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Original Research

Higher serum sPD-L1 levels after radiotherapy indicate poor outcome in hepatocellular carcinoma patients

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ABSTRACT

Background: Our preclinical research reveals that radiotherapy (RT) promoted PD-L1 upregulation in tumor tissues and that higher PD-L1 after RT worsened the prognosis through immunosuppression. We sought to validate our experimental results in clinical cohorts and promote clinical application.

Patients and methods: In cohort 1, formalin-fixed paraffin-embedded samples were obtained from 46 HCC patients, 23 of whom received preoperative RT and the other 23 received direct surgery. A prospectively collected database contained 122 HCC patients treated with liver RT were enrolled in cohort 2. Blood samples were taken a day before and two weeks after RT. Patients in cohort 2 were further divided into two groups, exploration (73 patients) and validation (49 patients) groups.

Results: In cohort 1, RT increased the expression of PD-L1 in tumor tissues ($p = 0.001$), and PD-L1 levels were associated with decreased cytotoxic T-cell infiltration and a trend toward poor prognosis ($p = 0.14$). Moreover, PD-L1 expression in tumor tissue positively correlated with soluble (s) PD-L1 in serum ($R = 0.421$, $p = 0.046$). Then, in cohort 2, we revealed RT increased sPD-L1 in serum ($p < 0.001$), which was associated with the number of circulating CD8+ T cells ($R = -0.24$, $p = 0.036$), indicating poor survival. Furthermore, patients with higher rate of sPD-L1 increase after RT have better treatment response ($p < 0.001$), PFS ($p = 0.032$) and OS ($p = 0.045$).

Conclusion: Higher post-RT serum sPD-L1, which may potentiate immune suppression effects, indicates a poor prognosis for HCC patients treated with RT.

Introduction

Hepatocellular Carcinoma (HCC) is still the leading cause of tumor-related mortality globally [1]. After considering the tumor stage and patients' liver status, HCC patients could be treated with local, locoregional and/or systemic therapy. Radiotherapy (RT) is recommended as a locoregional treatment option for unresectable HCC patients in the National Comprehensive Cancer Network guidelines. Historically, the curative effect of RT was attributed to its ability to cause DNA damage, which directly resulted in tumor cell death. Recent studies have shown that RT can reprogram the tumor microenvironment to produce a potent antitumor immune response [2]. Unfortunately, both preclinical and clinical observations suggest that RT can also induce immunosuppressive responses, which negates the benefit of RT for overall survival [3]. For instance, the recent preclinical work of our team revealed that RT promoted the upregulation of PD-L1 in HCC tumor cells, and irradiation-induced PD-L1 upregulation conferred tumor evasion by inhibiting cytotoxic T cell-mediated antitumor immunity. Furthermore, combining anti-PD-L1 with RT reversed the adverse immunosuppressive effects and provided a favorable prognosis [4].

The current clinical study was carried out to test the experimental results of our preclinical work. In this study, we found that PD-L1 expression was higher in irradiated tumor tissues from HCC patients than in non-irradiated tumor tissues. Higher PD-L1 levels after RT were negatively associated with the infiltration of CD8+ T cells and indicated a dim prognosis trend. In clinical practice, histology samples of HCC are difficult to obtain, especially for patients undergoing RT. Moreover, the PD-L1 expression in various tissues in the same patient was heterogeneous to some extent. This may explain the inconsistent results in many clinical studies that explored the prognostic role of PD-L1 on tumor tissues after hepatectomy [5–7]. As a result, an applicable clinical surrogate of PD-L1 in tumor tissues is required.

In addition to the membrane-bound forms, soluble (s) PD-L1 recently has been detected in the blood of cancer patients [8,9]. Several studies

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disclosed that levels of sPD-L1 levels in serum positively correlated with the expression of PD-L1 in tumor cells [10,11]. Furthermore, recent studies have explored the prognostic role of sPD-L1 in various malignancies, including HCC [12,13]. Herein, we found that similar to tumor histology samples, RT significantly increased sPD-L1 serum levels in HCC patients. The higher post-RT sPD-L1 concentration may reflect an immunosuppressive environment and indicate a worse prognosis.

Materials and methods

The Ethics Committee of Zhongshan Hospital, Fudan University approved the study. All patients or their representatives gave their written informed consent for participation.

Patient characteristics

The current study included two independent cohorts. Cohort 1 included 46 HCC patients, 23 of whom received preoperative RT between July 2005 and March 2010 (obtained from a cohort of previous clinical trials that investigated the role of neoadjuvant RT in HCC patients at Zhongshan Hospital), and 23 control HCC patients who received surgical resection without RT during the same period. The following were the inclusion criteria for the preoperative RT group: (1) stage III HCC patients (American Joint Committee on Cancer, 7th); (2) received intrahepatic tumor RT; (3) treated with hepatic tumor resection within 4 weeks after RT completion. Tumor tissues were collected immediately following surgical tumor resection.

Cohort 2 was obtained from a prospectively maintained database of individuals enrolled between February 2011 to January 2018. The inclusion criteria were as follows: (1) patients with newly diagnosed locally advanced HCC (stage II-III, American Joint Committee on Cancer, 7th); (2) unsuitable for surgical resection; (3) received definitive liver RT in our institution; (4) complete follow-up information. The following were exclusion criteria: (1) patients with early-stage HCC treated with stereotactic ablative radiotherapy; (2) patients with extrahepatic metastasis, a second primary tumor, or serious internal medicine diseases; and (3) patients with insufficient blood samples in our biobank. Blood samples were taken a day before and two weeks after RT. Blood samples were centrifuged at 3000 rpm for 10 min at room temperature to collect serum. Aliquots of serum samples were made and stored at -80°C.

RT dose fraction

In cohort 2, either conventional fractionated RT (2.0 Gy / fraction (f), 25-30 f, total dose: 50-60 Gy, biological equivalent dose (BED): 60-72 Gy) or moderately hypo-fractionated RT (hypo-RT) (2.5-3.6 Gy, 15-20 f, total dose: 50-56 Gy, BED: 62.5- 73.4 Gy) was used to perform RT.

sPD-1 and sPD-L1 enzyme-linked immunosorbent assays (ELISAs)

ELISAs were used to quantify sPD-1 and sPD-L1 using Human Immuno-Oncology Checkpoint Protein Panel Magnetic Bead Panel kits (Merck, CHCKPMAG-11K, Darmstadt, Germany), based on the manufacturer's instructions. Briefly, MSD (Mesoscale, Diagnostics, Rockville, ML) high-bind microtiter plates were incubated with 25 μl/well capture antibodies (2 mg/ml), sealed and incubated overnight. The next day, plates were washed (3 × 200 ml/well PBS/0.05% Tween), blocked for 1 h (5% BSA in PBS) with shaking (700 rpm), washed again, and 25 μl of calibrators or patient samples added per well for 2 h with shaking. After another washing step, 25 μl/well-unlabeled detection antibodies (200 ng/ml for PD-1 and 100 ng/ml for PD-L1) were added and incubated for 2 h with shaking. After washing the plates, 25 μl/well streptavidin-sulfotag antibodies were added for 2 h. Finally, the reading buffer was added and the SQ120 QuickPlex reader (Mesoscale Diagnostics, Rockville, ML) was used to perform chemiluminescent measurements. Absolute sPD-1 and sPD-L1 (ng/ml) concentrations in patient samples were calculated using a four-point-fit calibration curve of standard dilutions.

Histological examination

Liver sections were stained with hematoxylin or immunohistochemical stained with anti-PD-L1 (1:100, Cat. # AB-228462, Abcam, Cambridge, MA) and visualized with a streptavidin-biotin staining kit (Changdao, Shanghai, China) and diaminobenzidine.

For immunofluorescence staining, paraffin-embedded sections were de-paraffinized and rehydrated, followed by antigen retrieval with Antigen Unmasking Solution (H-3300, Vector Laboratories, Burlingame, California) was done. Frozen sections were fixed with -20°C acetone before being rinsed with PBS. Non-specific binding was inhibited by incubating sections in 0.5% casein in PBS for 1 h at room temperature in 0.5%. Then sections were incubated overnight at 4°C using primary antibodies to PD-L1 (1:100, Cat. # AB-228462, Abcam) or CD8 (1:200 Cat. # AB-93278, Abcam). Slides were washed and then incubated with fluorochrome-conjugated secondary antibodies for 1 h. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MO, USA). Zeiss Axiovert (Zeiss, Wetzlar, Germany) and Metamorph imaging software (Molecular Devices, Sunnyvale, CA) were used to capture the Images.

Ten serial sections per tumor tissue were obtained for quantitation. An observer with no knowledge of the treatment group counted stained cells per high-powered field from five non-overlapping images. In cases where staining was less distinctly cellular, immunofluorescence intensity was quantified by measuring the total signal for the entire image and correcting for cell density by dividing by the total DAPI area using in-house programs (Fiji-Image J, NIH, Bethesda, MD).

Statistical methods

In this study, R software version 4.0.5. and Graphad prism 8.0 were used for statistical analysis. The short treatment response was evaluated based on the Response Evaluation Criteria in Solid Tumors (1.1). Overall Survival (OS) and Progression-Free Survival (PFS) were calculated starting from the first day of RT. Disease-Free Survival (DFS) was calculated starting from the surgery time. The function of "surv_cutpoint" in R (PFS or DFS was defined as the end event) was used to determine the best cut-off values of the continuous variables in survival analysis. The Kaplan-Meier (KM) method was used to estimate OS, PFS and DFS and the log-rank test was used to compare them. Variables with p-value < 0.2 in univariate Cox analysis were inputted into multivariate Cox regression models. To account for the potential confounders, the multivariate Cox regression models for PFS and OS were used. To compare the continuous variables between the two groups, the Student's or paired t-test was used. To compare the categorical variables between different groups, the Chi-square test or Fisher's examination was used. Pearson's or Spearman's correlation analysis was used to evaluate the relationship between different variables. The $p < 0.05$ was considered statistically significant in all analyses.

Results

RT-induced PD-L1 expression in irradiated tumor tissues correlated with poor survival

To determine whether RT influences PD-L1 in HCC expression, we examined tumors from a total of 23 patients who received preoperative RT before tumor resection (cohort 1). Non-irradiated HCC tumor tissues were used as controls. The patients who received RT and those who did not were completely matched (Supplemental Table 1). For immunohistochemical analysis, an FDA-approved clinical laboratory used PD-L1 antibody clone SP142 (Fig. 1A). When irradiated tumor tissues were compared to matched, non-irradiated control tumor tissues, there was a

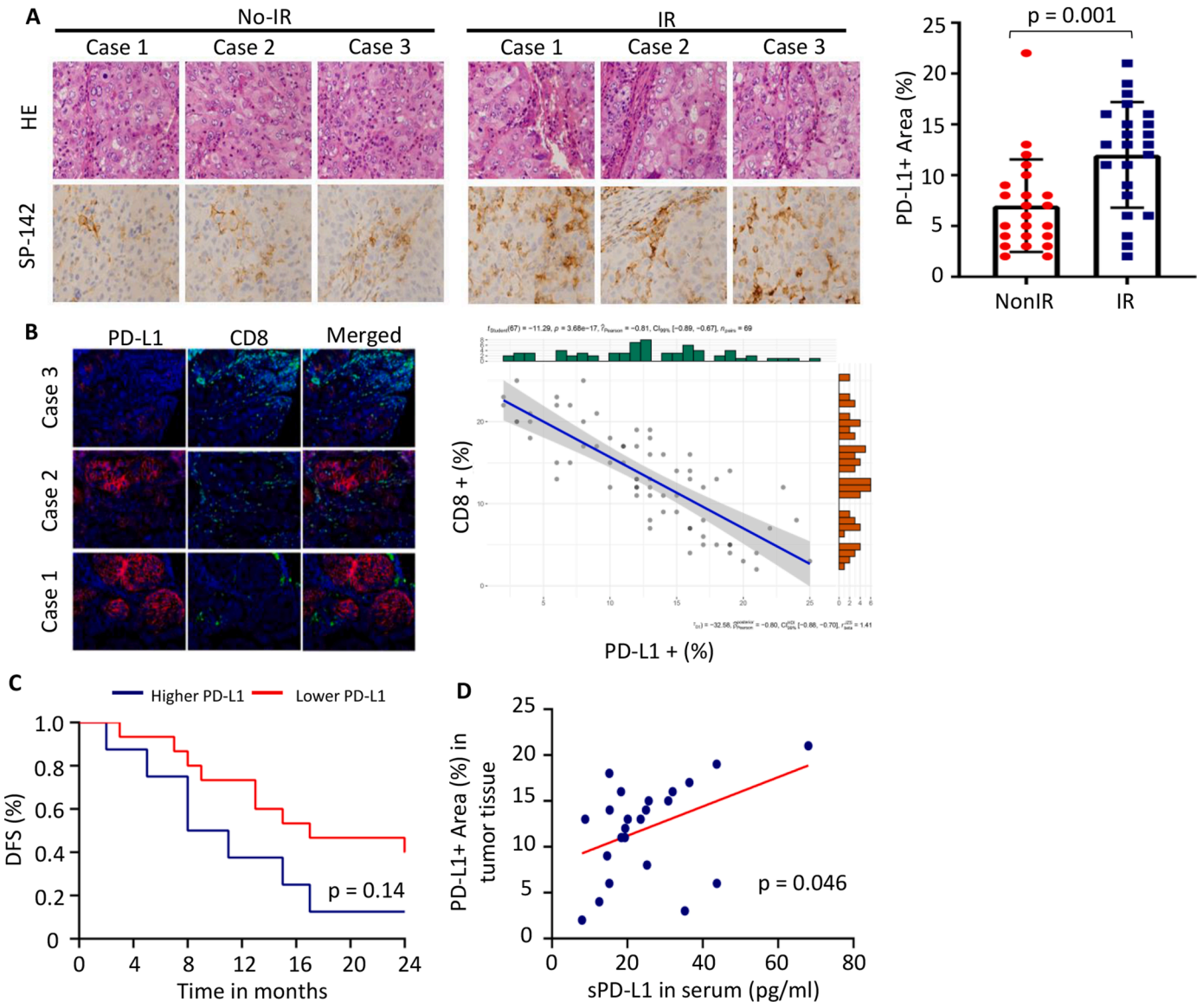

Fig. 1. (A) Representative H.E. (20 ×) and membranous PD-L1 (sp-142) expression (40 ×) images in irradiated or non-irradiated tumor tissues. Quantitative PD-L1+ area in tumor sections from irradiated or non-irradiated patients ($p = 0.001$). (B) Representative immunofluorescence images of irradiated tumor tissues from 3 individual patients. Blue channel: DAPI, Red channel: PD-L1+ cells, Green channel: CD8+ cells. Quantified PD-L1 intensity and CD8+ T cells in patient tumor tissues. The error bar represents the standard error (SD) from 10 serial sections of each patient. (C) Kaplan-Meier analysis of the disease-free survival (DFS) ($p = 0.14$). (D) PD-L1 expression in tumor tissues significantly associated with the sPD-L1 levels in serum ($R = 0.421$, $p = 0.046$).

significant increase in PD-L1 expression (Fig. 1A, $p = 0.001$). Samples were co-stained with PD-L1 and CD8 antibodies to further examine PD-L1 expression in tumor tissues with immune cell infiltration in irradiated tumor tissues. Higher PD-L1 staining areas had lower CD8+ T-cell infiltration (Fig. 1B). When PD-L1 staining intensity was quantitated, it was found to be negatively correlated with the number of cytotoxic CD8+ T cells (Fig. 1B). This is consistent with previous findings that tumor cell PD-L1 is sufficient for immune evasion in immunogenic tumors and inhibits CD8+ T-cell cytotoxicity [14]. The relationship between DFS and PD-L1 expression was noted to have a clear trend of poor DFS in higher PD-L1-staining tumor tissues ($p = 0.14$) (Fig. 1C). Furthermore, there was a significant relationship between PD-L1 expression in tumor tissues and the sPD-L1 levels in serum ($R = 0.421$, $p = 0.046$) (Fig. 1D), in line with previous studies [10,11].

HCC patient characteristics in cohort 2

Histology examination is invasive and can result in bleeding. Moreover, HCC can be diagnosed without pathology. Consequently, histology samples are scarce. We further investigated the prognostic role of serum sPD-L1 in HCC patients receiving RT to promote its clinical translation since PD-L1 expression in tumor tissue is positively associated with sPD-L1 levels in serum. From February 2011 to January 2018, our institution treated 278 HCC patients with liver RT. Among them, 57 had extrahepatic organ metastasis, 66 were early-stage HCC receiving stereotactic ablative radiotherapy, and 6 patients had insufficient blood samples. Among the last 149 patients, sPD-L1 or sPD-1 was not found in 27 patients. Finally, 122 patients enrolled in cohort 2. Patients in cohort 2 were further divided into exploration (73 patients) and validation cohorts (49 patients) based on the time of admission.

Chronic viral hepatitis B was the most common HCC etiology in the exploration cohort (79.45%). The 75.34% of patients had liver cirrhosis. All patients were classified as Child-Pugh score A. Transcatheter Arterial Chemoembolization (TACE) was used on 75.34% of the patients in this group, and targeted therapy was used on 38.36% of patients. After RT, no patients received adjuvant immune therapy.

In the validation group, 77.55% of patients had chronic viral hepatitis B, and 71.43% had liver cirrhosis. All patients were classified as Child-Pugh score A, and no patients received any adjuvant immune therapy in the initial stage of the treatment. Aside from RT, 67.35% of patients received TACE, while 40.82% of patients received targeted therapy. Supplemental Table 2 shows the detailed patient characteristics from both exploration and validation groups.

sPD-L1 levels and treatment outcomes in the exploration cohort

The Cox proportional hazards model was used to evaluate whether independent factors influence PFS and OS rates. In the case of PFS, variables with p values < 0.2 in univariate analysis were included in multivariate analysis. Multivariate analysis demonstrated that tumor number (HR = 0.44, $p = 0.006$), TACE (HR = 0.47, $p = 0.021$), sPD-L1 after RT (HR = 1.99, $p = 0.015$) and treatment response (partial remission: HR = 2.52, $p = 0.042$; stable disease: HR = 4.07, $p = 0.007$; progression disease: HR = 3.36, $p = 0.028$) were independent PFS prognostic factors. In multivariate analysis, after controlling for the known prognostic factors, only sPD-L1 after RT (HR = 2.01, $p = 0.047$) was found to be an independent prognostic factor for OS. In this study, pre-RT sPD-L1 and the levels of sPD-1 before and after RT were not shown to be prognostic variables. Detailed information about the results of univariate and multivariate analyses of PFS and OS were shown in Table 1.

Kaplan-Meier was used for survival analysis after RT, stratified by the serum sPD-L1. Fig. 2 shows that higher post-RT serum sPD-L1 level (>= 14.6 pg/ml) indicated inferior PFS and OS. The median PFS in the higher post-RT sPD-L1 group was 13.25 months and increased to 18.75 months in the lower sPD-L1 group ($p = 0.028$). The median OS in the higher and lower post-RT sPD-L1 groups was 25.03 and 36.33 months, respectively ($p = 0.033$).

Verify the prognostic role of post-RT serum sPD-L1 in the validation group

Survival analysis was done in a validation cohort to confirm the prognostic value of post-RT serum sPD-L1. After controlling for known survival predictors, multivariate analysis demonstrated that post-RT serum sPD-L1 maintained its negative independent relationship with both PFS (HR = 3.07, $p = 0.002$) and OS (HR = 2.70, $p = 0.019$) as shown in Table 2. Furthermore, in this validation cohort, pre-RT sPD-L1 and the concentrations of sPD-1 before and after RT were not significant factors.

Change of serum sPD-L1 in response to RT and the relationship between sPD-L1 and serum immune cells

To clinically validate the effect of RT on serum sPD-L1 levels, we analyzed the change of the sPD-L1 levels during the process of RT in 122 HCC patients from cohort 2. The baseline, pre-RT sPD-L1 levels (14.33 ± 11.05 pg/ml) increased two weeks after RT (19.42 ± 12.58 pg/ml) (Fig. 3A, $p < 0.001$). The serum sPD-L1 was upregulated in 76.23% of patients of all patients after RT but decreased in the last 23.77% of patients.

After a search from the database in our hospital, 79 of the 122 HCC patients in cohort 2 had blood immune cell analysis nearly at the same time as blood sample collection after RT (within 3 days). Subsequently, we assessed the relationship between post-RT sPD-L1 and the serum CD8+ T cell content. Fig. 3B shows that sPD-L1 expression was negatively correlated with cytotoxic CD8+ T cells ($R = -0.24$, $p = 0.036$).

The effect of an increased rate of post-RT sPD-L1 on short-term efficacy and long-term prognosis

Because elevated sPD-L1 caused by RT has a significant impact on prognosis, we further performed a subgroup analysis based on the increased rate of post-RT sPD-L1. All cohort 2 participants were divided into three groups according to their sPD-L1 increase rate (<50% group, 50-100% group, >100% group). As shown in Fig. 4A, the response rates in the three groups to RT (complete remission + partial remission) were 95.2%, 42.3% and 14.7%, respectively ($p < 0.001$). Further KM analysis demonstrated that higher post-RT sPD-L1 increase rate indicated poorer PFS (Fig. 4B, $p = 0.032$) and OS (Fig. 4C, $p = 0.045$).

Discussion

Significant research has been conducted over the past decades to improve our understanding of how irradiation interacts with our

Table 1
Univariate and multivariate analysis of PFS and OS in exploration cohort.

Characteristics	PFS						OS					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p
Age (>=51)	0.86	0.52 - 1.41	0.553				0.89	0.51 - 1.57	0.690			
Gender (male)	1.14	0.54 - 2.40	0.723				0.84	0.37 - 1.89	0.677			
KPS (<=80)	1.00	0.58 - 1.72	0.999				0.89	0.48 - 1.64	0.699			
Hbsag (positive)	0.65	0.36 - 1.16	0.146	0.66	0.35 - 1.24	0.193	1.14	0.55 - 2.36	0.726			
AFP (>=2290 ng/ml)	0.77	0.45 - 1.30	0.327				0.73	0.40 - 1.34	0.312			
Liver cirrhosis	1.15	0.66 - 1.98	0.622				1.18	0.64 - 2.20	0.592			
Tumor size (>=6.8 cm)	0.91	0.56 - 1.48	0.700				1.00	0.58 - 1.73	0.999			
Tumor number (single)	0.66	0.41 - 1.07	0.092	0.44	0.24 - 0.79	0.006	0.64	0.37 - 1.12	0.118	0.63	0.35 - 1.12	0.113
Thrombus (yes)	0.97	0.60 - 1.58	0.903				1.03	0.59 - 1.79	0.915			
Stage (II)	1.08	0.63 - 1.83	0.779				1.06	0.58 - 1.91	0.859			
RT modality (hypo-RT)	0.78	0.48 - 1.29	0.339				0.81	0.46 - 1.41	0.449			
BED (<71.68 Gy)	1.44	0.86 - 2.39	0.164	1.00	0.53 - 1.89	0.998	1.12	0.63 - 2.00	0.691			
PTV (<524.54 cm^3)	0.98	0.62-1.66	0.912				0.87	0.43-1.65	0.849			
TACE (yes)	0.63	0.36 - 1.09	0.099	0.47	0.25 - 0.89	0.021	0.67	0.35 - 1.29	0.227			
Targeted drugs (no)	1.15	0.70 - 1.90	0.578				1.18	0.68 - 2.06	0.556			
sPD-L1 before RT (>=16.34 pg/ml)	1.36	0.80 - 2.31	0.249				1.63	0.91 - 2.92	0.100	1.17	0.60 - 2.29	0.651
sPD-L1 after RT (>= 14.60 pg/ml)	1.76	1.06 - 2.92	0.029	1.99	1.15 - 3.46	0.015	1.88	1.04 - 3.38	0.035	2.01	1.01 - 4.02	0.047
sPD-1 before RT (>=378.54 pg/ml)	0.82	0.31 - 1.68	0.412				0.83	0.47 - 1.47	0.529			
sPD-1 after RT (>= 444.35 pg/ml)	0.53	0.30 - 0.93	0.028	0.60	0.28 - 1.26	0.176	0.74	0.40 - 1.40	0.359			
Response (pr)	2.20	1.01 - 4.79	0.046	2.52	1.03 - 6.15	0.042	1.51	0.65 - 3.50	0.334	1.86	0.79 - 4.40	0.155
Response (sd)	2.29	0.93 - 5.66	0.073	4.07	1.47 - 11.3	0.007	1.85	0.72 - 4.75	0.202	2.41	0.91 - 6.39	0.076
Response (pd)	5.62	2.07 - 15.29	0.001	3.36	1.14 - 9.89	0.028	2.42	0.83 - 7.10	0.107	2.63	0.84 - 8.18	0.100

Abbreviations:BED = biological equivalent dose; TACE = transcatheter arterial chemoembolization; pr = partial remission; sd = stable disease; pd = progression disease.

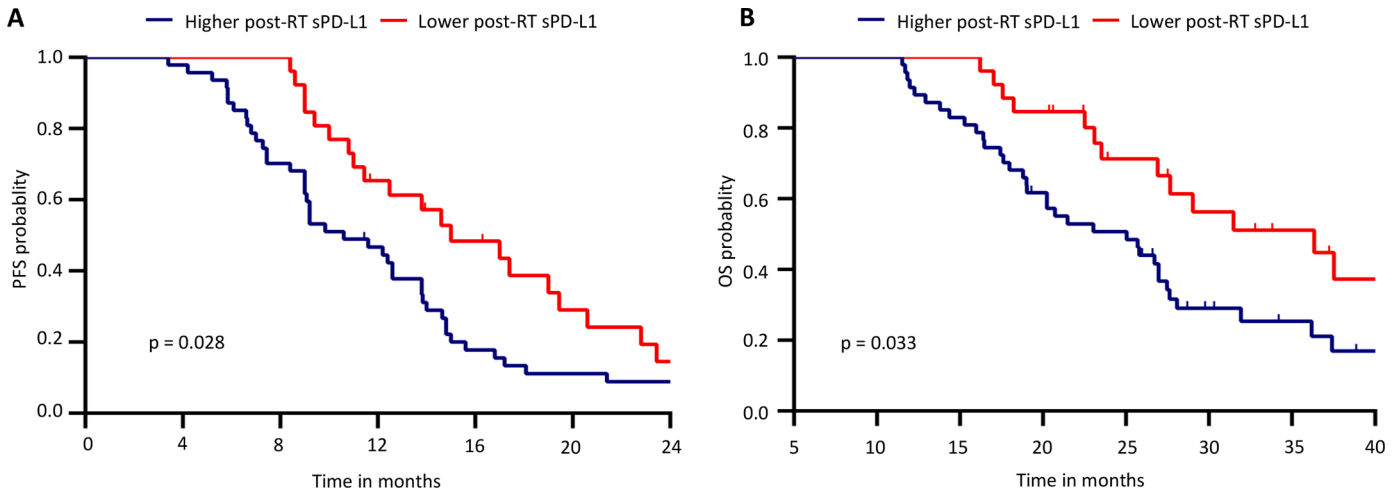

Fig. 2. Kaplan-Meier analyses of progression-free survival (PFS) and overall survival (OS) in the exploration group. Higher post-RT serum sPD-L1 concentrations (>= 14.60 pg/ml) was correlated with poor PFS (A, $p = 0.028$) and OS (B, $p = 0.033$).

Table 2
Univariate and multivariate analysis of PFS and OS in the validation cohort.

Characteristics	PFS						OS					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p
Age (>=51)	0.89	0.46 - 1.73	0.727				0.83	0.41 - 1.69	0.610			
Gender (male)	1.20	0.53 - 2.73	0.656				1.21	0.50 - 2.95	0.672			
KPS (<=80)	1.32	0.69 - 2.53	0.397				1.18	0.57 - 2.44	0.655			
Hbsag (positive)	0.42	0.20 - 0.89	0.024	0.55	0.24 - 1.26	0.156	0.32	0.15 - 0.70	0.004	0.43	0.19 - 0.97	0.041
AFP (>=2290 ng/ml)	0.85	0.44 - 1.64	0.636				0.80	0.39 - 1.67	0.556			
Liver cirrhosis	2.43	1.22 - 4.86	0.012	2.15	0.94 - 4.91	0.068	2.18	1.02 - 4.66	0.044	1.44	0.61 - 3.4	0.4006
Tumor size (>=6.8 cm)	0.45	0.23 - 0.87	0.017	0.60	0.30 - 1.18	0.139	0.47	0.23 - 0.98	0.045	0.60	0.28 - 1.29	0.1888
Tumor number (single)	1.24	0.68 - 2.24	0.486				1.26	0.65 - 2.42	0.495			
Thrombus (yes)	1.32	0.73 - 2.37	0.360				1.36	0.71 - 2.60	0.352			
Stage (II)	1.63	0.87 - 3.06	0.124	2.41	1.04 - 5.56	0.040	1.46	0.74 - 2.89	0.274			
RT modality (hypo-RT)	0.96	0.52 - 1.75	0.884				1.09	0.57 - 2.10	0.797			
BED (<71.68 Gy)	0.67	0.35 - 1.27	0.217				0.54	0.26 - 1.13	0.100	0.93	0.39 - 2.19	0.8608
PTV (<524.54 cm^3)	0.88	0.72-1.77	0.822				0.76	0.35-1.64	0.712			
TACE (yes)	1.33	0.71 - 2.48	0.371				1.20	0.61 - 2.35	0.602			
Targeted drugs (no)	1.00	0.56 - 1.82	0.987				1.04	0.54 - 1.99	0.906			
sPD-L1 before RT (>=16.34 pg/ml)	0.61	0.31 - 1.21	0.256				0.95	0.47 - 1.91	0.889			
sPD-L1 after RT (>= 14.60 pg/ml)	3.59	1.87 - 6.87	0.000	3.07	1.49 - 6.34	0.002	3.11	1.55 - 6.26	0.000	2.70	1.18 - 6.19	0.019
sPD-1 before RT (>=378.54 pg/ml)	0.70	0.35 - 1.38	0.304				1.11	0.59 - 2.11	0.738			
sPD-1 after RT (>= 444.35 pg/ml)	0.98	0.55 - 1.76	0.950				0.88	0.42 - 1.81	0.725			
Response (sd+pd)	1.95	1.07-3.56	0.030	1.93	0.86 - 4.31	0.1088	2.36	1.21 - 4.59	0.012	1.94	0.95 - 3.96	0.0705

Abbreviations:BED = biological equivalent dose; TACE = transcatheter arterial chemoembolization; pr = partial remission; sd = stable disease; pd = progression disease.

immune system and tumor microenvironment. On one hand, RT increases the infiltration of natural killer cells, effector T cells, and other leukocytes that retard tumor growth [15]. Remarkably, RT can also stimulate immunity by cGAS-STING activating dendric cells through interferon signaling and promoting the antitumor adaptive immune system [16–19]. Conversely, RT can also attract immunosuppressive cells into the tumor microenvironment, such as regulatory T cells, myeloid-derived suppressor cells, tumor-associated macrophages, and so on [3]. Meanwhile, when confronted with the threat of irradiation, the tumor attempts to adjust to avoid immune eradication. The current study found that PD-L1 expression in clinical HCC tumor tissues was upregulated after RT, which was consistent with our preclinical data. Moreover, the sPD-L1 level in serum was increased, indicating that the immunomodulatory effects of RT are not only localized but also systemic.

Immunoregulatory molecules exist as cell membranes as well as soluble forms. Many soluble co-stimulatory and co-inhibitory molecules, including sCTLA-4, sCD80, sCD86, sB7-H3, and sLAG-3, have been detected in the blood of cancer patients [20–25]. Recently, it was found that a soluble form of PD-L1 can be detected in the sera of patients. The exact source of sPD-L1 in serum was unknown at the time. Nevertheless, several studies found that the concentration of serum sPD-L1 was associated with the amount of PD-L1 expressing cells [11,13,26]. In our study, we also demonstrated a close positive correlation between them, corroborating the findings of PD-L1 in tumor tissues. The RT significantly regulated the levels of sPD-L1, with higher post-RT sPD-L1 noted poor prognosis. All these findings point to the close relationship between the two issues, however, further research is required to identify the veracious source of sPD-L1.

The immunosuppression caused by membrane PD-L1 has been well studied [27]. For instance, PD-L1 expressed on tumor cells locally inhibited CD 8+ T cell activity and protected tumor cells from eradication by the immune system [14], consistent with our finding of a negative association of sPD-L1 and CD 8+ T cells in peripheral blood. The negative associations between them have previously been discovered, which corroborates our results [13]. Furthermore, previous studies revealed that sPD-L1 in HCC patients positively correlated with sCD163 content, Treg cells, IL-10 and sIL-2R, all of which were key molecules

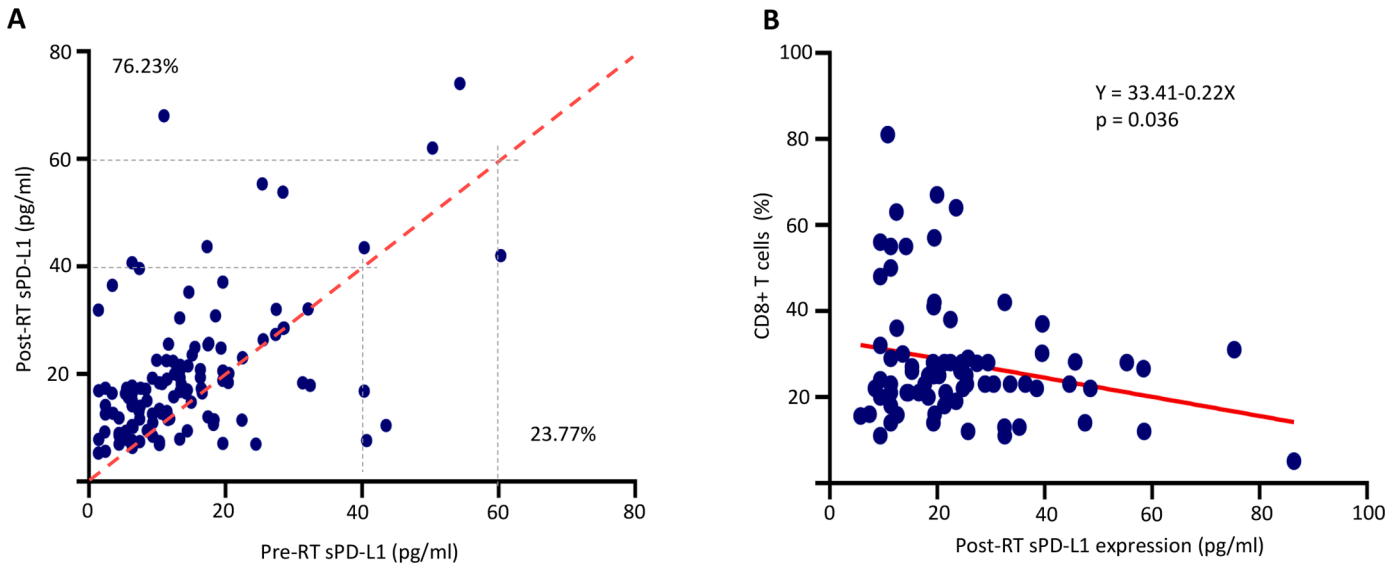

Fig. 3. (A) Changes in serum sPD-L1 levels during the RT process in 122 HCC patients of cohort 1 ($p < 0.001$). (B) The relationship between post-RT serum sPD-L1 levels and blood CD8+ T cells content in 79 HCC patients in cohort 1 (R = -0.24, p = 0.036).

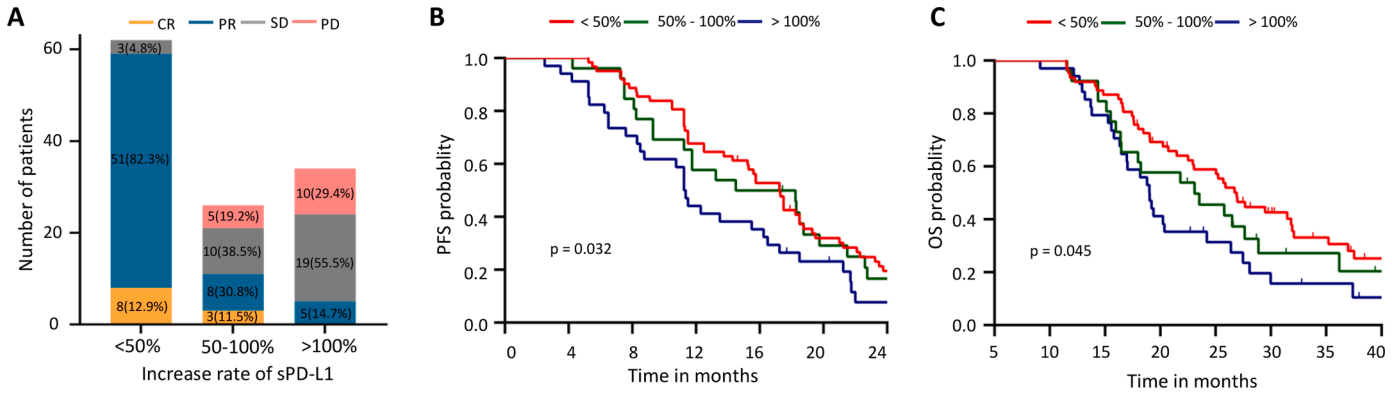

Fig. 4. Patients with higher increase rates of sPD-L1 during the process of RT noted inferior short-term response rates (A, $p < 0.001$), poor PFS (B, $p = 0.032$) and OS (C, $p = 0.045$).

participating in immune suppression [12,13]. The findings support the idea that patients with high sPD-L1 levels tend to have an immunosuppressive environment, which then hampers the host anti-tumor response, resulting in a poor prognosis after RT. These patients may benefit from a combined RT and anti-PD-L1 immunotherapy strategy.

In our study, post-RT sPD-L1 was significantly correlated with prognosis but not pre-RT sPD-L1. The interaction between sPD-L1 and tumor microenvironment always exists, however, we hypothesize that the interaction between them was relatively mild and equilibrium without any stimulation. Contrary to immunotherapy or chemotherapy, which could decrease the expression levels of sPD-L1 [28], RT significantly promotes the sPD-L1 levels upregulation. Although it was unclear whether increased sPD-L1 after RT improved the immunosuppressive effect, we did observe that patients with higher increase rates of sPD-L1 had worse short and long-term efficacy. Hence, we hypothesized that the phenomenon of post-RT sPD-L1, impairing the treatment outcome, rather than pre-RT sPD-L1, could be attributed to the amplified immunosuppressive effect induced by high sPD-L1 levels after RT. However, more research needs to be done to understand this phenomenon.

In comparison to tumor histology examination, sPD-L1 detection has its advantages of being non-invasive, low cost, easy determination, and reproducible. All these factors contribute to the use of sPD-L1, especially in HCC patients, who can be diagnosed without tissue biopsy [29]. Over the past two decades, the development of novel effective therapies and immune-based combinations has improved the management of advanced HCC [30,31]. A subset of HCC patients may benefit from the combination of RT and these systematic therapies. However, the population should be carefully selected [32]. Serum sPD-L1, as a potential surrogate biomarker for monitoring therapeutic responses, will surely enhance early decision-making and effective personalized therapy.

There are several limitations in this study as well. First, this was a single-center study and the sample size was not very large. Secondly, due to the undesirable group heterogeneity of our study, the data may be subject to confounding bias. Currently, our center is conducting a prospective, randomized, multicenter study to compare the efficacy of TACE combined with or without RT in patients with unresectable but confined liver HCC (NCT03116984). In this multicenter large clinical cohort, the role of sPD-L1 will be re-evaluated.

Conclusion

Our clinical cohort study found that sPD-L1 levels in serum after RT could be a prognostic factor for HCC patients. The outcomes of the combination of RT and anti-PD-L1 immunotherapy, particularly for HCC patients with higher post-RT sPD-L1, need to be further investigated.

Data available

The data are available from the corresponding authors on reasonable request.

CRediT authorship contribution statement

Yang Zhang: Methodology, Writing – original draft, Investigation. **Zongjuan Li:** Conceptualization, Visualization, Writing – original draft, Investigation. **Yixing Chen:** Writing – original draft, Investigation. **Ping Yang:** Data curation, Software. **Yong Hu:** Validation, Data curation. **Zhaochong Zeng:** Supervision, Conceptualization, Methodology, Writing – review & editing. **Shisuo Du:** Resources, Supervision, Conceptualization, Methodology, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Supplementary materials

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