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ORIGINAL RESEARCH

Distinct Roles of HHLA2 and PD-L1 in the Immune Cell and Prognosis of Hepatocellular Carcinoma

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Background: HHLA2, a member of the B7 family, is extensively expressed in various cancers and plays a pivotal role in modulating the immune microenvironment. However, its prognostic significance in hepatocellular carcinoma (HCC) remains poorly understood. This study aims to elucidate the expression patterns of HHLA2 and PD-L1 in HCC, their associations with tumor-infiltrating lymphocytes (TILs), and their impact on clinical outcomes.

Methods: Immunohistochemistry (IHC) was employed to evaluate HHLA2 and PD-L1 expression in 547 HCC tissue samples. PD-L1 positivity was defined as ≥1% membranous or cytoplasmic staining. Hematoxylin and eosin (H&E) staining was utilized to quantify TILs (percentage/area), while IHC was used to measure the densities of CD3+, CD4+, and CD8+ TILs (cells/mm²).

Results: HHLA2 and PD-L1 exhibited similar positivity rates. HHLA2 positivity was associated with older age, lower alpha-fetoprotein (AFP) levels, well-differentiated tumors, and improved overall survival (OS). HHLA2 expression was inversely correlated with stromal TIL density. In contrast, tumor cell (TC)-PD-L1 and inflammatory cell (IC)-PD-L1 positivity were positively correlated with higher stromal TIL density and increased levels of CD3+, CD4+, and CD8+ TILs. Patients with HHLA2(+)/PD-L1(-) status demonstrated the longest OS. A novel classification system based on HHLA2/PD-L1 expression identified distinct immune profiles and prognostic subgroups.

Conclusion: HHLA2 significantly influences the immune microenvironment of HCC and serves as an independent prognostic marker. The combined assessment of HHLA2 and PD-L1 expression facilitates risk stratification, providing a framework to optimize immunotherapy strategies. These findings contribute to the advancement of precision medicine in the management of HCC.

Keywords: HHLA2, PD-L1, TILs, prognosis, HCC

Introduction

Hepatocellular carcinoma (HCC) accounts for 90% of all primary liver tumors and is considered to be the second most frequent cancer in the world.[1] HCC is the second most common cause of cancer-related death and the fourth most prevalent malignancy in China.[2] Patients with early-stage HCC can be cured using current therapeutic options, such as liver transplantation or surgical resections.[3,4] However, approximately 80% of patients were diagnosed with HCC at an advanced stage, which is mostly attributable to the absence of symptoms and efficient HCC screening methods.[5] Due to limited treatment options, systemic drug doses are often used, particularly when surgical sectioning is not possible. Therefore, there is an urgent need for efficient therapeutic approaches that can dramatically increase the survival of HCC.

Immunotherapy augments anti-tumor immune responses through the modulation of immune tolerance mechanisms and the optimization of the tumor microenvironment. Programmed cell death protein 1 (PD-1) and its ligand PD-L1, which are expressed on tumor cells and tumor-infiltrating lymphocytes (TILs), facilitate immune evasion by inhibiting

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T-cell-mediated cytotoxicity.[6] Immune checkpoint inhibitors (ICIs) targeting the PD-1/PD-L1 axis have demonstrated clinical efficacy in the treatment of multiple metastatic malignancies;[7] however, PD-L1 expression alone has been shown to be an inadequate predictive biomarker for therapeutic response.[8] The liver's inherently immunosuppressive microenvironment fosters the proliferation of malignant hepatocytes and compromises immune surveillance, rendering hepatic tumors particularly amenable to immunotherapeutic interventions.[9] Recent studies have highlighted the potent anti-tumor activity of PD-1/PD-L1-targeting agents in hepatocellular carcinoma (HCC).[10,11] Despite these advances, the response rates to PD-1/PD-L1 blockade in HCC remain suboptimal, with reported rates ranging from 17% to 22%.[11–13] To expand immunotherapeutic options for HCC patients who are ineligible for anti-PD-1/PD-L1 therapy, further investigation into the expression and functional roles of alternative immune checkpoints is warranted.

Human Endogenous Retrovirus-H Long Repeat-Associating 2 (HHLA2), a recently identified immune checkpoint protein belonging to the B7 family,[14,15] has been demonstrated to inhibit the proliferation and cytokine production of CD4+ and CD8+ T lymphocytes upon T-cell receptor (TCR) stimulation.[15,16] While numerous studies have established HHLA2's role in modulating T-lymphocyte function, its precise functional mechanisms remain controversial. HHLA2 is frequently overexpressed in multiple malignancies, including renal, intrahepatic biliary tract, breast, lung, thyroid, and melanoma.[17–19] Emerging evidence suggests that HHLA2 exhibits dualistic, context-dependent roles in tumor biology. For instance, Xu et al reported that HHLA2 inhibits proliferation in epithelial ovarian cancer and is associated with improved survival.[20] Similarly, elevated HHLA2 expression in pancreatic and ampullary tumors correlates with favorable postoperative prognosis,[21] consistent with findings in kidney renal clear cell carcinoma (KIRC), where HHLA2 overexpression confers clinical benefits.[22] However, the functional implications of HHLA2 in hepatocellular carcinoma (HCC) appear more complex. While Luo et al observed that HHLA2 overexpression in HCC is associated with poor prognosis,[23,24] Liao et al reported that HHLA2 expression correlates with increased CD8+ T-cell infiltration and improved outcomes.[25] Notably, these HCC studies were limited by small sample sizes and lacked comprehensive analysis of HHLA2 expression patterns, their relationship with PD-L1 expression, and their impact on the tumor immune microenvironment.

In this study, we investigated the correlation between HHLA2 and PD-L1 expression in 547 hepatocellular carcinoma (HCC) cases using immunohistochemistry (IHC). Furthermore, we conducted a comprehensive comparative analysis of HHLA2 and PD-L1 expression patterns, their clinical significance, and their associations with tumor-infiltrating CD3+, CD4+, and CD8+ immune cells in the HCC cohort following curative resection. Additionally, we assessed the prognostic implications of HHLA2 and PD-L1 expression in patients. This research establishes a foundational framework for identifying novel immunotherapeutic targets in HCC by complementing PD-L1 expression analysis and offers potential predictive insights into patient outcomes.

Materials and Methods

Patients and Samples

This study was approved by the Institute Research Medical Ethics Committee of Sun Yat-sen University Cancer Center. A total number of 547 paraffin-embedded primary HCC samples and corresponding non-tumor tissues were obtained from Sun Yat-sen University Cancer Center (SYSUCC). HCC patients who underwent hepatectomy from January 2000 to December 2010 were included. None of these patients had received radiotherapy or chemotherapy prior to surgery. All pathological specimens were collected along with complete clinical and pathological data. Archived paraffin-embedded specimens were reembedded into new paraffin blocks for tissue microarray (TMA). All samples were deidentified, and all patients signed informed consents. The study methodologies conformed to the standards set by the Declaration of Helsinki.

Patients Follow Up

Follow-up of patients' survival data was obtained by means of retrieving medical records, email, and direct communication by phone. All patients were followed up until death or January 2020. The endpoint of this study was overall survival (OS) and disease-free survival (DFS). The follow-up period was defined as the time interval from the date of

surgery to the last follow-up. The cancer-specific overall survival (OS) was defined as the time between the surgery and cancer-related death or the censored at the date of the last follow-up, and the disease-free survival (DFS) was defined as the time between the surgery and recurrence/metastasis or the censored at the date of the last follow-up. The median follow-up time was 21.8 months, and the sample included 483 (88.3%) males and 64 (11.7%) females. The mean age was 49 years, ranging from 13 to 77 years.

Tumor Infiltrating Lymphocytes (TILs) were evaluated on the hematoxylin and eosin (H&E) sections of the tumor following the guidelines of the international TILs working group.[26] The TILs were evaluated within the invasive border and a percentage, as well as a quantification of TILs in square millimeter, was given. An average percentage and quantification of TILs were documented for each case. The percentage (≥5%) of the Tils counts is used as a cutoff value.

Hematoxylin and Eosin (HE) and Immunohistochemistry (IHC) Staining

HCC tissues and adjacent non-tumorous hepatic tissue samples were collected and constructed for TMA. Primary antibodies (anti-HHLA2: Sigma-Aldrich, HPA 055478; anti-PD-L1: Roche, SP263; anti-CD3: ZSGB-Bio, ZM-0417; anti-CD4: ZSGB-Bio, ZA-0519; anti-CD8: ZSGB-Bio, ZM-0508) were incubated at 4°C, washed three times with phosphate-buffered saline, incubated with biotinylated goat anti-mouse antibodies, and then stained with DAKO liquid 3,3′-diaminobenzidine tetrahydrochloride (DAB) and finally with Mayer's hematoxylin. TMA slides stained with HHLA2 and PD-L1 were observed under a microscope, and the protein expression levels of HHLA2 and PD-L1 were assessed by two independent pathologists (Chunhua Wang and Lili Liu). The HHLA2 positively stained samples were scored as follows: "0" (less than 5% positively stained cells), "1" (6–24% of positively stained cells), "2" (25–49% of positively stained cells), "3" (50–74% of positively stained cells) and "4" (75–100% of positively stained cells). Intensity was scored according to the standard: 0, negative staining; 1, weak staining; 2, moderate staining; and 3, strong staining. The final score was served by multiplying the percentage score by the staining intensity score. The median IHC score of HHLA2 was chosen as the cutoff value for defining high and low expression.

For tumor PD-L1 expression (TC), the percentages of cells demonstrating membranous staining for PD-L1 among total tumor cells were quantified, consistent with our previous studies.[3,27] For inflammatory cell PD-L1 expression (IC), any expression (≥1%) of PD-L1 on tumor infiltrating and stromal immune cells was considered present. The tumor and inflammatory cell PD-L1 positivity threshold was defined as at least 1% displaying membranous PD-L1 staining of any intensity. The density of inflammatory cells was manually counted in five separate fields under × 200 high-power magnification. The inflammatory cell positivity threshold was defined as at least 1/mm^2. Quantification was conducted independently by two experienced pathologists who were blinded to the clinical data of patients, any discrepancies in scoring were adjudicated.

Quantification of CD3+, CD4+ and CD8+ positive TILs were performed with digital imaging analysis (Halo imaging analysis software; Indica Labs, Corrales, NM). The software counted the number of positive immune cells in the tumor areas of the whole slides, while the two pathologists counted the positive immune cells through the digital scan of the slides separately. Consensus was reached between the two authors if there was a discrepancy among the collected data. The median of the CD3+, CD4+ and CD8+ counts was used as a cutoff value.

Statistical Analysis

SPSS 19.0 was used to perform statistical analyses (SPSS, Chicago, IL, USA). Student's *t*-test was used to assess the significance of differences in HHLA2 and PD-L1 expression. The Chi-square (χ^2) test was used to analyze the correlation between HHLA2 expression and clinicopathological parameters in HCC patients. Pearson's χ^2 test was used to analyze the correlation between the expression of HHLA2 and PD-L1. OS and DFS were analyzed by Kaplan–Meier analysis and compared by Log rank test. Univariate and multivariate Cox regression analyses were used to analyze prognostic correlations. $P < 0.05$ was considered statistically significant.

Results

Baseline Characteristics of HCC Patients

A total of 547 surgically resected FFPE HCC samples who underwent primary tumor resection were analyzed in this study. The clinicopathological characteristics were listed in Table 1. In 547 HCC cases, the median OS time was 21.8 months (range 1.0–146.6 months), and the median DFS time was 12.6 months (range 0.4–125.3 months).

PD-L1 and HHLA2 Expression Pattern in HCC

Typical microphotographs of PD-L1 expression were listed in Figure 1A. The positive rate of PD-L1 on tumor cells (TC) was 39.7% (217/547) (Figure 1B Left). And 53.4% (292/547) HCC patients were PD-L1 positive on inflammatory cells (IC) (Figure 1B Right). As detailed in Figure 1C, negative to strong expression of HHLA2 was observed in HCC TMAs. The HHLA2 stained area was mostly in cytoplasm, on the membrane of tumor cells. The median IHC score of the HHLA2 expression in HCC tumor tissue was 4, and patients with IHC score ≥4 was considered to be high HHLA2 expression and their counterparts with IHC score <4 were defined as low HHLA2 expression. In 547 HCC cases, 31.8% (174/547) and 68.2% (373/547) of the patients were classified as high HHLA2 expression and low HHLA2 expression, respectively (Figure 1D). The expression between PD-L1 TC and HHLA2 was not identified significant correlation (P = 0.711, Table 1). In 12.9% (71/547) cases, both immune checkpoints were detected. In PD-L1 TC or IC-negative HCC, 31.2% (103/330) and 32.5% (65/255) patients were observed to express elevated HHLA2, respectively (Figure 1E).

Relationship of HHLA2 and PD-L1 Expression and Clinicopathological Features

As illustrated in Table 1, high HHLA2 expression was more likely to associate with old age ($P = 0.017$), low serum AFP level ($P = 0.002$) and well tumor differentiation ($P = 0.035$). Clinicopathological features of PD-L1 TC-positive and IC-positive patients were shown in Table S1. PD-L1 positive on TC or IC was associated with cirrhosis ($P = 0.027$ and $P < 0.001$, respectively). Moreover, PD-L1 positive on TC was associated with old age ($P = 0.005$), and PD-L1 positive on IC was associated with poor tumor differentiation ($P = 0.023$).

Prognostic Significances of HHLA2 and PD-L1

The results of univariate and multivariate analyses for OS were presented in Table 2. In univariate analysis for OS, tumor multiplicity ($P < 0.001$), tumor size ($P < 0.001$), TNM stage ($P < 0.001$), poor tumor differentiation ($P = 0.019$), vascular invasion ($P < 0.001$), microvascular invasion (MVI) ($P < 0.001$), incomplete tumor capsule ($P < 0.001$), LN metastasis ($P < 0.001$), stromal TILs ($P = 0.005$), low HHLA2 expression ($P = 0.001$; Figure 2A), positive PD-L1 on TC ($P = 0.046$; Figure 2B) and positive PD-L1 on IC ($P = 0.030$; Figure 2C) were found to have a significant correlation with unfavorable OS. In multivariate analysis, tumor multiplicity ($P = 0.028$, hazard ratio [HR] = 1.279, 95% CI 1.027–1.593), tumor size ($P < 0.001$, HR = 1.634, 95% CI 1.275–2.094), MVI ($P < 0.001$, HR = 2.286 95% CI 1.488–3.511), stromal TILs ($P = 0.001$, HR = 1.418, 95% CI 1.115–1.742) and HHLA2 expression ($P = 0.046$, HR = 0.814, 95% CI 0.664–0.990) continued to be prognostic indicators for OS. In terms of DFS, only MVI ($P = 0.037$) was found to be prognostic indicators in univariate analysis, and high HHLA2 expression ($P = 0.238$) failed to stratify DFS (Table S2). To sum up, high HHLA2 expression was identified as an independent prognostic factor for OS, but not for DFS. PD-L1 could well stratified OS ($P = 0.046$ and $P = 0.030$ for TC and IC expression, respectively; Figure 2B and C; Table 2), but not for DFS ($P = 0.859$ and $P = 0.450$ for TC and IC expression, respectively; Additional file 2: Table S2).

Co-Expression of HHLA2 and PD-L1 in HCC and Prognostic Significance

Considering that both HHLA2 and PD-L1 were belonged to the B7 family and had an inhibitory function on CD4+ or CD8+ T cells, we attempted to explore the prognostic impact of HHLA2/PD-L1 (TC or IC) co-expression in HCC. In view of the high expression of HHLA2 or low expression of PD-L1 (TC or IC) predict a good prognosis, patients were divided into three groups: group I, HHLA2 (+)/ PD-L1 TC (-); group II, both positive HHLA2 (+)/PD-L1 TC (+) or both negative HHLA2 (-)/PD-L1 TC (-); and group III, HHLA2 (-)/PD-L1 TC (+). Kaplan–Meier analysis demonstrated that only OS, but not DFS in group I was significantly increased compared with those in group II and group III (Figure 3A).

Table 1 Correlation Between HHLA2 Expression and Baseline Clinicopathological Features in HCC

Characteristic	All Patients (n = 547)				
	Patients		HHLA2 Expression		
	No.	%	Low	High	*P*-value
All patients	547	100	373	174	
Gender					0.887
Male	483	88.3	330	153	
Female	64	11.7	43	21	
Age (years)					**0.017**
<49	268	49.0	196	72	
≥49	279	51.0	177	102	
Tumor multiplicity					0.555
Single	374	68.4	258	116	
Multiple	173	31.6	115	58	
Tumor size (cm)					0.090
<5	135	24.7	84	51	
≥5	412	75.3	289	123	
HBV					0.328
Negative	93	17.0	59	34	
Positive	454	83.0	314	140	
AFP (ng/mL)					**0.002**
<20	124	22.7	70	54	
≥20	423	77.3	303	120	
TNM[a] stage					0.459
I–II	314	57.4	210	104	
III–IV	233	42.6	163	70	
Differentiation					**0.035**
Well-Moderate	48	8.8	26	22	
Poor	499	91.2	347	152	
Cirrhosis					0.221
No	92	16.8	68	24	
Yes	455	83.2	305	150	
Vascular invasion					0.114
No	454	83.0	303	151	
Yes	93	17.0	70	23	
MVI					0.168
No	505	92.3	340	165	
Yes	42	7.7	33	9	
Tumor capsule					0.308
Complete	236	43.1	155	81	
Incomplete	311	56.9	218	93	
LNM					0.999
No	521	95.2	355	166	
Yes	26	4.8	18	8	
PD-L1 (TC)					
<1%	330	60.3	225	105	0.711
≥1%	217	39.7	146	71	

(*Continued*)

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Table 1 (Continued).

Characteristic	All Patients (n = 547)				
	Patients		HHLA2 Expression		
	No.	%	Low	High	*P*-value
Tils					
Low	379	69.3	244	135	**0.004**
High	168	30.7	129	39	
Cytological type					0.799
Hepatic	477	87.2	326	151	
Clear cell	36	6.6	26	10	
Others	34	6.2	21	13	

Notes: [a]TNM stage was classified according to the AJCC 7th TNM staging system. $P < 0.05$ (bold values) was considered statistically significant.
Abbreviations: TNM, tumor node metastasis stage; MVI, microvascular invasion; LNM, lymph node metastasis.

Importantly, multivariate analysis revealed that HHLA2 (+)/PD-L1 TC (-) still had a significant impact on OS ($P = 0.007$, HR = 1.211, 95% CI 1.053–1.392; Table S3). Similarly, combined HHLA2 with PD-L1 on IC, patients were also divided into three groups: group I, HHLA2 (+)/PD-L1 IC (-); group II, both positive HHLA2 (+)/PD-L1 IC (+) or both negative HHLA2 (-)/PD-L1 IC (-); and group III, HHLA2 (-)/PD-L1 IC (+). Kaplan–Meier analysis also demonstrated that only OS, but not DFS in group I was significantly increased compared with those in group II and group III (Figure 3B). Furthermore, multivariate analysis revealed that HHLA2 (+)/PD-L1 IC (-) also had a significant impact on OS ($P = 0.008$, HR = 1.203, 95% CI 1.050–1.379; Table S4).

Tumor Infiltrating T Cells and Their Relationship with HHLA2 and PD-L1 Expression

Microphotographs of CD3+, CD4+ and CD8+ TILs, which represent overall T cells, T helper cells and cytotoxic T cells (CTLs), respectively, were presented in Figure 4A. As detailed in Table S5, high expression of HHLA2 was associated with low density of stromal TILs ($P = 0.004$), and with low density of CD4+ TILs counts ($P = 0.020$; Figure 4B). Moreover, no significant correlation was observed between the counts of CD3+ or CD8+ TILs and HHLA2 expression. Furthermore, PD-L1 expression on TC was correlated with high density of stromal TILs ($P = 0.037$; Table S6), CD3+ and CD8+ TILs counts ($P < 0.001$; Figure 4C; Table S6). Similarly, PD-L1 expression on IC was also correlated with high density of stromal TILs, CD3+, CD4+ and CD8+ TILs counts (Figure 4D; Table S7). The prognostic significances of the variables concerning on the intratumoral infiltrations of different T cell subsets for HCC were evaluated via univariate analysis, low stromal TILs, high CD4+ or CD8+ TILs counts were found to be significantly associated with OS, but not with DFS (Table S8).

Discussion

Our investigation revealed that the expression levels of HHLA2 and PD-L1 in HCC exhibited comparable rates yet demonstrated no significant correlation between these two immune checkpoint molecules. Notably, both HHLA2 and PD-L1 expression on tumor cells (TC) or immune cells (IC) emerged as independent prognostic factors for overall survival (OS). Moreover, the co-expression of HHLA2 and PD-L1 on TC or IC served as an independent predictor of OS. Importantly, the expression patterns of these molecules were associated with distinct immune cell infiltration profiles: PD-L1-positive tumors displayed elevated densities of stromal tumor-infiltrating lymphocytes (TILs), CD3+ TILs, and CD8+ TILs, while HHLA2 overexpression correlated with reduced densities of stromal TILs.

Members of the B7 family frequently have multiple roles depending on the immunological environment, as well as their interaction with different receptors or receptor engagement or blockade.[28] Xiao et al showed that as a member of B7 family, HHLA2 had either co-inhibitory or co-stimulatory properties, depending on the malignancy type.[29] Several recent

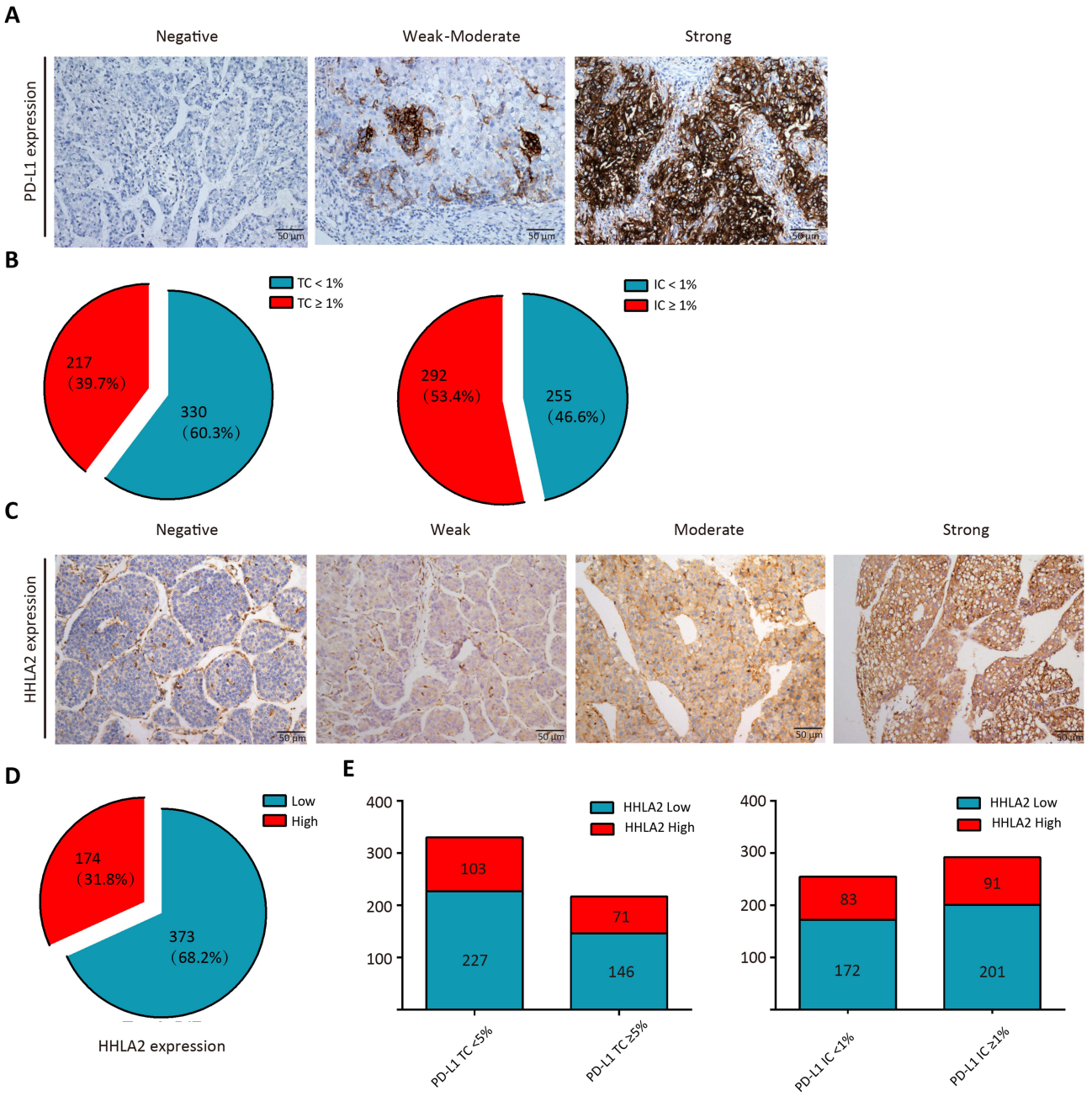

Figure 1 PD-L1 and HHLA2 expression in HCC tissue samples. Representative micrographs of PD-L1 expression within tumor (scale bar, 50 μm) (**A**). The positive rate of PD-L1 on TC and IC were 39.7% and 53.4%, respectively (**B**). Representative micrographs of HHLA2 expression within tumor (scale bar, 50 μm) (**C**). HHLA2 was elevated in 31.8% HCC cases (**D**). No significant correlation was found between HHLA2 and PD-L1 expression (**E**).

studies found that HHLA2 was a significant prognostic factor in a subset of tumors, but there was significant heterogeneity in prognostic value across tumor types, with some being protective and others being unfavorable or lacking significant prognostic factors. For example, Luo et al showed that HHLA2 was upregulated in 55 HCC tissues, and may play a major role in the development and progression of HCC, supporting a tumor progression role in HCC, meanwhile, the expression of HHLA2 was related to poor prognosis of HCC patients (202 cases) in her study.[24] Meanwhile, some studies also suggest that HHLA2 promotes the malignant progression of HCC in vitro.[30,31] Similarly, based on The Cancer Genome Atlas (TCGA) database, it is indicated that high expression of HHLA2 suggests a poor prognosis for HCC patients (Supplementary Figure 1A). However, according to our data, elevated expression of HHLA2 was frequently associated with good prognosis and good clinicopathological features, such as with low AFP level, and

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Table 2 Univariate and Multivariate Analysis of Prognostic for Overall Survival

Variables	Overall Survival		
	Univariate *P* value	**Multivariate *P* value**	**Multivariate HR (95% CI)**
Overall survival			
Gender (male vs female)	0.360	NA	NA
Age (≥49 vs <49 years)	0.415	NA	NA
Tumor multiplicity (multiple vs Single)	**0.000**	**0.028**	1.279 (1.027–1.593)
Tumor size (cm) (≥5 vs <5)	**0.000**	0.000	1.634 (1.275–2.094)
HBV (positive vs negative)	0.312	NA	NA
AFP (ng/mL) (≥20 vs <20)	0.081	NA	NA
TNM stage (III–IV vs I–II)	**0.000**	0.096	1.223 (0.965–1.550)
Differentiation (moderate-poor vs well)	**0.019**	0.387	1.115 (0.837–1.581)
Cirrhosis (yes vs no)	0.816	NA	NA
Vascular invasion (yes vs no)	**0.000**	0.224	1.222 (0.884–1.690)
MVI (yes vs no)	**0.000**	**0.000**	2.286 (1.488–3.511)
Tumor capsule (complete vs incomplete)	**0.000**	0.058	0.829 (0.682–1.006)
LN metastasis (yes vs no)	**0.000**	0.151	1.389 (0.887–2.176)
Cytological type (liver cell vs clear cell vs fatty-rich vs giant cell)	0.237	NA	NA
PD-L1 expression (TC ≥ 1% vs TC < 1%)	**0.046**	0.359	1.116 (0.883–1.411)
PD-L1 expression (IC ≥ 1% vs IC < 1%)	**0.031**	0.367	1.112 (0.882–1.402)
Stromal TILs (≥5% vs <5%)	**0.005**	**0.001**	1.418 (1.115–1.742)
HHLA2 expression (high vs low)	**0.003**	**0.046**	0.814 (0.664–0.990)

Abbreviations: TNM, tumor-node-metastasis staging; HR, hazard ratio; CI, confidence interval; MVI, microvascular invasion; TILs tumor infiltrating lymphocytes.

well tumor differentiation in a larger cohort of 547 HCC cases. The basis for our results may be mainly on the positive correlations between HHLA2 expression and low serum AFP levels, as well as tumor differentiation. AFP that typically represent tumor load both at source locations and in the circulation for HCC, and well tumor differentiation suggested less tumor aggressively, indicating that patients with HHLA2 overexpression are less likely to vulnerable to recurrence and metastasis.[29] In view of the different conclusions, there are several situations need to be considered, different detecting level (including DNA, mRNA, and proteins), different antibody source manufacturers, different HCC cases, different statistical analysis, most importantly, the cutoff value of HHLA2 expression was different. Moreover, our data

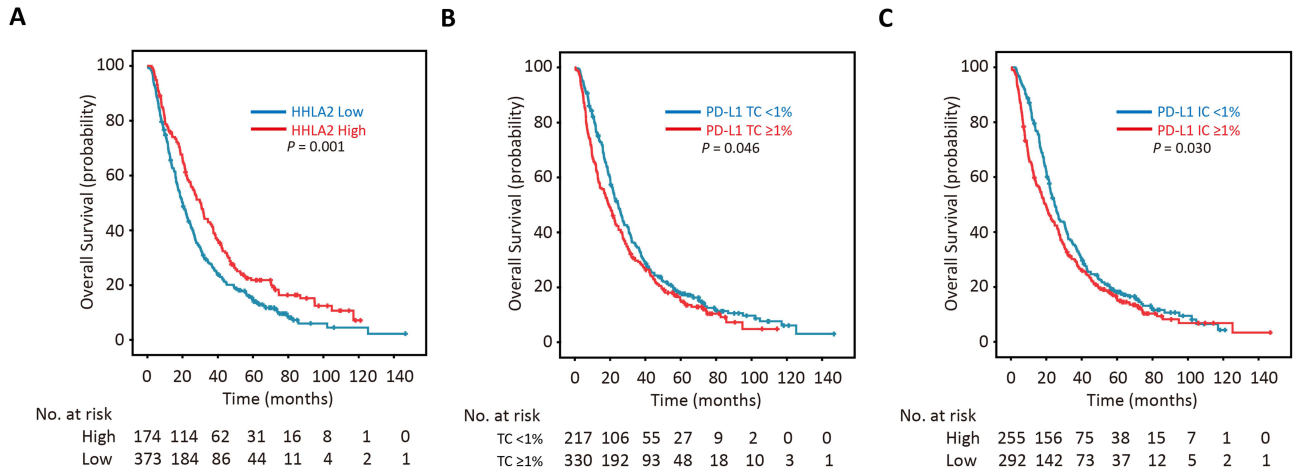

Figure 2 Kaplan Meier survival curves for OS of HCC patients according to HHLA2 and PD-L1 expression. High HHLA2 expression was significantly associated with good overall survival (OS) in HCC patients (**A**). High PD-L1 expression on TC (**B**) and IC (**C**) were both associated with poor overall survival (OS) in HCC patients. The *P*-values were determined via Log rank test.

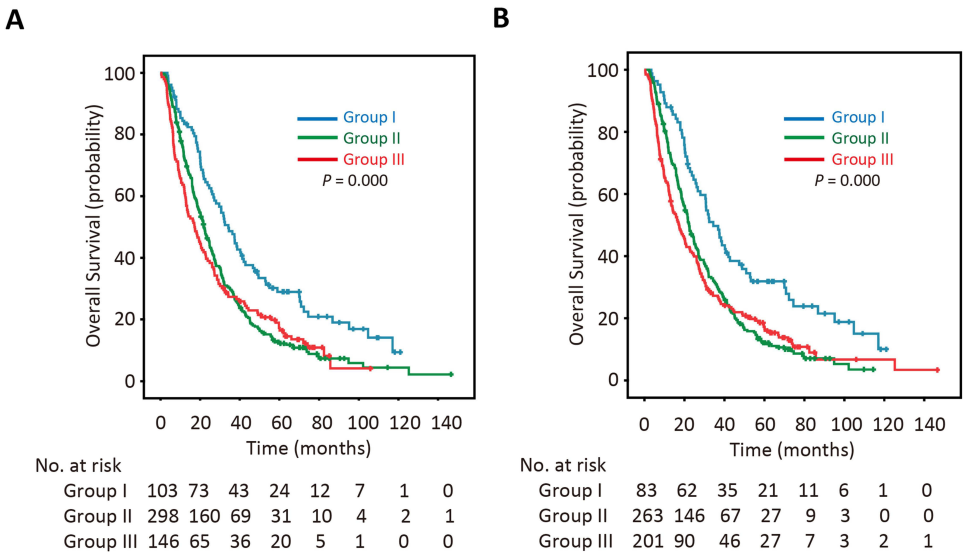

Figure 3 Kaplan Meier survival curves for OS of HCC patients according to HHLA2 and PD-L1 co-expression. (**A**) OS according to a combination of HHLA2/PD-L1 TC co-expression. Group I, HHLA2 (+)/ PD-L1 TC (-); Group II, both positive HHLA2 (+)/PD-L1 TC (-) or both negative HHLA2 (-)/PD-L1 TC (-); and Group III, HHLA2 (-)/PD-L1 TC (+). (**B**) OS according to a combination of HHLA2/PD-L1 IC co-expression. Group I, HHLA2 (+)/ PD-L1 IC (-); Group II, both positive HHLA2 (+)/PD-L1 IC (-) or both negative HHLA2 (-)/PD-L1 IC (-); and Group III, HHLA2 (-)/PD-L1 IC (+).

was in line with other studies indicating that HHLA2 was overexpressed and associated with better prognosis, such as in another HCC study,[25] in epithelial ovarian cancer,[20] in pancreatic and ampullary cancers[21] and in kidney renal clear cell carcinoma patients.[22] Collectively, we suggested our data were more representative to demonstrate the role of HHLA2 in HCC.

Previous research on the relationships between HHLA2 expression and immune cell infiltration among various cancer types produced inconsistent findings. High CD8+ TILs count and HHLA2 expression were independently associated with ovarian cancer.[20] However, there was no discernible connection between HHLA2 expression and TILs presence in osteosarcoma.[32] Additionally, a negative correlation between HHLA2 over-expression and cytotoxic T cells was found in Intrahepatic cholangiocarcinoma.[19] In addition, several studies have shown that HHLA2 serves as a T-cell co-inhibitory molecule and inhibits both human CD4+ T cells and CD8+ T cells in terms of proliferation and cytokine production.[15,16] Thus, it suggests that a high amount of HHLA2 may play a significant role in the development of tumors by inhibiting the immune system. In this study, we found that HHLA2 over-expression was related to low density of stromal TILs in HCC, which was in line with the result of many studies and indicated that HHLA2 may inhibit the proliferation of T cells.

In our study, PD-L1 positive rate on TC and IC in 547 HCC cases was 39.7% and 53.4%, respectively. This outcome was somewhat higher than what has previously been reported in the literature.[11–13] Different PD-L1 immunohistochemistry assays, various scoring systems, and evaluation of different tumor compartments may be the reasons for this observed diversity. In our study, positive PD-L1 either on TC or on IC was found to have a significant correlation with unfavorable OS, and these were in line with several previous HCC studies.[33,34] However, with findings from The Cancer Genome Atlas (TCGA) database, reduced level of PD-L1 mRNA (CD274) demonstrates a potential association with unfavorable prognosis in hepatocellular carcinoma (HCC) patients, although this correlation did not reach statistical significance (P = 0.095, Supplementary Figure 1B). Moreover, we discovered that PD-L1 (TC and IC) positive cases exhibited extensive T cell infiltration, which was in line with some previous studies,[19,33] but contradicted with the report from Schalper et al about lung cancer.[35] PD-L1 expression, based on the molecular basis, was speculated to have a negative impact on survival, whereas its positive connection with T cell infiltration mainly exerts an opposing impact and makes prognostic significance ambiguous.[36] The infiltration of T cells, especially CD8+ T cells, was a favorable survival factor and which was also confirmed in our cohort (Table S8). According to Schalper et al, the inconsistent prognostic significances of PD-L1 may not preclude its potential use as a therapeutic target and a biomarker for

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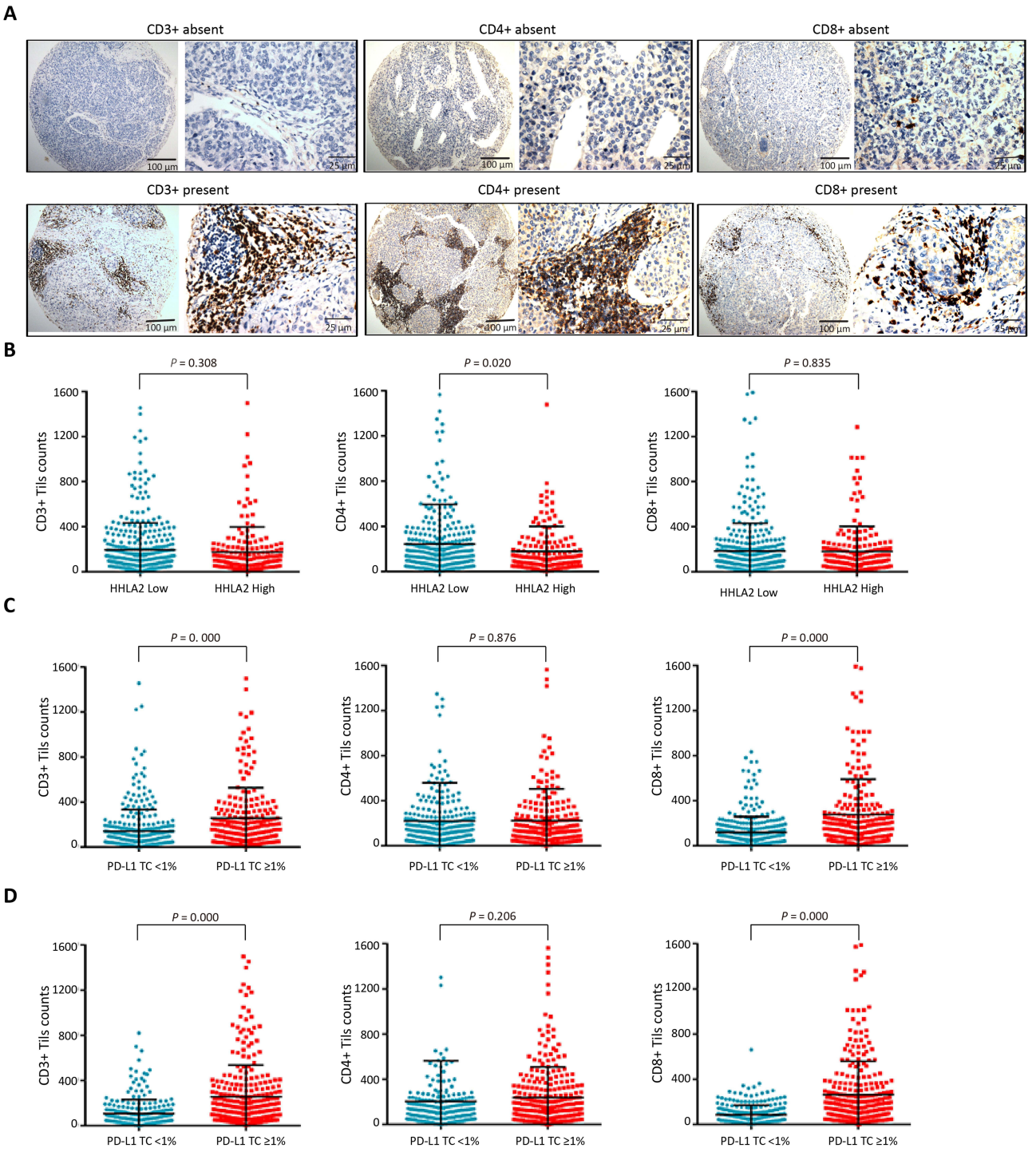

Figure 4 Tumor-infiltrating lymphocytes, helper T cell (Ths) and cytotoxic T cells (CTLs) and their correlation between HHLA2 and PD-L1 expression. Images of positive CD3+, CD4+, CD8+ staining and the corresponding intra-tumor negative controls (**A**). Magnification × 100 for full views and × 400 for zoomed-in views (scale bar, 25 μm). Scatter plot depicted the correlation between classic subsets of T cells and HHLA2 expression (**B**), PD-L1 TC expression (**C**), PD-L1 IC expression (**D**). *P* -values were generated by Mann–Whitney *U*-test. Error bars indicate median and interquartile range.

predicting treatment response.[35] Tumor-associated macrophages (TAMs) have been identified as pivotal components within the immunosuppressive tumor microenvironment, where they exert significant influence on immune evasion mechanisms and facilitate the malignant progression of HCC.[37–40] In our study, the correlation between PD-L1 expression and TAMs warrants further investigation, however, our analysis did not establish a statistically significant

correlation between PD-L1 expression levels and CD68+ macrophage infiltration. In summary, our study suggested that PD-L1 expression was considerable in HCC and was associated with higher densities of stromal TILs and poor prognosis.

Although the expression of both HHLA2 and PD-L1 is induced by related immunoregulatory factors, the limited overlap between the expression of PD-L1 and HHLA2 indicates their potential non-redundant biological functions or distinct spatial and/or temporal contributions to immune evasion. A previous study[18] indicated that co-expression of HHLA2 and PD-L1 was linked to a poor prognosis in individuals with clear cell renal cell carcinoma. Likewise, in our study, we discovered that the proportions of HHLA2 (+)/PD-L1 TC (-) and HHLA2 (-)/PD-L1 TC (+) cells in HCC patients were 18.8% (103/547) and 26.7% (146/547), respectively. Immune checkpoint inhibitor (ICI) therapy that targets anti-PD-1 or its ligand (anti-PD-L1) is the basis of different combination regimens intended to improve the objective response and survival of patients with HCC.[41] Similar to PD-L1,[35] it is possible that HHLA2 will serve as a therapeutic target and a biomarker that predicts therapeutic response despite its sporadic prognostic significances. Our investigation indicates that HHLA2 may be a potential immunotherapeutic target because it was related to a TME (tumor microenvironment) that was inhibitive and characterized by a decrease in TILs.

Despite the large number of cases in our cohort and the satisfactory results obtained in our research, there were also some limitations. Firstly, this is a single-center study conducted in China, and further multi-center validation is required to determine whether the results are applicable to other populations. Additionally, our study is subject to selection bias. Secondly, this is a retrospective study, HHLA2 expression and biological function in HCC are warranted to further elucidate. Thirdly, although immunohistochemistry was employed to evaluate the relationships among HHLA2, PD-L1, and tumor-infiltrating lymphocytes (TILs), variations in experimental methodologies and statistical analyses could lead to divergent outcomes. Consequently, the establishment of standardized protocols and multi-center validation is imperative before HHLA2 can be considered for clinical applications. Finally, the use of TMA sections in this investigation may weaken tumor representation or heterogeneity of the markers.

Conclusion

In summary, the expression of HHLA2 and PD-L1 in tumor cells (TC) or immune cells (IC) was frequently observed in HCC and demonstrated significant prognostic value. Notably, the HHLA2 (+)/PD-L1 TC (-) subgroup exhibited a more favorable prognosis compared to other groups. Elevated HHLA2 levels were associated with reduced stromal TIL density, whereas positive PD-L1 TC (or IC) expression correlated with increased stromal TIL density, higher CD4+ T cell (Th) counts, and greater CD8+ T cell (CTL) infiltration. These findings suggest that HHLA2 may function as a co-stimulatory ligand in HCC, positioning it as a promising immunotherapeutic target alongside PD-L1, with potential utility in predicting patient outcomes.

Abbreviations

HHLA2, Human endogenous retro virus-H Long repeat-associating 2; HCC, hepatocellular carcinoma; TILs, Tumor Infiltrating Lymphocytes; TNM, Tumor-Node-Metastasis; OS, overall survival; DFS, Disease-free survival.

Data Sharing Statement

The authenticity of this article has been validated by uploading the key raw data onto the Research Data Deposit public platform (https://www.researchdata.org.cn) with the approval RDD number as RDDA2024486748.

Ethics Approval and Consent to Participate

This study has been approved by the Institutional Review Board and Human Ethics Committee of SYSUCC. Written consent to use the samples for research purposes was obtained from all the patients before surgery.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare that they have no competing interests. This paper has been uploaded to ResearchSquare as a preprint: https://www.researchsquare.com/article/rs-2319886/v1.

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