HDAC9 + PDL1 expression	2.115	1.101–4.062	0.024	2.164	1.1–4.257	0.025
HDAC2 expression	1.011	0.53–1.927	0.973	–	–	–
HDAC2 + PDL1 expression	0.993	0.498–1.977	0.983	–	–	–
Age, year	0.814	0.427–1.552	0.532	–	–	–
Gender, male *vs*. female	2.112	0.508–8.785	0.304	–	–	–
Tumor size, cm	5.784	2.247–14.892	0	4.108	1.536–10.984	0.005
AFP, μg/L	1.569	0.689–3.573	0.284	–	–	–
Distant metastasis, yes *vs*. no	5.912	2.836–12.323	0	2.409	1.101–5.273	0.028
Microvascular invasion, yes *vs*. no	3.648	1.764–7.547	0	2.701	1.265–5.769	0.01
Involucrum, complete *vs*. incomplete	0.495	0.239–1.023	0.058	–	–	–
HBV infection, yes *vs*. no	0.621	0.3–1.283	0.198	–	–	–
Cirrhosis, yes *vs*. no	0.857	0.449–1.634	0.639	–	–	–
Differentiation, III/IV *vs*. I/II	0.427	0.151–1.205	0.108	–	–	–

HR, hazard ratio; CI, confidence interval; OS, overall survival.

HDAC9 overexpression promotes HCC cell growth in vivo

Whether HDAC9 had any direct effect on HCC cells? Next, lentiviral vectors encoding HDAC9 and GFP (control) were constructed and infected into MHCC-97L cells. The expression levels of HDAC9 in the infected cells were detected by western blot analysis (*Figure 5A*). Subsequently, the HDAC9-overexpressed and control cells were used to establish xenografts model on nude mice to assess tumor growth *in vivo*. As shown in *Figure 5B* and *C*, mice in the HDAC9-overexpressed group exhibited accelerated tumor growth and increased tumor volume compared with the control group. Furthermore, IHC staining revealed upregulated PD-L1 expression in the HDAC9-overexpressed tumors (*Figure 5D*). Taken together, the aforementioned findings suggested that HDAC9 overexpression in HCC cells could promote tumor growth and PD-L1 expression *in vivo*.

Discussion

The expression of PD-L1 varies among different cancer types. It has been demonstrated that PD-L1 is overexpressed in several types of tumors, including melanoma, ovarian cancer, breast cancer, lung cancer and hematological malignancies (30). Currently, the expression profile of PD-L1 in patients with HCC has been poorly investigated. Herein, a cohort of 109 HCC specimens were analyzed. The results showed that PD-L1 was overexpressed in the majority of HCC (76.1%) tumor tissues compared with matched normal peritumoral tissues. However, the survival analysis failed to reveal any significant prognostic association between PD-L1 expression with OS, which was consistent with a previous analysis by Xu *et al.* (31). These findings indicated that PD-L1 expression alone could not serve as a prognostic marker for HCC.

Emerging evidence has demonstrated that the expression levels of PD-L1 related to the response of PD-1/PD-L1 blockade immunotherapy in a variety types of cancer (32). The FDA has approved PD-L1 expression as a companion diagnostic indicator for anti-PD-1 therapy in NSCLC (33,34). By contrast, several studies revealed no association between PD-L1 expression and response to anti-PD-1 therapy (35,36). Therefore, PD-L1 is not considered as

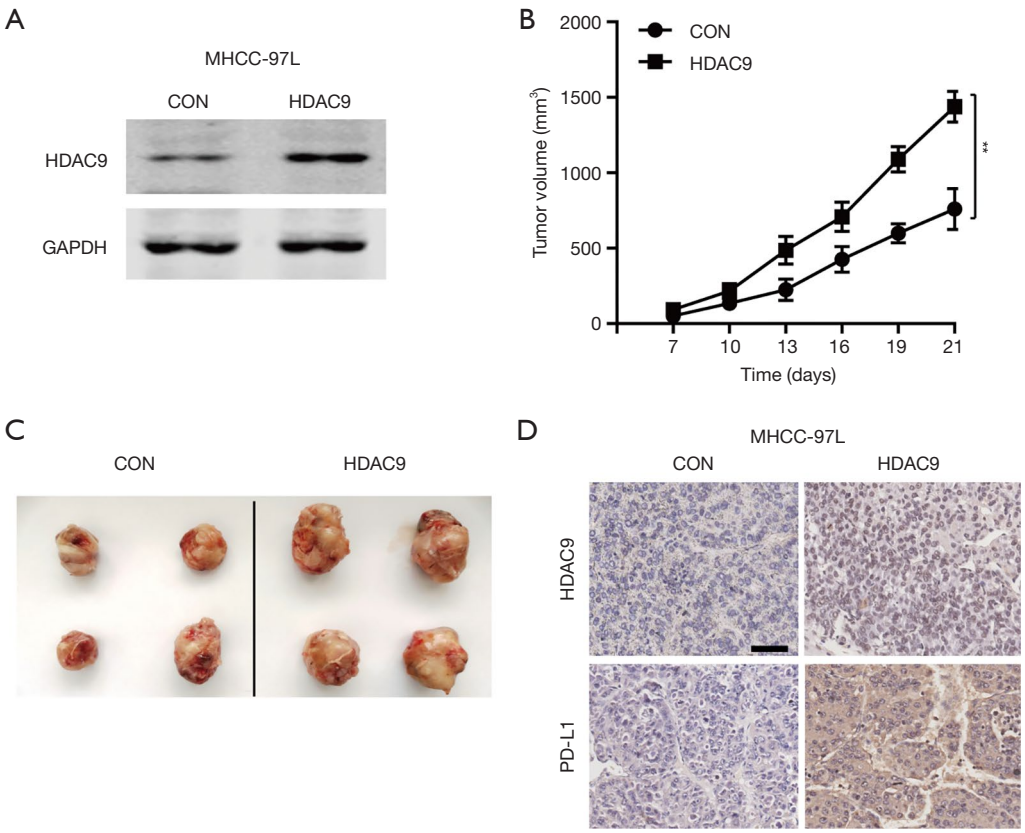

Figure 5 HDAC9 overexpression promotes HCC cell growth *in vivo*. (A) Western blot of HDAC9 in MHCC-97L cells transduced with a lentiviral vector encoding HDAC9 or the corresponding controls. (B) The MHCC-97L cell line overexpressing HDAC9 and control cells were inoculated subcutaneously into nude mice. The tumor volumes were measured, and the results are expressed as the mean ± standard deviation (SD). **, P<0.01. (C) Representative images of the xenograft tumors. (D) IHC staining of HDAC9 and PD-L1 in the tumor tissues from each group (scale bar, 50 μm). HDAC, histone deacetylase; HCC, hepatocellular carcinoma; PD-L1, programmed death-ligand 1; IHC, immunohistochemistry.

an appropriate predictive biomarker for selecting patients predisposed to respond to immunotherapy. Additional predictive biomarkers or combined indicators are urgently needed for selecting patients most likely to benefit from PD-1-based therapy.

Recently, several studies have shown that treatment with HDAC inhibitors induce the expression of checkpoint inhibitors in tumor cells. For example, Woods *et al.* (29) reported that HDAC inhibitors administration in melanoma-bearing mice lead to enhanced expression of PD-L1 and PD-L2mediated by increased histone acetylation. In the present study, RNA expression data from TCGA database revealed positive correlation between PD-L1 and HDAC expression. More specifically, the HDAC9 and HDAC2 expressions were most significantly correlated with PD-L1 expression. Additionally, the protein expression levels of HDAC9 and HDAC2 were notably upregulated in HCC samples, which was consistent with the PD-L1 mRNA expression profile. Correlation analysis revealed positive correlation between PD-L1 expression and both HDAC9 and HDAC2. This correlation was stronger with HDAC9 expression. Furthermore, survival analysis showed that the combined high PD-L1/HDAC9 levels, but not those of HDAC2, were significantly correlated with worse prognosis. These data strongly indicated that PD-L1/HDAC9 expression could serve as a novel prognostic marker for HCC. The current study suggested a potential role of HDAC9 in modulating PD-L1 expression. However, this mechanism needs to be further investigated.

To detect whether HDAC9 had any effect on PD-L1

expression in human HCC cells, we injected HDAC9-transfected MHCC-97L cells and control cells into immunodeficient nude mice. The results further confirmed that HDAC9 in human HCC cells promoted PD-L1 expression *in vivo*. As a crucial immune-checkpoint receptor, PD-1 could be expressed by several immune cells, for example $CD8^+$ T cells, NK cells and macrophages. By upregulating PD-L1 expression, HDAC9-transfected HCC cells possibly block antitumor immune induced by multiple immunocytes, and thus yield a bigger tumor size, even in T-cell-deficient nude mice. As a member of the class IIa histone deacetylases, HDAC9 catalyzes the deacetylation of histones and transcription factors to suppress or activate gene transcription. Many studies show that HDAC9 regulates the expression of cancer related genes by altering chromatin structures of promotor region or the transcriptional activities, such as STAT5, Myc and β-catenin, some of which control PD-L1 gene transcription. Therefore, HDAC9 may promote PD-L1 expression through activating its transcription factors, and more mechanistic experiments are needed to validate the hypothesis in future.

Despite PD-1/PD-L1 blockade is emerging as a promising approach in advanced cancer therapy, the response rate of patients to PD-1/PD-L1 targeted monotherapy remains still unsatisfactory in HCC. Therefore, the development of combination strategies to improve the effectiveness of HCC treatment is urgently needed. Previous findings have shown that HDAC inhibitors can enhance the response to anti-PD-1 therapy in mouse models. Herein, the results in human HCC specimens supported that the treatment with combined inhibition of HDAC9 and PD-1/PD-L1 could be considered as a potential approach to benefit patients with HCC.

Conclusions

The present study demonstrated that PD-L1 was upregulated and highly associated with HDAC9 and HDAC2 expression in HCC. Survival analysis showed that PD-L1/HDAC9 expression levels could be used as a powerful prognostic marker for patients with HCC. These results highlighted the strong association between HDACs and immunotherapy. However, further studies are necessary to investigate the potential clinical application of the combined therapy with specific HDAC9 inhibitors and PD-1 blockade in treating HCC.

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Footnote

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Data Sharing Statement: Available at http://dx.doi.org/10.21037/tcr-20-3415

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr-20-3415). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethic Committee of Eastern Hepatobiliary Surgery Hospital (No.: SHDC12017122) and informed consent was taken from all the patients. Experiments were performed under a project license (No.: 2018ZX10723204) granted by institutional ethics committee of Second Military Medical University (Shanghai, China), in compliance with the institutional guidelines for the care and use of animals.

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Supplementary

Table S1 Clinicopathologic features of 109 HCC patients

Patient number	Age (years)	Gender	Tumor size (cm)	AFP (ng/mL)	Distant metastasis	Vascular invasion	Involucrum	HBsAg	Cirrhosis	TNM stage	Recurrence
Case 1	45	Male	7	1000	No	Yes	Absent	Negative	Yes	II	No
Case 2	43	Male	3.5	26.1	No	Yes	Incomplete	Positive	Yes	II	No
Case 3	58	Male	4	3.8	No	Yes	Incomplete	Positive	Yes	IIIa	No
Case 4	65	Male	2.5	6.5	No	Yes	Incomplete	Positive	Yes	II	Yes
Case 5	56	Male	11	38	No	Yes	Absent	Positive	Yes	II	Yes
Case 6	43	Male	12	1000	Yes	Yes	Absent	Positive	No	II	Yes
Case 7	52	Male	4	5.4	No	Yes	Incomplete	Positive	Yes	II	No
Case 8	69	Male	14	1000	Yes	Yes	Complete	Positive	Yes	II	Yes
Case 9	55	Male	3	8	No	Yes	Absent	Positive	Yes	II	No
Case 10	62	Female	6	2.1	No	No	Incomplete	Negative	No	II	Yes
Case 11	61	Male	8	1000	Yes	Yes	Absent	Positive	Yes	II	Yes
Case 12	72	Male	3.5	29	No	Yes	Incomplete	Positive	Yes	II	Yes
Case 13	55	Male	2.5	1001	No	No	Complete	Positive	Yes	II	No
Case 14	48	Male	11	1001	Yes	Yes	Absent	Positive	Yes	II	Yes
Case 15	46	Male	6	115	No	No	Complete	Positive	No	IIIa	No
Case 16	49	Male	1.5	170	No	No	Complete	Positive	Yes	II	No
Case 17	56	Male	3	4	No	No	Absent	Negative	No	II	No
Case 18	41	Male	12	1000	No	Yes	Incomplete	Positive	No	II	No
Case 19	48	Male	4	77	No	Yes	Incomplete	Negative	No	II	Yes
Case 20	44	Male	12	273	Yes	Yes	Complete	Positive	Yes	II	Yes
Case 21	22	Male	3	842	No	Yes	Complete	Positive	No	II	No
Case 22	31	Male	15	209	No	Yes	Absent	Positive	Yes	II	Yes
Case 23	74	Male	12	5.2	No	No	Incomplete	Positive	No	II	No
Case 24	69	Female	6	24	No	Yes	Complete	Positive	Yes	II	Yes
Case 25	51	Male	7	86	No	Yes	Absent	Positive	No	IIIa	No
Case 26	41	Male	4.5	1001	No	No	Complete	Positive	Yes	I	No
Case 27	28	Male	16	1001	No	Yes	Absent	Positive	Yes	II	Yes
Case 28	53	Male	3.5	31	No	No	Incomplete	Positive	No	I	Yes
Case 29	45	Male	13	1000	No	Yes	Incomplete	Negative	No	II	No
Case 30	72	Male	5	14	No	Yes	Absent	Positive	Yes	II	Yes
Case 31	36	Male	11	81	No	No	Complete	Positive	Yes	IIIa	No
Case 32	50	Male	4.5	9.6	No	No	Incomplete	Positive	Yes	II	No
Case 33	78	Male	5.5	57	No	No	Incomplete	Negative	Yes	I	No
Case 34	45	Male	13	1000	No	Yes	Absent	Negative	No	II	Yes

Table S1 (*continued*)

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Table S1 (*continued*)

Patient number	Age (years)	Gender	Tumor size (cm)	AFP (ng/mL)	Distant metastasis	Vascular invasion	Involucrum	HBsAg	Cirrhosis	TNM stage	Recurrence
Case 35	62	Male	11	266	Yes	No	Absent	Negative	No	II	Yes
Case 36	60	Male	6.5	26	No	Yes	Incomplete	Positive	Yes	IIIa	Yes
Case 37	64	Male	3.5	4	No	Yes	Complete	Positive	No	II	Yes
Case 38	52	Male	1.5	123	No	No	Complete	Positive	Yes	I	No
Case 39	48	Female	1.5	1001	No	Yes	Absent	Positive	No	II	No
Case 40	50	Male	4	1000	No	No	Absent	Positive	No	II	Yes
Case 41	58	Male	5.5	19	No	No	Complete	Negative	Yes	I	Yes
Case 42	32	Male	5.5	54	No	No	Complete	Negative	Yes	IIIa	No
Case 43	37	Male	10	1001	No	Yes	Absent	Positive	No	II	No
Case 44	54	Female	1.8	12	No	No	Complete	Positive	Yes	I	No
Case 45	51	Male	6	1001	No	Yes	Absent	Positive	No	II	Yes
Case 46	34	Male	1.4	1086	No	No	Complete	Positive	Yes	I	No
Case 47	40	Male	9	112	No	Yes	Absent	Positive	No	II	Yes
Case 48	54	Male	10	569	No	Yes	Incomplete	Positive	No	II	Yes
Case 49	37	Male	20	1000	No	Yes	Absent	Negative	No	IIIa	No
Case 50	46	Male	5.5	1000	No	Yes	Complete	Negative	No	II	Yes
Case 51	34	Male	2.5	1000	No	No	Complete	Positive	Yes	II	No
Case 52	50	Male	6	56	No	Yes	Incomplete	Positive	No	II	Yes
Case 53	38	Male	13	1000	No	Yes	Absent	Positive	No	II	No
Case 54	58	Male	5	1001	Yes	Yes	Incomplete	Negative	No	II	Yes
Case 55	70	Male	4.5	191	No	No	Complete	Positive	No	II	No
Case 56	54	Male	4	1001	No	No	Incomplete	Negative	No	I	No
Case 57	66	Male	8	30	Yes	Yes	Absent	Positive	Yes	IIIa	Yes
Case 58	28	Male	6.5	732	No	Yes	Absent	Positive	No	IIIb	No
Case 59	51	Male	2.5	507	No	No	Complete	Positive	No	I	No
Case 60	31	Male	3	1000	No	No	Absent	Positive	No	I	No
Case 61	55	Male	1.5	226	No	Yes	Absent	Positive	Yes	I	Yes
Case 62	45	Male	2.8	36	No	No	Absent	Positive	Yes	IIIa	Yes
Case 63	56	Female	6	1000	No	Yes	Complete	Positive	No	II	No
Case 64	49	Female	2	208	No	No	Complete	Positive	No	II	No
Case 65	65	Male	12	267	No	No	Incomplete	Positive	Yes	IV	No
Case 66	76	Female	4	46	No	No	Complete	Positive	Yes	II	No
Case 67	47	Male	4	21	No	Yes	Complete	Positive	Yes	II	No
Case 68	40	Male	8	545	No	Yes	Absent	Negative	No	II	Yes

Table S1 (*continued*)

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Table S1 (*continued*)

Patient number	Age (years)	Gender	Tumor size (cm)	AFP (ng/mL)	Distant metastasis	Vascular invasion	Involucrum	HBsAg	Cirrhosis	TNM stage	Recurrence
Case 69	42	Male	3	8	No	No	Incomplete	Positive	Yes	I	No
Case 70	48	Male	9	347	No	Yes	Complete	Negative	No	II	No
Case 71	56	Female	2.5	9.2	No	No	Incomplete	Positive	Yes	I	No
Case 72	42	Male	2	19.8	No	No	Complete	Positive	Yes	I	Yes
Case 73	39	Male	5	12	No	Yes	Complete	Positive	Yes	II	Yes
Case 74	68	Male	5	1000	No	Yes	Absent	Positive	No	II	No
Case 75	45	Male	4.3	1001	No	Yes	Complete	Positive	Yes	II	No
Case 76	56	Male	1.2	1001	No	No	Absent	Negative	No	I	No
Case 77	57	Male	2.5	70	No	No	Complete	Negative	No	I	No
Case 78	39	Male	10	1001	Yes	Yes	Complete	Positive	No	III	Yes
Case 79	62	Male	5	11	No	No	Complete	Positive	No	I	Yes
Case 80	41	Male	14	16	Yes	No	Incomplete	Positive	No	I	Yes
Case 81	67	Male	9.5	12	No	No	Complete	Positive	No	IIIa	No
Case 82	40	Male	9	62	No	No	Complete	Positive	No	I	No
Case 83	50	Male	6	2.5	No	No	Complete	Positive	Yes	IIIa	No
Case 84	34	Male	1.6	1001	No	No	Complete	Positive	Yes	I	No
Case 85	66	Male	6	24	Yes	Yes	Complete	Positive	Yes	II	Yes
Case 86	47	Male	8	1001	No	Yes	Absent	Positive	Yes	II	Yes
Case 87	53	Male	2.8	18	No	No	Complete	Positive	No	I	No
Case 88	51	Male	8	12	No	No	Incomplete	Negative	No	I	No
Case 89	53	Male	2	1001	No	No	Complete	Negative	No	I	No
Case 90	54	Female	11	15	No	Yes	Incomplete	Negative	No	IIIa	No
Case 91	49	Male	5.5	179	No	No	Absent	Positive	No	IIIa	No
Case 92	40	Male	3	1001	No	No	Complete	Positive	Yes	I	No
Case 93	44	Male	11	1000	Yes	Yes	Absent	Positive	Yes	II	Yes
Case 94	64	Male	7	40	No	No	Incomplete	Positive	Yes	IIIa	No
Case 95	42	Male	7	17	No	No	Absent	Negative	Yes	I	Yes
Case 96	43	Male	10	63159	No	No	Absent	Positive	No	I	No
Case 97	63	Female	5	247	No	No	Absent	Positive	No	I	No
Case 98	57	Male	7.5	7.1	No	No	Absent	Positive	No	I	No
Case 99	61	Male	10	21	No	No	Absent	Positive	Yes	I	No
Case 100	71	Male	4.5	1001	No	No	Complete	Positive	No	I	No
Case 101	53	Male	9	10	No	No	Complete	Positive	Yes	IIIa	Yes
Case 102	48	Male	6.5	574	No	No	Complete	Positive	Yes	I	No

Table S1 (*continued*)

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Table S1 (*continued*)

Patient number	Age (years)	Gender	Tumor size (cm)	AFP (ng/mL)	Distant metastasis	Vascular invasion	Involucrum	HBsAg	Cirrhosis	TNM stage	Recurrence
Case 103	50	Male	6.5	1001	No	No	Absent	Positive	Yes	IIIa	Yes
Case 104	62	Female	7	1001	No	No	Absent	Positive	Yes	I	No
Case 105	74	Male	7	1001	No	No	Absent	Positive	Yes	IIIc	Yes
Case 106	56	Male	22	3	No	Yes	Absent	Positive	No	II	Yes
Case 107	47	Male	2.7	94	No	No	Complete	Positive	No	IIIa	No
Case 108	57	Male	9	1000	No	No	Absent	Positive	Yes	IIIa	No
Case 109	57	Male	2	50	No	Yes	Complete	Positive	No	IIIa	Yes

HCC, hepatocellular carcinoma.

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Table S2 Relationship between intratumoral PD-L1 expression and clinicopathologic features

Variable	All cases	PD-L1 density		P value
		Low (n=54)	High (n=55)	
Age (years)				0.537
≤50	53	26	27	
>50	56	28	28	
Gender				0.487
Female	11	6	5	
Male	98	48	50	
HBsAg				0.223
Positive	87	41	46	
Negative	22	13	9	
AFP (ng⁄mL)				0.173
<20	27	16	11	
≥20	82	38	44	
Cirrhosis				0.538
Yes	55	27	28	
No	54	27	27	
Tumor size (cm)				0.531
<5	43	21	22	
≥5	66	33	33	
TNM stage				0.48
I–II	86	42	44	
III–IV	23	12	11	
Distant metastasis				0.189
Yes	12	4	8	
No	97	50	47	
Vascular invasion				0.54
Yes	52	26	26	
No	57	28	29	
Involucrum				0.24
Complete	43	19	24	
Incomplete or absent	66	35	31	
Recurrence				0.04
Yes	45	20	25	
No	64	34	30	

Patients with HCC were divided into PD-L1 Low expression group (whose final density was lower than the median) and High expression group (whose final density was higher than the median). The patient and disease profiles in each group were compared.

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Table S3 Relationship between intratumoral HDAC2 + PD-L1 expression and clinicopathologic features

Variable	All cases	HDAC2 + PD-L1 density		P value
		Low (n=35)	High (n=35)	
Age (years)				0.405
≤50	32	17	15	
>50	38	18	20	
Gender				0.5
Female	5	3	2	
Male	65	32	33	
HBsAg				0.383
Positive	56	29	27	
Negative	14	6	8	
AFP (ng⁄mL)				0.5
<20	19	10	9	
≥20	51	25	26	
Cirrhosis				0.236
Yes	38	21	17	
No	32	14	18	
Tumor size (cm)				0.229
<5	26	11	15	
≥5	44	24	20	
TNM Stage				0.281
I–II	55	26	29	
III–IV	15	9	6	
Distant metastasis				0.367
Yes	10	4	6	
No	60	31	29	
Microvascular invasion				0.316
Yes	37	17	20	
No	33	18	15	
Involucrum				0.309
Complete	25	11	14	
Incomplete or absent	45	24	21	
Recurrence				0.594
Yes	34	17	17	
No	36	18	18	

Patients with HCC were divided into Low HDAC2 + PD-L1 expression group and High expression group. The patient and disease profiles in each group were compared. HCC, hepatocellular carcinoma.

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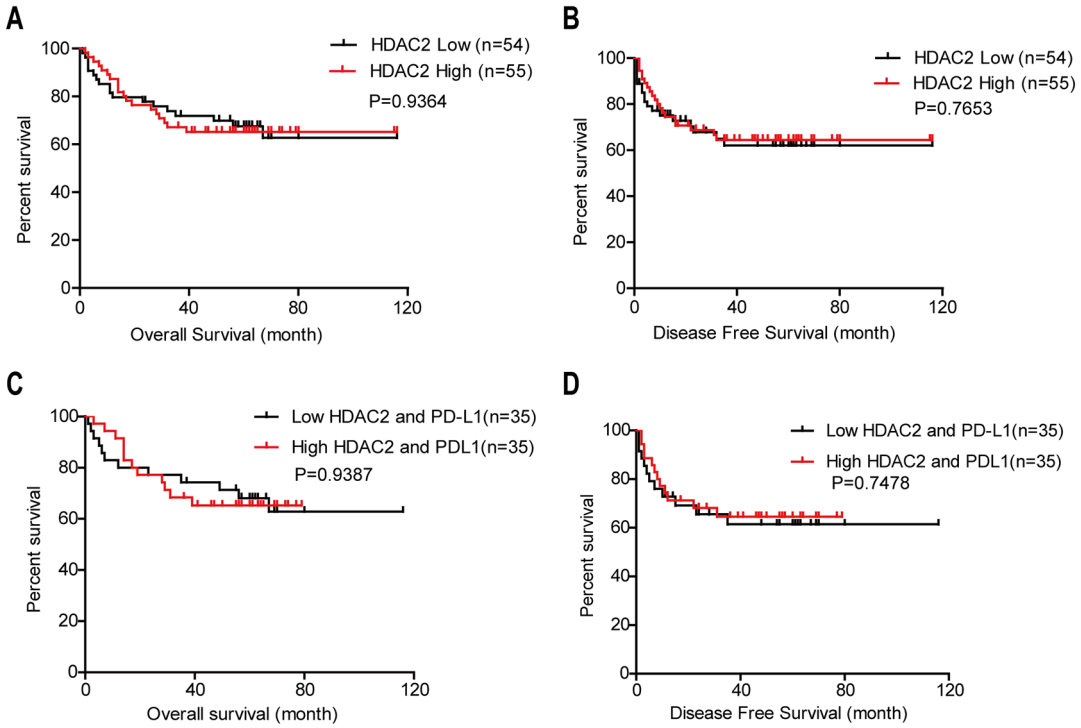

Figure S1 Overall survival (A) and disease-free survival (B) for the high and low HDAC2 expression groups were analyzed by Kaplan-Meier survival analysis. Overall survival (C) and disease-free survival (D) for the combined high and low HDAC2 and PD-L1 expression groups were analyzed by Kaplan-Meier survival analysis.

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