Synergistic Effect of PD-L1 and HLA Class I On Prognosis of Patients With Hepatocellular Carcinoma

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Abstract

Background: Up-regulating the expression of PD-L1 and down-regulating the expression of HLA class I are the two main means of tumor-induced immune tolerance. The purpose of this study is to explore whether there is synergistic effect of up-regulation of PD-L1 and down-regulation of HLA-I on prognosis in hepatocellular carcinoma (HCC).

Methods: A cohort of 185 consecutive HCC patients was included in this study. According to the expression of PD-L1 and HLA class I, patients were divided into three subgroups: group A was PD-L1 negative + HLA class I high expression, group B was PD-L1 positive + HLA class I high expression or PD-L1 negative + HLA class I low expression, group C was PD-L1 positive + HLA class I low expression.

Results: PD-L1 positive was significantly associated with cirrhosis and tumor-infiltrating lymphocytes (P = 0.026; P= 0.000, respectively). Neither of PD-L1 positive and HLA class I low expression was significantly associated with shorter survival patients (P = 0.116; P = 0.171, respectively). The overall survival time of group C was significantly lower than that of group A and group B (31 months vs 58 months vs 49 months, P = 0.004), which was further confirmed by multivariate Cox regression analysis (group A/B vs group C, HR 3.652, 95%CI 1.627-8.200, P= 0.002).

Conclusions: The synergistic effect of PD-L1 positive and HLA class I low expression could result into a significant reduction in survival of patients with HCC, providing theoretical support for the combination of immunotherapy in future.

Introduction

Cancer-immunity cycle indicates that to recognize tumors cells and kill tumor cells constitute tow bolstering pillars in tumor-specific immune response[1]. Human leukocyte antigen (HLA) class I molecules participate in providing tumor-specific antigens to CD^{8+} T cell and activating $CD8^+$ T cells. $CD8^+$ T cells participate in killing target tumor cells. Dysfunction of HLA-I class molecules and $CD8^+$ T cells can result in immune toleranc[2]. The immune checkpoint, PD-1/PD-L1 (programmed cell death protein-1/ programmed death-ligand), plays an important role in inhibiting the function of $CD8^+$ T cells in hepatocellular carcinoma (HCC)[3].

Whether PD-L1 up-regulated by HCC can significantly reduce survival remains controversial[4, 5]. The results of phase I/II clinical trial (Checke040) showed that status of PD-L1 had no significant impact on objective response rate among patients with advanced HCC who received the treatment of Nivolumab[6], an anti-PD-1 monoclonal antibody to blocke the immune checkpoint interaction between PD-1and PD-L1. Phase III trial (KEYNOTE-240) did not yield a satisfactory result about pembrolizumab (anti-PD-1 monoclonal antibody) as second-line treatment in patients with advanced HCC[7]. It could be speculated by these results of clinical trials that PD-L1 expressed by HCC has a limited impact on prognosis in

patient with HCC. Rational underlying such speculation is multiple pathways involved in HCC-induced immune tolerance.

Down-regulation of HLA class I molecules gives rise to failure of HCC-associated antigen presentation and subsequent inability of immune system to recognize HCC[2]. Dysfunction of HLA class I molecules on HCC cells may be indicative of a gloomy prognosis, although the results of previous studies were not consistent in other tumors[8, 9]. There are sparse studies about mechanism of down-regulation of HLA I antigen in HCC[10, 11]. Moreover, prognostic information about HLA class I molecules in HCC is very limited.

Combination of PD-L1 up-regulation and HLA class I antigen down-regulation might bring out a synergistic impact on prognosis in HCC. Therefore, we conducted a retrospective study to investigate the value of PD-L1 and HLA class I antigen in HCC, and their synergistic impact on survival.

Materials And Methods

Study population

This study was approved by the review board of Peking University First Hospital. Written informed consent before collecting tissue samples was obtained from all patients. We retrospectively reviewed the medical registry at our institution and identified all patients diagnosed with HCC between November 2011 and December 2017. The eligibility criteria for inclusion were as follows: (1) underwent surgical resection; (2) definite pathologic diagnosis of HCC; (3) HCC treatment-naive before surgery. Patients who died within one month after surgery were excluded.

There were 185 patients, 29 females and 156 males, with a mean age of 58 years (rang, 27 to 80 years), who met the above criteria. Clinical characteristics, including age, gender, risk factors (HBV or HCV infection), liver cirrhosis, preoperative serum alpha-fetoprotein (AFP) levels, tumor size, vascular invasion, Child-Pugh classification, were retrieved from patients' medical records. Postoperative treatments and surveillance followed a uniform guideline. Survival time was calculated from the date of surgery to the date of death or last follow-up. During the follow-up, 109 patients were censored, and 76 were dead. The median follow-up was 32 months (rang, 2 to 91 months).

Immunohistochemical staining

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tumor tissue sections following a standard protocol [12]. PD-L1 expression was detected using rabbit monoclonal antibody (ab205921, ABCAM, 1:400 dilution ratio). HLA class I expression was detected using mouse monoclonal antibody (ab70328, ABCAM, 1:100 dilution ratio). Briefly, 4-μm sections were deparaffinized in xylene and dehydrated in an ethanol series, followed by heat-mediated antigen retrieval with EDTA buffer in an autoclave and deactivation of endogenous peroxidases with 3% H_2O_2. All sections were incubated with anti-PD-L1 or anti-HLA class I monoclonal antibody overnight at 4°C. Subsequently, the

sections were rinsed, incubated with second antibodies (horseradish peroxidase/Fab polymer conjugated; PV-6000, ZSGB-BIO). Reaction products were visualized with 3,3'-Diaminobenzidine (ab64238, ABCAM) and counterstained with hematoxylin. Human tonsil tissue was used as positive control. Negative controls were treated identically but without the addition of primary antibodies.

A tumor cell was considered PD-L1 or HLA class I positive when the cell membrane is stained, regardless of cytoplasmatic staining [13]. Samples with membranous expression of PD-L1 on ≥1% of the total cells were defined as tumors PD-L1 positive[6] (Figure 1). Both staining intensity and percentage of positive tumor cells were considered to assess the expression HLA class I antigen[8]. HLA class I expression was consider as low when the score was less than 5. The scores were calculated based on staining intensity grade (0: no staining;1: week; 2: moderate;3: strong) and staining percentage grade(0 for 0%; 1 for < 10%; 2 for < 30%; 3 for < 80%; 4 for ≥80%) (Figure 2). The number of tumor-infiltrating lymphocytes (TILs) was counted under a magnification of ×400, infiltration with ≥100 lymphocytes was defined was TILs positive[14] (Figure 3). The expression of PD-L1 and HLA class I was independently evaluated by two experienced pathologists without knowledge of any clinical information on the samples, and any discrepancy in expression level was resolved by a mutual discussion.

Statistical analysis

Categorical data were presented as number (n) or percentage, and any differences between the two groups were analyzed by chi-squared test. Alternatively, Fish's exact test or continuity correction was used when the chi-square test was violated. Survival curves were assessed by Kaplan-Meier method and compared by log-rank test. Univariate and multivariate regression analysis for hazard ratios (HR) was performed using the Cox proportional hazards model. All of the statistical tests and p-value were two tailed and p-values of <0.05 were considered statistically significant. All analyses were performed using the SPSS 16.0 (IL, USA).

Results

Of the 185 patients enrolled, PD-L1 positive was found in 41 (22.2%) patients, and HLA class I antigen low expression was found in 60 (32.4%) patients. TILs positive was found in 12 patients (6.5%). Based on the immunohistochemical results of PD-L1 and HLA class I antigen, the 185 patients were classified into three subgroups: A group (PD-L1 negative/HLA class I antigen high expression), B group (PD-L1 negative/HLA class I antigen low expression or PD-L1 positive/HLA class I antigen high expression) and C group (PD-L1 positive/HLA class I antigen low expression).

Association of PD-L1 and HLA class I antigen with clinical characteristics

As is shown in Table 1, PD-L1 expression is significantly associated with cirrhosis (P = 0.016) and TILs (P = 0.000). The remaining clinical characteristics, including gender, age, virus infection, AFP, tumor size and vascular invasion, were not significantly associated with PD-L1 expression (P > 0.05). None of clinical characteristics, including gender, age, virus infection, cirrhosis, AFP, tumor size and vascular invasion

were significantly associated with HLA class I antigen expression ($P > 0.05$). Although low expression of HLA class I antigen was more likely to exhibit low level of AFP and vascular invasion compared with high expression of HLA class I antigen, difference did not reach statistical significance ($P = 0.095$, $P = 0.052$). In addition, there was no significant difference among the three subgroups (A group, B group and C group) in clinical characteristics (Table 2).

Table 1
Association of PD-L1 and HLA class I with clinical chararateristics

Characteristics	No.	PD-L1 (%)		*P*	HLA class I (%)		*P*
		Negative	Positive		Low	High	
Gender				0.237			0.299
female	29	25 (17.4)	4 (9.8)		7 (11.7)	22 (17.6)	
male	156	119 (82.6)	37 (90.2)		53 (88.3)	103 (82.4)	
Age				0.262			0.321
<60	93	77 (53.6)	16 (39.0)		27 (45.0)	66 (52.8)	
≥60	92	67 (46.5)	25 (61.0)		33 (45.0)	59 (47.2)	
Cirrhosis				0.026			0.420
no	63	55 (38.2)	8 (19.5)		18 (30.0)	45 (36.0)	
yes	122	89 (61.8)	33(80.5)		42 (70.0)	80 (64.0)	
HBV/HCV				0.213			0.888
no	35	30 (20.8)	5 (12.2)		11 (18.3)	24 (19.2)	
yes	150	114 (79.2)	36 (87.8)		49 (81.7)	101 (80.8)	
AFP				0.277			0.095
<200	104	84 (58.3)	20 (48.8)		39 (65.0)	65 (52.0)	
≥200	81	60 (41.7)	21 (51.2)		21 (35.0)	60 (48.0)	
Tumor size				0.158			0.757
<5 cm	77	56 (38.9)	21 (51.2)		24 (40.0)	53 (42.4)	
≥5 cm	108	88 (61.1)	20 (48.8)		36 (60.0)	72 (57.6)	
Vascular invasion				0.797			0.052
no	137	106 (73.6)	31 (75.6)		39 (65.0)	98 (78.4)	
yes	48	38 (26.4)	10 (24.4)		21 (35.0)	27 (21.6)	
TILs				0.000			0.375
negative	173	142 (98.6)	31 (75.6)		58 (96.7)	115 (92.0)	
positive	12	2 (1.4)	10 (24.4)		2 (3.3)	10 (8.0)	

Table 2
Clinical characteristics in subgroups

Characteristics	No.	Subgroups (%)			*P*
		A	B	C	
Gender					0.107
female	29	20 (20.8)	7 (9.1)	2 (16.7)	
male	156	76 (79.2)	70 (90.9)	10 (83.3)	
Age					0.177
<60	93	54 (56.2)	35 (45.5)	4 (33.3)	
≥60	92	42 (43.8)	42 (54.5)	8 (66.7)	
Cirrhosis					0.077
no	63	38 (39.6)	24 (31.2)	1 (8.3)	
yes	122	58 (60.4)	53 (68.8)	11 (91.7)	
HBV/HCV					0.567
no	35	20 (20.8)	14 (18.2)	1 (8.3)	
yes	150	76(79.2)	63 (81.8)	11 (91.7)	
AFP					0.750
<200	104	53 (55.2)	43 (55.8)	8 (66.7)	
≥200	81	43 (44.8)	34 (44.2)	4 (33.3)	
Tumor size					0.657
<5 cm	77	37 (38.5)	35 (45.5)	5 (41.7)	
≥5 cm	108	59 (61.5)	42 (54.5)	7 (58.3)	
Vascular invasion					0.409
no	137	75 (78.1)	54 (70.1)	8 (66.7)	
yes	48	21 (21.9)	23 (29.9)	4 (33.3)	

Association of PD-L1 and HLA class I antigen with survival

Although patients with positive PD-L1 had a shorter survival than those with negative PD-L1, difference was not statistically significant (P = 0.116) (Fig. 4a). There was also a trend that survival was shorter in patients with low expression of HLA class I antigen than in those with high expression of HLA class I antigen (P = 0.171) (Fig. 4b). However, coexistence of PD-L1 positive and HLA class I antigen low

expression was significantly associated with worse survival (Fig. 4c), C group had a shorter survival than A group and B group (31 months vs 58 months vs 49 months, P = 0.004).

Hazard ratios were assessed by the Cox proportional hazards model are shown in Table 3. Variables with $p < 0.05$ in the univariate analysis were entered into the multivariate Cox model to investigate their independent contribution. In univariate analysis, tumor size ≥ 5 cm, vascular invasion and coexistence of PD-L1 positive and HLA class I antigen low expression were significantly associated with worse survival (tumor size ≥ 5cm: HR 1.713, 95%CI 1.061–2.766, P = 0.028; vascular invasion: HR 3.414, 95%CI 2.130–5.472, P = 0.000; coexistence of PD-L1 positive and HLA class I antigen low expression: HR 3.461, 95%CI 1.556-7.700, P = 0.002), respectively. In multivariate analysis, both of vascular invasion and coexistence of PD-L1 positive and HLA class I antigen low expression were independent and significant prognostic factors (vascular invasion: HR 3.468, 95%CI 2.160–5.569, P = 0.000; coexistence of PD-L1 positive and HLA class I antigen low expression: HR 3.652, 95%CI 1.627-8.200, P = 0.002). The mortality risk of subgroup C was more than 3 times that of subgroup A/B.

Table 3. Univariate and multivariate analysis of prognostic factors

Characteristics	Univariate analysis			Multivariate analysis		
	Hazard Ratio	95%CI	*P*	Hazard Ratio	95% CI	*P*
Gender			0.372			-
female	1			-		
male	1.354	0.696-2.637		-	-	
Age			0.071			-
<60	1			-		
≥60	1.523	0.965-2.404		-	-	
Cirrhosis			0.660			-
no	1			-		
yes	1.113	0.690-1.797		-	-	
Virus infection			0.460			-
no	1			-		
yes	1.253	0.689-2.278		-	-	
AFP			0.631			-
<200	1			-		
≥200	1.117	0.711-1.756		-	-	
Tumor size			0.028			0.130
<5cm	1			1		
≥5cm	1.713	1.061-2.766		1.457	0.896-2.371	
Vascular invasion			0.000			0.000
no	1			1		
yes	3.414	2.130-5.472		3.468	2.160-5.569	
HLA/PD-L1						
A/B	1		0.002	1		0.002
C	3.461	1.556-7.700		3.652	1.627-8.200	
TILs			0.142			

negative	1	
positive	0.420	0.132-1.335
Abbreviation: CI, confidence interval.		

Discussion

Hepatocellular carcinoma (HCC) is one of the most common cancers, which ranks as the second leading cause of cancer-related death worldwide[15]. Is spite of recent advances in treatment options, prognosis remains quite poor, especially in patients with advanced HCC. Patients with advanced HCC have a median survival of less than one year[16]. In light of poor prognosis and resistance to chemotherapy and radiotherapy, other treatment strategies have been investigated extensively. The multikinase inhibitor (sorafenib) was the first systemic agent to show a significant improvement in overall survival for patients with advanced HCC[17]. However, anti-angiogenic agents (sorafenib and lenvatinib) only yield a modest improvement of about 3 months in overall survival[18]. Therefore, immunotherapy, which aims to interrupt immune checkpoint interaction and break immune tolerance, has come under in the spotlight.

Experimental evidence has demonstrated that PD-L1 on tumor cells can delivery inhibitory signals to PD-$1^{+}CD8^{+}$ T cells, resulting in suppression of immune response by inducing apoptosis, anergy and functional exhaustion of $CD8^{+}$ T cells[19]. Further pathologic study showed that PD-L1 positive HCC was significantly associated with biological aggressiveness[20], including vascular invasion, poor differentiation, satellite nodules and high AFP levels; nevertheless, whether PD-L1 expression can influence prognosis in patients with HCC remains open to debate. Results from several studies investigating prognostic significance of PD-L1 in HCC are inconsistent[4, 5, 21]. A meta-analysis indicated that PD-L1 positive was predictive for shorter overall survival and disease-free survival[22]. However, such meta-analysis suffers several limitations[23]. First the meta-analysis did not screen our all studies in this field. Second, one included study used serum rather than tumor samples to assess status of PD-L1. Third, included patients received different treatment. All of these limitations increase heterogeneity and undermine the reliability of the results.

Our present study showed that patients with positive PD-L1 had a shorter survival than those with negative PD-L1. However, difference was not statistically significant (P = 0.116). PD-L1 expression is significantly associated with cirrhosis (P = 0.016) and TILs (P = 0.000). The remaining clinical characteristics, including gender, age, virus infection, AFP, tumor size and vascular invasion, were not significantly associated with PD-L1 expression (P > 0.05). Clinical trials of Checkmate 040 and KEYNOTE 240 indicated that anti-PD-1 monoclonal antibody could not significantly increase survival and PD-L1 expression did not influence objective response rate in patients with advanced HCC[6, 7], which might suggest that PD-L1 expression in HCC exert a modest impact on prognosis in patients with HCC. Previous studies have showed that TILs can trigger PD-L1 up-regulation in tumor cells by secreting interferon-γ[24], which is further confirmed by the result that PD-L1 positive is significantly with TILs in our present study.

There has been reported that genetic variations of PD-1 predisposed patients with chronic HBV infection to cirrhosis[25]. Whether an association of tumor PD-L1 positivity with cirrhosis is a causal relationship needs to be further investigated.

The value of HLA class I molecules in HCC was rarely investigated. Down-regulation of HLA class I antigen is one of strategies for HCC-induced immune tolerance[1]. A direct evidence of tumor escape from T cell immunity cause by MHC-I down-regulation is the facial cancer in Tasmanian devil, which is transmissible to histo-incompatible companions[26, 27]. The underlying mechanism is that this cancer silenced the genes for antigen presentation at the epigenetic level. Nevertheless, our present study showed that survival was shorter in patients with low expression of HLA class I antigen than in those with high expression of HLA class I antigen, but the difference did not reach a statistical significance (P = 0.171). None of clinical characteristics, including gender, age, virus infection, cirrhosis, AFP, tumor size and vascular invasion were significantly associated with HLA class I antigen expression (P > 0.05).

Multiple pathways are involved in HCC-induced immune tolerance[1]. Logically, two immune pathways may exert a synergistic impact on immune tolerance, which recommend combination immunotherapy in cancers. Combination of immunotherapy (ipilimumab/nivolumab) also yields a better survival than single immunotherapy (ipilimumab or nivolumab) in advanced melanoma[28]. Our present study showed that coexistence of PD-L1 positive and HLA class I antigen low expression was significantly associated with worse survival (P = 0.004), which provide a rational for combination of immunotherapy in HCC. To date, there are two star drugs against PD-1/PD-L1 checkpoint (nivolumab and pembrolizumab), which have been applied clinically. Nevertheless, effective and safe drugs to recover HLA class I antigen remains under investigation, which may be a major target for future studies[29].

In conclusion, PD-L1 positive and HLA class I antigen low expression have no significant impact on prognosis when they were analyzed alone, only when they are analyzed together can they yield a significant synergistic impact on prognosis in patients with HCC. Such conclusion provide a combining immunotherapy strategy of inhibiting PD-1/PD-L1 and recovering HLA class I antigen for patients with HCC.

Declarations

Availability of data and materials The data generated during the current study are available to any scientist wishing to use them for non-commercial purpose from the corresponding author on reasonable request. However, the clinical data might be available without the privacy data of participates in the current study.

Conflict of interest statement: The authors declare that they have no conflict of interest.

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Ethical approval and informed consent: This study was approved by Institutional Review Board of Yijishan Hospital of Wannan Medical College. All patients signed an informed consent form.

Conflict of Interest: *none*

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Figures

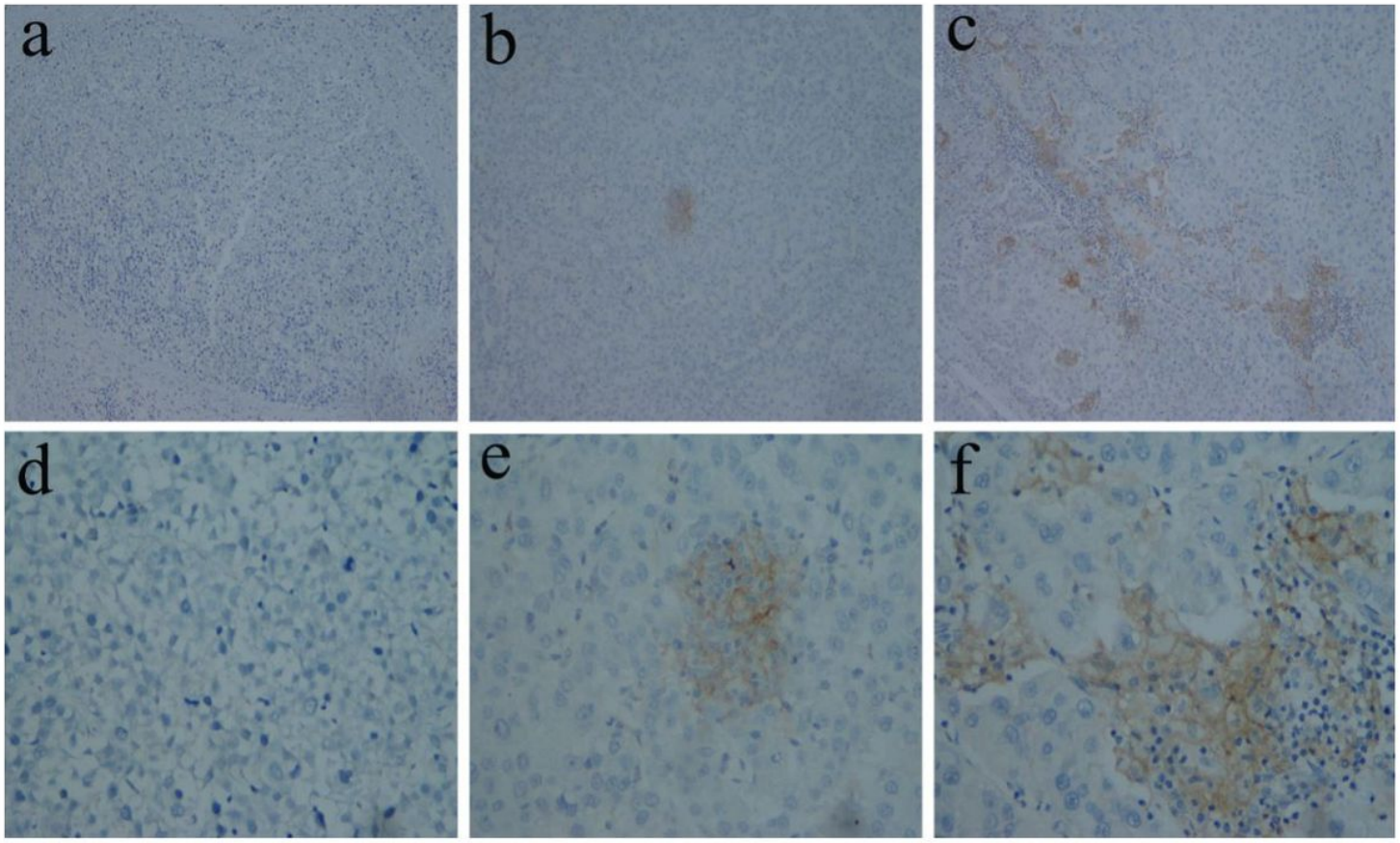

Figure 1

Immunohistochemical assessment of PD-L1 expression in HCC surgical specimen (a-c, 100×magnification; d-f, 400×magnification). PD-L1 expression was scored as 0% (a and d); PD-L1 expression was scored as <1%(b and e); PD-L1 expression was scored as ≥1%(c and f).

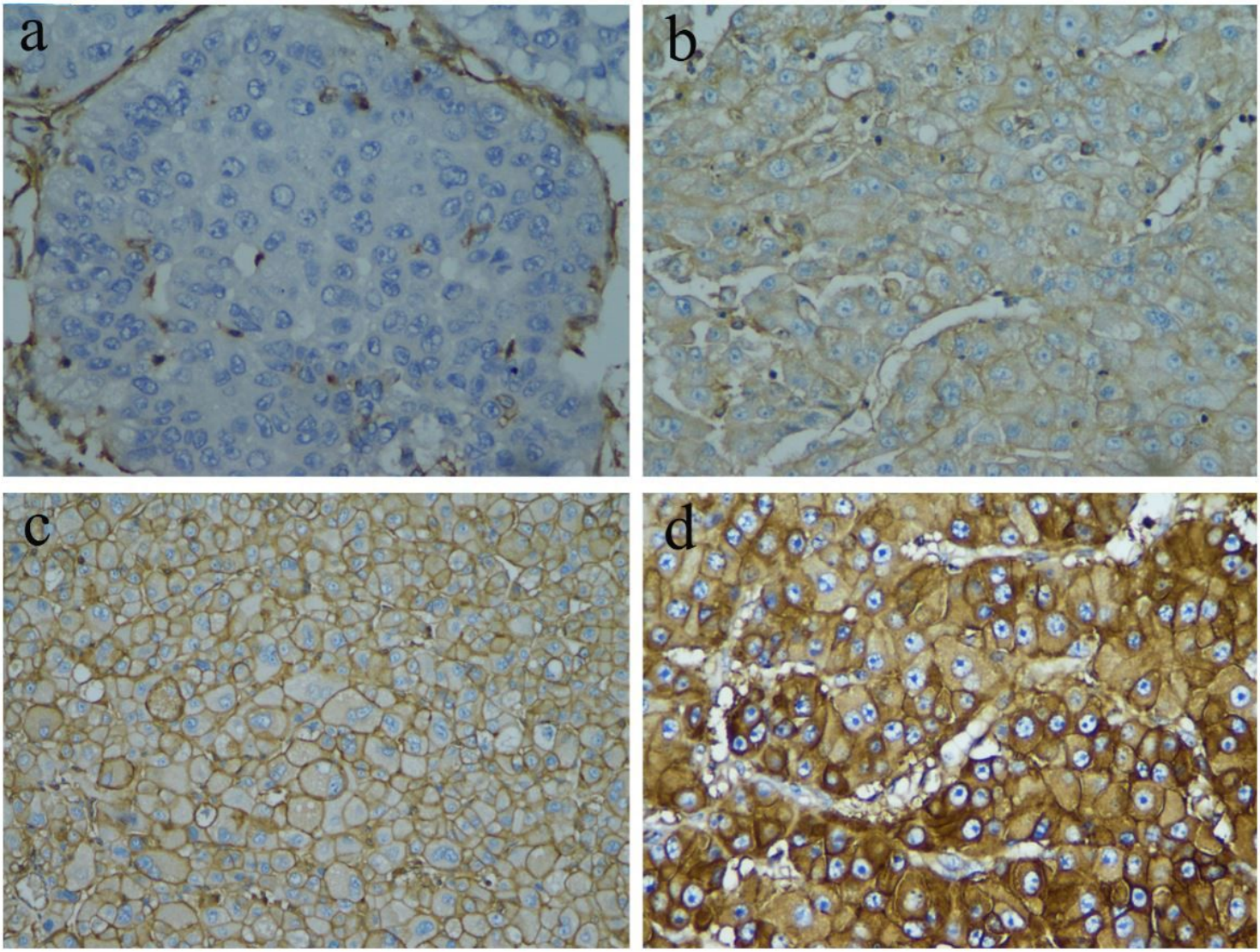

Figure 2

Immunohistochemical staining intensity of HLA class I in HCC specimen (a for negative, b for weak, c for moderate, d for strong).

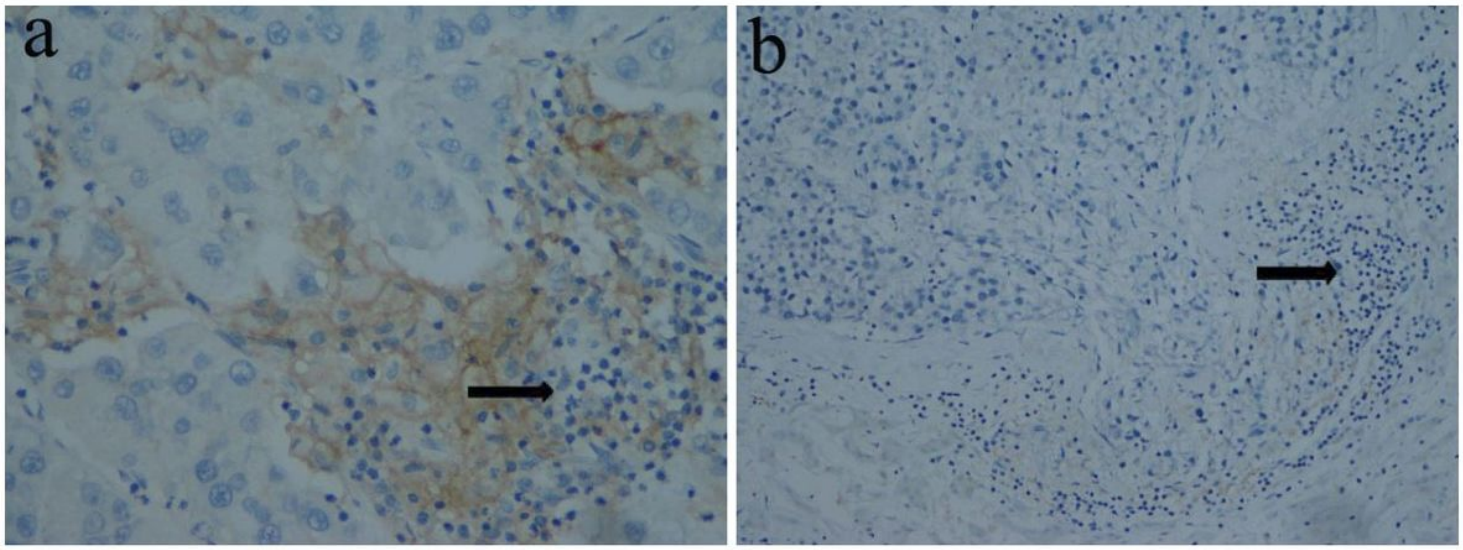

Figure 3

Tumor-infiltrating lymphocytes (black arrows) in the presence of PD-L1 (a) and in the absence of HLA-class I (b).

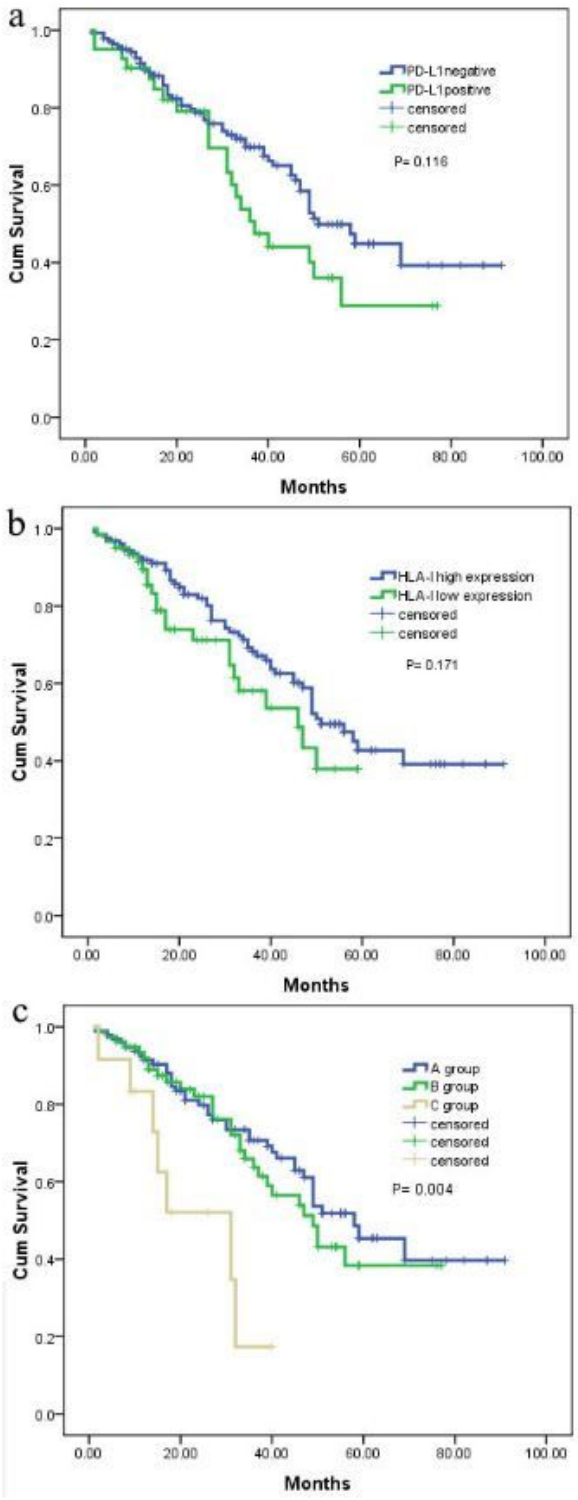

Figure 4

Overall survival for subgroups analyzed by Kaplan–Meier method and Log-rank test.