



PD-L1 and CD47 co-expression in pulmonary sarcomatoid carcinoma: a predictor of poor prognosis and potential targets of future combined immunotherapy

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Abstract

Purpose Combined immunotherapy with anti-programmed cell death-ligand 1 (PD-L1) and an inhibitor of cluster of differentiation 47 (CD47) have exhibited preliminary anti-tumor effect. Our study attempted to describe the PD-L1/CD47 expression status in pulmonary sarcomatoid carcinoma (PSC), and explore its survival impact and relevance with cytotoxic T lymphocytes and macrophages infiltration.

Methods 148 patients with PSC who underwent surgeries were retrospectively reviewed. Tissue microarrays were conducted for immunohistochemistry (IHC) of PD-L1, CD47, CD8 and CD68.

Results 54 (36.5%) and 78 (52.7%) cases were positive for PD-L1 and CD47, respectively, and 36 (24.3%) of them demonstrated PD-L1/CD47 co-expression. There was a significant correlation between PD-L1 and CD47 expression ($P=0.011$). The median overall survival (OS) was 22.5 months (range 0.9–102.4 months). The univariate analysis demonstrated a significantly worse OS in cases with CD47 expression (hazard ratio [HR], 1.66; 95% CI, 1.14–2.42, $P=0.008$) and PD-L1/CD47 co-expression (HR, 1.75; 95% CI, 1.15–2.67, $P=0.009$). The multivariate analysis demonstrated PD-L1/CD47 co-expression (HR, 1.83; 95% CI, 1.17–2.87, $P=0.008$), T stage, M stage, completeness of resection and adjuvant therapy were independent prognostic factors for OS. There was a significant relevance between PD-L1 expression and PD-L1/CD47 co-expression with higher densities of CD8-positive T lymphocytes ($P=0.004$, 0.012, respectively) and CD68-positive macrophages ($P=0.026$, 0.034, respectively).

Conclusion We demonstrated the PD-L1/CD47 co-expression status in PSC. PD-L1 expression correlated with CD47 expression, and PD-L1/CD47 co-expression correlated with poorer prognosis and may serve as a predictive biomarker for combined dual-targeting immunotherapy in PSC patients.

Keywords Pulmonary sarcomatoid carcinoma · PD-L1 · CD47 · Prognosis · Immunotherapy

Purpose

Pulmonary sarcomatoid carcinoma (PSC) is an uncommon subtype of lung carcinoma, with an incidence of approximately 0.4% of all lung cancers, presenting a relatively worse prognosis compared to other subtypes of non-small-cell lung cancers (NSCLCs), as reported in previous studies (Yendamuri et al. 2012; Martin et al. 2007). Due to its strong aggressiveness and non-specific symptoms, the stage is often late at diagnosis, making it difficult to perform a complete resection. Moreover, the high relapse rate and the poor response to chemotherapy aggravated the intractability of PSC treatment (Martin et al. 2007; Vieira et al. 2013; Bae et al. 2007). Therefore, it is urgent to explore novel therapeutic strategies.

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Recently, checkpoint inhibitors targeting programmed cell death-1 (PD-1)/programmed cell death-ligand 1 (PD-L1) have become promising in many types of cancers, including NSCLCs (Garon et al. 2015; Herbst et al. 2014; Topalian et al. 2012). The PD-L1 expression has been utilized as a predictive biomarker, and multiple researches have demonstrated a high PD-L1 expression in PSC, as compared to other subtypes of NSCLCs (Velcheti et al. 2013; Kim et al. 2015; Vieira et al. 2016; Pecuchet et al. 2017). However, there were still at least 70% of patients who failed to obtain a satisfactory response towards anti-PD-1/PD-L1 treatment (Topalian et al. 2012), suggesting that this mono-immunotherapy was not potent enough, calling for combined therapies.

Recently, cluster of differentiation 47 (CD47), a ubiquitously expressed transmembrane glycoprotein, has been proved to suppress phagocytosis from macrophages and help tumor cells evade from immune system. Anti-CD47 therapy has been under investigation in pre-clinical and early clinical trials, demonstrating preliminary promising anti-tumor effect (Weiskopf et al. 2013; Chao et al. 2010; Gholamin et al. 2017; Sikic et al. 2019; Ansell et al. 2016). In addition, several studies have presented the synergistic anti-tumor effect of anti-PD-1/PD-L1 therapy and CD47 blockade therapy (Sokolosky et al. 2016; Ingram et al. 2017), raising the possibility and feasibility of dual-targeting PD-L1 and CD47 in clinical practice.

In this study, we attempted to summarize the clinicopathological characteristics, describe the PD-L1 and CD47 expression status in a series of PSC patients, and explore the survival impact of PD-L1/CD47 co-expression and its relevance with cytotoxic T lymphocytes and macrophages infiltration in order to briefly depict the microenvironment. This study may serve as a useful reference for selecting beneficiaries from future dual-targeting immunotherapy for PSC patients.

Methods

Patients

A total of 148 patients were diagnosed with PSC according to the 2015 World Health Organization classification and underwent intent-to-treat surgeries in Cancer Hospital, Chinese Academy of Medical Sciences between August 2008 and November 2016. The surgeries included pneumonectomy and lobectomy if the clinical condition permitted, or sublobectomy otherwise, and all patients underwent systematic or lobe-specific lymph node dissection. This study was approved by the ethics committee of National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical

College. The patients' medical records and tumor tissues were retrospectively obtained. Clinical characteristics extracted included age, gender, smoking status, family history, tumor size, T staging, N staging, M staging, TNM staging, surgical procedures, completeness of resection, postoperative treatments and overall survival. The staging criteria were based on the American Joint Committee on Cancer (AJCC) staging system (the 8th version). All 148 of the patients were followed up until Dec 20th of 2018 or until the time of death. The follow-up information was obtained according to their medical records or by getting in contact with their family.

Tissue microarray and immunohistochemical analysis

Tissue microarrays (TMAs) were manufactured and conducted for immunohistochemistry (IHC) of PD-L1, CD47, CD8 and CD68. All the tissues obtained by surgery were fixed with 10% formalin and then embedded in paraffin. Two cores of 2 mm were taken from each sample, constituting the TMA. Then, 4- μ m-thick TMA sections were prepared and incubated with the primary antibodies against PD-L1 (28-8, Abcam), CD47 (EPR21794, Abcam), CD8 (D8A8Y, CST) and CD68 (KP1, Abcam), and then with the secondary antibodies and 3,3'-diaminobenzidine (DAB). The results of IHC were examined by two independent pathologists blinded to the clinical information. PD-L1 and CD47 expression were both scored by membranous tumor proportion score (TPS), with a definition of positive as $TPS \geq 1\%$ for PD-L1 and $TPS \geq 10\%$ for CD47. Co-expression of PD-L1 and CD47 was defined as PD-L1 positive and CD47 positive. The CD8-positive tumor-infiltrating lymphocytes (TILs) and CD68-positive macrophages were averaged over the counts of six high-power fields for each case, and the density of CD8-positive TILs and CD68-positive macrophages was divided into high and low group with the median count as the cut-off value.

Statistical analysis

All data analyses were performed using SPSS version 22 software. The statistical analysis of categorical data was performed using Fisher's exact test. Overall survival (OS) was defined as the interval between the date of surgery to death of any cause or the time of last follow-up, at when patients known to be alive were censored. Survival analysis was performed using Kaplan–Meier curves, and univariate and multivariate analyses were performed using Cox proportional hazards models. $P < 0.05$ was defined as statistically significant.

Results

Clinicopathological characteristics and PD-L1/CD47 expression status

A total of 148 patients with PSC were included. The clinicopathological characteristics are demonstrated in Table 1. The median age of all patients was 62.5 years (range 36–81 years). 117 (79.1%) of them were male, and 104 (70.3%) were smokers. 107 (72.3%) patients had no tumor family history. At diagnosis, 48.6% and 5.4% of these patients had regional lymph node metastasis and distant metastases, respectively, and there were 69 (46.6%) stage I–II patients, 71 (48.0%) stage III patients and 8 (5.4%) stage IV patients. All patients underwent pulmonary surgery including 127 (85.8%) lobectomy/bilobectomy, 18 (12.2%) pneumonectomy and 3 (2.0%) sublobar resection. 142 (95.9%) patients had a complete resection (R0) and 6 (4.1%) patients had an incomplete resection (R1 and R2, or R+). 64 (43.2%) of these patients received adjuvant therapies including chemo- and radiotherapies. A total of 54 (36.5%) cases and 78 (52.7%) cases were positive for PD-L1 expression and CD47 expression, respectively, and 36 (24.3%) of them demonstrated co-expression of PD-L1/CD47. The representative immunohistochemical images for PD-L1 and CD47 are shown in Fig. 1. The association between PD-L1, CD47, and PD-L1/CD47 co-expression status and other clinicopathological factors was analyzed, demonstrating a significant correlation between CD47 and clinical stage ($P=0.028$), while no extra correlation was observed for other clinicopathological characteristics, as described in Table 1. In addition, the association between PD-L1 and CD47 expression was analyzed, demonstrating a statistically significant correlation ($P=0.011$).

The univariate and multivariate analysis of survival

Follow-up was performed for all patients. At the time of data extraction, 113 (76.4%) patients died and 35 (23.6%) were still alive. The median OS for all patients was 22.5 months (range 0.9–102.4 months). We performed the survival analysis using Kaplan–Meier curve stratified by PD-L1 expression status, CD47 expression status, and the co-expression status of PD-L1/CD47, demonstrating that positive CD47 expression (hazard ratio [HR], 1.66; 95% CI, 1.14–2.42, $P=0.008$; log-rank $P=0.007$) and PD-L1/CD47 co-expression (HR, 1.75; 95% CI, 1.15–2.67, $P=0.009$; log-rank $P=0.008$) exhibited a significantly worse prognosis, as shown in Fig. 2, while no association between PD-L1 and OS was observed (HR, 1.36; 95% CI,

0.93–1.98). Moreover, the greater tumor size (log-rank $P<0.001$), advanced T stage (T3–4) (log-rank $P<0.001$), distant metastasis (log-rank $P=0.003$), more advanced stage (log-rank $P<0.001$) and incomplete resection (log-rank $P<0.001$) also exhibited a significantly worse prognosis in univariate analysis, as shown in Fig. 2. In addition, the multivariate analyses for clinicopathological characteristics were performed, as described in Table 2, demonstrating advanced T stage (T3–4) ($P<0.001$), the presence of distant metastasis ($P=0.036$), the incomplete resection ($P=0.001$), the deficiency of adjuvant therapy ($P=0.027$), and PD-L1/CD47 co-expression (HR, 1.83; 95% CI, 1.17–2.87, $P=0.008$) were independent prognostic factors for shorter OS.

Correlation between PD-L1/CD47 expression and CD8-positive TIL density and CD68-positive macrophage density

IHC for CD8 and CD68 were performed, with the representative images shown in Fig. 3. The median CD8 and CD68 densities were 34.7 (range 0–324.3) and 20.6 (range 0–184.5), respectively, and 74 (50%) patients each were assigned to the infiltrated CD8-positive TIL-high group and infiltrated CD68-positive macrophages-high group, with the median density as the cut-off value. There was a significant association between PD-L1 expression with high CD8-positive TIL density and high CD68-positive macrophage density ($P=0.004$ and 0.026, respectively), and a significant association between PD-L1/CD47 co-expression with high CD8-positive TIL density and high CD68-positive macrophage density ($P=0.012$ and 0.034, respectively), while no significance between CD47 expression and high CD8-positive TIL density or high CD68-positive macrophage density was observed ($P=0.622$ and 0.138, respectively), as demonstrated in Table 3.

Discussion

To the best of our knowledge, this is the largest cohort of PSC patients and the first study focusing on PD-L1 and CD47 co-expression of PSC patients.

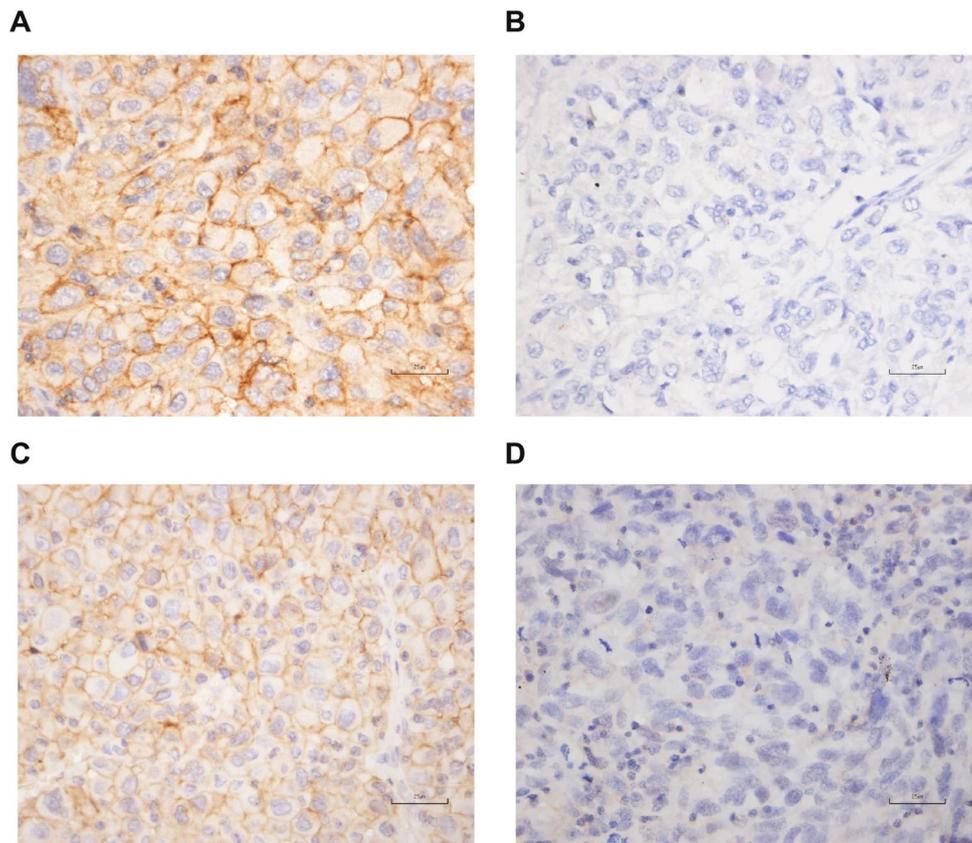
The median age, high percentage of males and smokers demonstrated in our PSC cohort were similar to previous reports (Maneenil et al. 2018; Hou et al. 2018; Lin et al. 2016; Gu et al. 2015; Huang et al. 2013; Zehani et al. 2014). At diagnosis, 5.4% of these patients had distant metastasis, nevertheless, all underwent surgeries. The reasons why patients with distant metastasis also received surgery were unexpected pleural metastasis discovered during surgery, intolerable respiratory symptoms that could not be alleviated by other interventions, and accidentally incomplete

Table 1 Association between PD-L1 and CD47 expression status and clinicopathological factors

Factors	N (%)		PD-L1, N (%)		CD-47, N (%)		PD-L1/CD47, N (%)		P
	Positive	Negative	Positive	Negative	Positive	Negative	Co-expression	Others	
Age (years)									
<60	50 (33.8%)	31 (20.9%)	19 (12.8%)	31 (20.9%)	25 (16.9%)	25 (16.9%)	13 (8.8%)	37 (25.0%)	0.840
≥60	98 (66.2%)	63 (42.6%)	35 (23.6%)	63 (42.6%)	53 (35.8%)	45 (30.4%)	23 (15.5%)	75 (50.7%)	
Gender									
Male	117 (79.1%)	71 (48.0%)	46 (31.1%)	71 (48.0%)	63 (42.6%)	54 (36.5%)	28 (18.9%)	89 (60.1%)	0.817
Female	31 (20.9%)	23 (15.5%)	8 (5.4%)	23 (15.5%)	15 (10.1%)	16 (10.8%)	8 (5.4%)	23 (15.5%)	
Smoking history									
Smokers	104 (70.3%)	65 (43.9%)	39 (26.4%)	65 (43.9%)	56 (37.8%)	48 (32.4%)	24 (16.2%)	80 (54.1%)	0.676
Non-smokers	44 (29.7%)	29 (19.6%)	15 (10.1%)	29 (19.6%)	22 (14.9%)	22 (14.9%)	12 (8.1%)	32 (21.6%)	
Tumor family history									
Yes	41 (27.7%)	23 (15.5%)	18 (12.2%)	23 (15.5%)	25 (16.9%)	16 (10.8%)	14 (9.5%)	27 (18.2%)	0.092
No	107 (72.3%)	71 (48.0%)	36 (24.3%)	71 (48.0%)	53 (35.8%)	54 (36.5%)	22 (14.9%)	85 (57.4%)	
Tumor size									
≤5 cm	90 (60.8%)	59 (39.9%)	31 (20.9%)	59 (39.9%)	45 (30.4%)	45 (30.4%)	21 (14.2%)	69 (46.6%)	0.845
>5 cm	58 (39.2%)	35 (23.6%)	23 (15.5%)	35 (23.6%)	33 (22.3%)	25 (16.9%)	15 (10.1%)	43 (29.1%)	
T									
T1–2	75 (50.7%)	48 (32.4%)	27 (18.2%)	48 (32.4%)	34 (23.0%)	41 (27.7%)	17 (11.5%)	58 (39.2%)	0.703
T3–4	73 (49.3%)	46 (31.1%)	27 (18.2%)	46 (31.1%)	44 (29.7%)	29 (19.6%)	19 (12.8%)	54 (36.5%)	
N									
N0	76 (51.4%)	50 (33.8%)	26 (17.6%)	50 (33.8%)	39 (26.4%)	37 (25.0%)	18 (12.2%)	58 (39.2%)	1.000
≥N1	72 (48.6%)	44 (29.7%)	28 (18.9%)	44 (29.7%)	39 (26.4%)	33 (22.3%)	18 (12.2%)	54 (36.5%)	
M									
M0	140 (94.6%)	89 (60.1%)	51 (34.5%)	89 (60.1%)	71 (48.0%)	69 (46.6%)	33 (22.3%)	107 (72.3%)	0.403
M1	8 (5.4%)	5 (3.4%)	3 (2.0%)	5 (3.4%)	7 (4.7%)	1 (0.7%)	3 (2.0%)	5 (3.4%)	
TNM stage									
I–II	69 (46.6%)	45 (30.4%)	24 (16.2%)	45 (30.4%)	30 (20.3%)	39 (26.4%)	14 (9.5%)	55 (37.2%)	0.414
III	71 (48.0%)	44 (29.7%)	27 (18.2%)	44 (29.7%)	41 (27.7%)	30 (20.3%)	19 (12.8%)	52 (35.1%)	
IV	8 (5.4%)	5 (3.4%)	3 (2.0%)	5 (3.4%)	7 (4.7%)	1 (0.7%)	3 (2.0%)	5 (3.4%)	
Total									
	140 (94.6%)	94 (63.5%)	54 (36.5%)	94 (63.5%)	78 (52.7%)	70 (47.3%)	36 (24.3%)	112 (75.7%)	

PD-L1 programmed cell death-ligand 1, CD47 cluster of differentiation 47

Fig. 1 Representative images of immunohistochemical staining for PD-L1 and CD47 in tissue microarrays from patients with pulmonary sarcomatoid carcinoma. **a** Positive membrane staining for PD-L1. **b** Negative membrane staining for PD-L1. **c** Positive membrane staining for CD47. **d** Negative membrane staining for CD47. Scale bar: 25 μ m. PD-L1, programmed cell death-ligand 1; CD47, cluster of differentiation 47



pre-surgical examination. The median OS of our cohort was 22.5 months, which was slightly longer than the median survival range of 3.5–19 months reported by previous studies (Maneenil et al. 2018; Hou et al. 2018; Lin et al. 2016; Vieira et al. 2016; Huang et al. 2013), which might be explained by a lower percentage of stage IV patients (5.4%), and all patients in our cohort underwent surgeries. T stage and M stage were demonstrated to be independent prognostic factors instead of N stage, consistent with previous studies (Maneenil et al. 2018; Hou et al. 2018; Yuki et al. 2007). The possible reason why N stage could not independently influence prognosis might be explained by the high percentage of vascular invasion regardless of lymphatic invasion (Yuki et al. 2007; Suda et al. 2014; Vieira et al. 2014; Lococo et al. 2017a). It was expected that the incomplete resection would lead to a worse clinical outcome because of the residual disease, and our result did demonstrate that R+ status was an independent prognostic factor, consistent with previous study (Lococo et al. 2017a). Adjuvant therapy was also an independent prognostic factor for longer OS, consistent with previous studies (Martin et al. 2007; Maneenil et al. 2018; Hou et al. 2018), suggesting postoperative adjuvant treatment should be applied in PSC. However, the existence of several confounding selection biases in the adjuvant setting should be considered, for example, frail patients were usually not addressed to adjuvant treatments.

In consideration of the high mutation rates suggesting a strong tumor immunogenicity reported by previous study (Vieira et al. 2016; Fallet et al. 2015) and the absence of efficient treatment options for PSC, novel therapeutic strategies such as immunotherapy should be taken into account. A previous study has exhibited a significant correlation between tumor mutation load and PD-L1 expression (Lococo et al. 2017b), which has been reported to be a predictive biomarker to anti-PD-1/PD-L1 therapies, further indicating the theoretical feasibility of this type of therapy in PSC. Several previous studies have demonstrated that PD-L1 expression in PSC is higher than other subtypes of NSCLCs, exhibiting a positive PD-L1 rate of 69.2% (9/13), 90.2% (37/41), and 53% (40/75), respectively (Velcheti et al. 2013; Kim et al. 2015; Vieira et al. 2016). In our cohort, 36.5% of patients had positive expression of PD-L1, which seems to be lower than previous studies. The reason might be the limited sample size of previous studies and the discrepancies between TMAs and sections from surgical specimens. The univariate analysis demonstrated that PD-L1 was not associated with any clinicopathological characteristics and could not influence prognosis either, which was consistent with previous studies (Velcheti et al. 2013; Kim et al. 2015). Although immune checkpoint blockade has exhibited a promising and durable anti-tumor activity, a large proportion of patients still failed to benefit from anti-PD-1/PD-L1 treatment

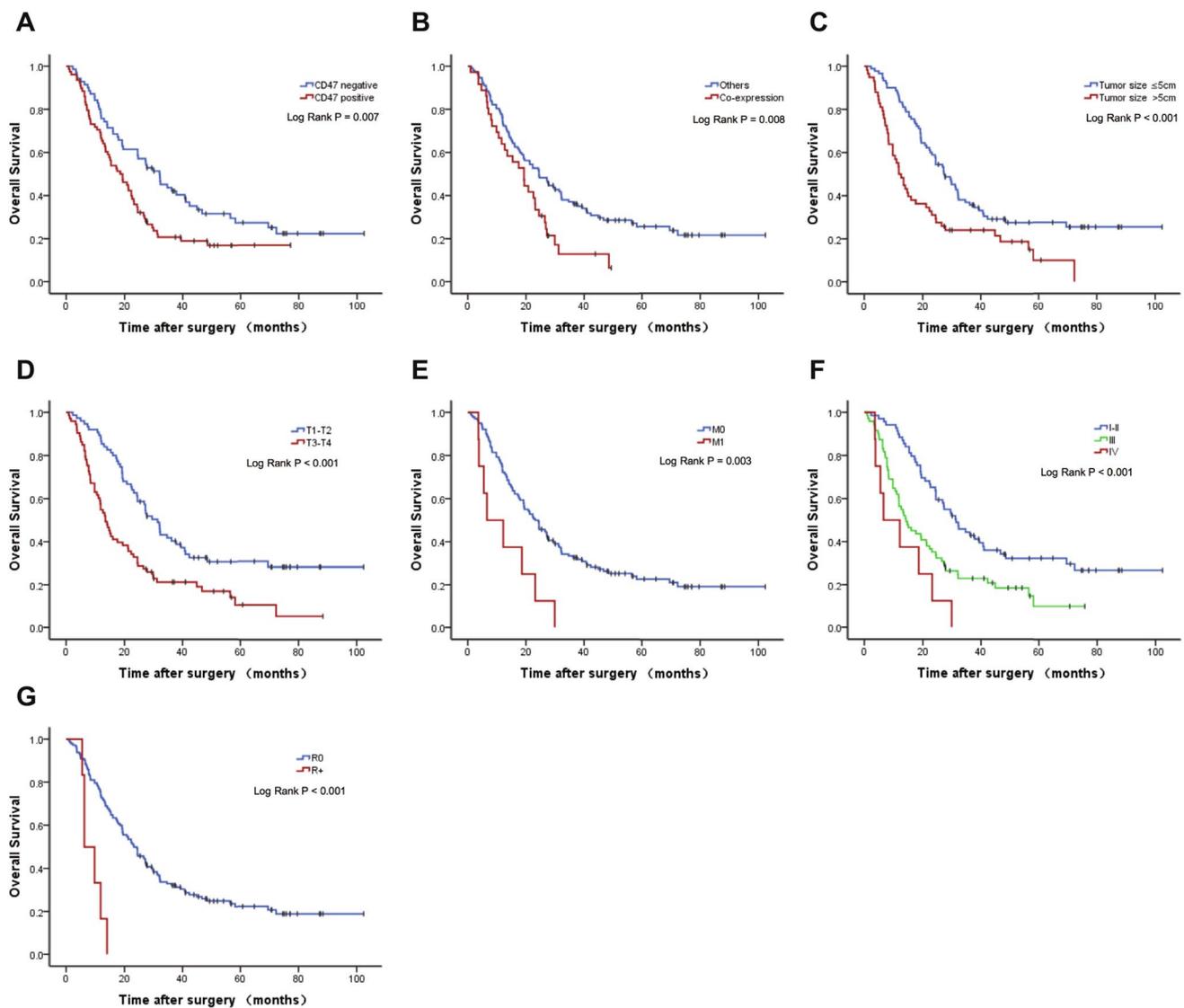


Fig. 2 Kaplan–Meier curves demonstrating OS of patients with pulmonary sarcomatoid carcinoma according to CD47 expression status (a), PD-L1/CD47 co-expression status (b), tumor size (c), T stage

(d), M stage (e), TNM stage (f) and completeness of resection (g). OS overall survival, CD47 cluster of differentiation 47, PD-L1 programmed cell death-ligand 1

(Topalian et al. 2012), suggesting that anti-PD-1/PD-L1 monotherapy was not potent enough.

Recently, several novel targets were identified, such as CD47 (also named integrin-associated protein, or IAP), a transmembrane glycoprotein expressed in normal cells that can provide a “do not eat me” signal by binding to the signal regulatory protein alpha (SIRP α) on macrophages and dendritic cells and suppress phagocytosis (Weiskopf et al. 2013). Therefore, CD47 on tumor cells has played an important role in immune escape in progression of tumor. In our cohort, 52.7% of the patients had a positive expression status of CD47. Further analysis demonstrated that CD47 expression status was associated with clinical stage, and notably, stage IV patients had an obviously higher CD47 positive

rate (7/8, 87.5%). Zhao et al. (2016) reported that CD47 expression in NSCLC patients significantly associated with clinical staging, while lymph node metastasis and distant metastasis were also relevant factors in their study. Moreover, the univariate analysis exhibited a significant association between CD47 expression and worse prognosis. Yoshida et al. (2015) also observed a significantly lower overall 5-year survival rate for CD47-positive patients than CD47-negative ones in gastric cancer. Several studies have exhibited a negative impact of CD47 expression on prognosis (Li et al. 2017; Galli et al. 2015; Xiao et al. 2015; Weiskopf et al. 2016). Recently, several anti-CD47 inhibitors including mono-antibodies and bispecific antibodies have been under investigation in patients with leukemia, myelodysplastic

Table 2 The univariate and multivariate analyses of factors associated with overall survival

Variable		Univariate analysis		Multivariate analysis	
		HR (95% CI)	<i>P</i> value	HR (95% CI)	<i>P</i> value
Age	< 60	0.85 (0.57–1.26)	0.420	1.05 (0.66–1.67)	0.828
	≥ 60				
Gender	Male	1.02 (0.65–1.59)	0.940	0.94 (0.46–1.92)	0.869
	Female				
Smoking status	Smoker	1.16 (0.77–1.75)	0.470	1.33 (0.69–2.56)	0.395
	Non-smoker				
Tumor family history	Yes	0.95 (0.63–1.43)	0.794	0.87 (0.56–1.33)	0.506
	No				
Tumor size	≤ 5 cm	0.51 (0.35–0.74)	<0.001		
	> 5 cm				
T stage	T1–2	0.48 (0.33–0.70)	<0.001	0.46 (0.31–0.67)	<0.001
	T3–4				
N stage	N0	0.71 (0.49–1.03)	0.071	0.71 (0.48–1.06)	0.093
	≥N1				
M stage	M0	0.35 (0.17–0.72)	0.004	0.42 (0.19–0.95)	0.036
	M1				
TNM stage	I+II	0.24 (0.11–0.52)	<0.001		
	III	0.49 (0.23–1.04)	0.062		
	IV				
Completeness of resection	R0	0.20 (0.08–0.47)	<0.001	0.22 (0.09–0.54)	0.001
	R+				
Treatment	Surgery	1.25 (0.86–1.81)	0.252	1.65 (1.06–2.58)	0.027
	Surgery plus adjuvant therapy				
PD-L1	Positive	1.36 (0.93–1.98)	0.116		
	Negative				
CD47	Positive	1.66 (1.14–2.42)	0.008		
	Negative				
PD-L1/CD47	Co-expression	1.75 (1.15–2.67)	0.009	1.83 (1.17–2.87)	0.008
	Others				

PD-L1 programmed cell death-ligand 1, *CD47* cluster of differentiation 47

syndrome (MDS) and advanced solid tumors, with some coming into early-phase clinical trials including Hu5F9-G4 and CC-90002, etc., demonstrating preliminary promising therapeutic effect (Chao et al. 2010; Gholamin et al. 2017; Sikic et al. 2019; Ansell et al. 2016).

As the investigation on immunotherapy goes further, scientists discovered that the reason why certain proportion of patients could not benefit from anti-PD-1/PD-L1 treatment might be the failure in tumor-specific T lymphocytes priming (Lesokhin et al. 2015) in those patients other than the lack of ideal predictive biomarkers that could screen for more suitable beneficiaries. Desirably, while anti-PD-1/PD-L1 treatment plays a role in the adaptive immunity, CD47 as an innate-immunity checkpoint has drawn great attention and exhibited appealing potential in combined immunotherapy with anti-PD-1/PD-L1 treatment. Recently,

several underlying mechanisms of dual-targeting treatment have been revealed. In addition to the synergetic function in suppressing tumor evasion from immune system, CD47-SIRP α binding on dendritic cells could inhibit tumor antigen presentation to CD8-positive T cells (Latour et al. 2001; Liu et al. 2015) and the binding on macrophages could inhibit the action of effector T cells (Weiskopf 2017), therefore anti-CD47 treatment could act as a T cell recruiter and solve the obstacle of lacking tumor-specific T cells priming in mono-immunotherapy. Afterwards, several scientists have proved the synergistic effect of anti-PD-L1 treatment and CD47 blockade therapy (Sokolosky et al. 2016; Ingram et al. 2017). Recently, the development of fusion protein has been under active investigation, and the predictive biomarker which could identify possible beneficiaries would be one of the most important orientations in future researches.

Fig. 3 Representative images of immunohistochemical staining for CD8 and CD68 in tissue microarrays from patients with pulmonary sarcomatoid carcinoma. **a** High density of CD8-positive lymphocytes infiltration. **b** Low density of CD8-positive lymphocytes infiltration. **c** High density of CD68-positive macrophages infiltration. **d** Low density of CD68-positive macrophages infiltration. Scale bar: 25 μ m. *CD8* cluster of differentiation 8, *CD68* cluster of differentiation 68

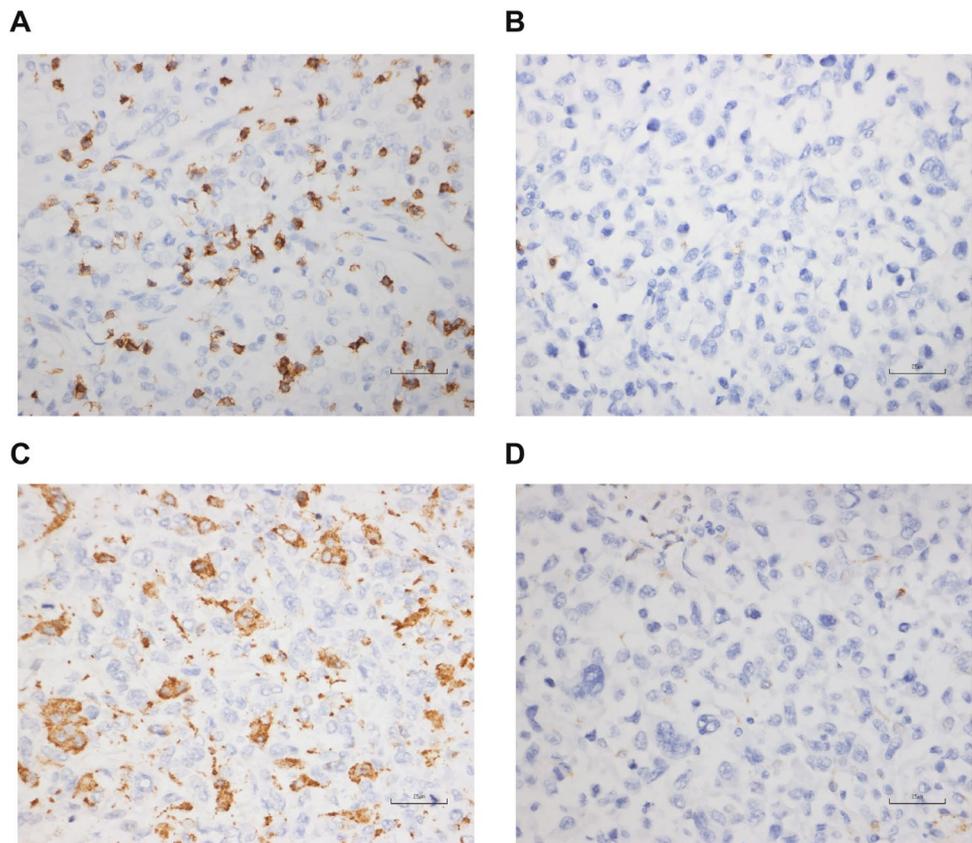


Table 3 Associations between CD8-positive T cells and CD68-positive macrophages density with PD-L1 expression, CD47 expression, and PD-L1/CD47 co-expression

Factors	N (%)	PD-L1, N (%)			CD47, N (%)			PD-L1/CD47, N (%)			
		Positive	Negative	P value	Positive	Negative	P value	Co-expression	Others	P value	
CD8	Low	74 (50%)	18 (12.2%)	56 (37.8%)	0.004	37 (25.0%)	37 (25.0%)	0.622	11 (7.4%)	63 (42.6%)	0.012
	High	74 (50%)	36 (24.3%)	38 (25.7%)		41 (27.7%)	33 (22.3%)		25 (16.9%)	49 (33.1%)	
CD68	Low	74 (50%)	20 (13.5%)	54 (36.5%)	0.026	34 (23.0%)	40 (27.0%)	0.138	12 (8.1%)	62 (41.9%)	0.034
	High	74 (50%)	34 (23.0%)	40 (27.0%)		44 (29.7%)	30 (20.3%)		24 (16.2%)	50 (33.8%)	

PD-L1 programmed cell death-ligand 1, *CD47* cluster of differentiation 47, *CD8* cluster of differentiation 8, *CD68* cluster of differentiation 68

In our cohort, we analyzed co-expression rate of PD-L1 and CD47, in consideration of the potential possibility of co-expression status as predictive marker for future next-generation immunotherapies, and we presented 24.3% of the patients had co-expression of PD-L1 and CD47, and there was a significant correlation between the expression of PD-L1 and CD47 ($P=0.011$). The mutual correlation was consistent with the study of Casey et al. (2016) which found that MYC was an important oncogenic transcription factor that could simultaneously regulate elevated expression levels of PD-L1 and CD47 on the surface of tumor cells, therefore again exhibiting the theoretically feasible application prospect of dual-targeting therapy. We also demonstrated that the PD-L1/CD47 co-expression was significantly relevant with

a worse prognosis in either univariate ($P=0.009$) or multivariate analysis ($P=0.008$), which could be explained by the synergetic function of immune evasion. Several studies have demonstrated co-expression status of PD-L1 and CD47 in cell lines and in some melanoma patients in clinic (Liu et al. 2018a, b), while no enough data could be searched on the co-expression status in real-world and larger-scaled populations to the best of our knowledge. These results suggested that quite a proportion of patients with PSC might have the opportunity to benefit from dual-targeting immunotherapy in the future, if co-expression status could be proved to be a predictor.

Moreover, we analyzed expression status of CD8 and CD68, as the specific marker of killer T cells and

macrophages, respectively, in order to briefly depict the immune microenvironment. We demonstrated a significant relevance of PD-L1 expression with higher CD8-positive TIL density and CD68-positive macrophage density ($P = 0.004$, 0.026 , respectively), and PD-L1/CD47 co-expression with higher CD8-positive TIL density and CD68-positive macrophage density ($P = 0.012$, 0.034 , respectively), while no association between CD47 expression and CD8-positive TIL or CD68-positive macrophage density was observed. The significant relevance between PD-L1 and CD8 was consistent with previous studies. Kim et al. (2015) and Vieira et al. (2016) exhibited more CD8-positive T lymphocyte infiltration in PD-L1-positive PSCs in comparison with PD-L1-negative ones ($P = 0.006$ and 0.001 , respectively). This correlation could be explained by an underlying mechanism that CD8 T lymphocytes could increase PD-L1 expression by releasing interferon- γ (Theate et al. 2015; Zhai et al. 2015). The significant relevance between PD-L1 and macrophages was consistent with previous studies, which exhibited PD-L1-positive PSC patients had a significantly higher infiltration of CD163-positive macrophages compared to PD-L1-negative ones ($P = 0.002$) (Kim et al. 2015; Vieira et al. 2016). Gordon et al. (2017) has demonstrated that infiltrated tumor-associated macrophages (TAMs) could be induced to express PD-1, and the binding to PD-L1 on tumor cells would suppress the phagocytic potency of TAMs. Therefore, infiltrated macrophages, just as infiltrated cytotoxic T lymphocytes, might have a function of immuno-screening, which could induce tumor cells to develop adaptability in immune microenvironment, thus resulting in an association between PD-L1 and higher density of infiltrated macrophages and cytotoxic T lymphocytes, and the same hypothesis could also explain those associations in PD-L1/CD47 co-expression cases. Notably, PSC has been demonstrated to harbor high mutation rates, suggesting a strong tumor immunogenicity, resulting in rich inflammatory reaction where high amount of infiltrated immune cells could be observed. Therefore, PD-L1 and CD47 co-expression might be common in PSC patients, which might be a predictive biomarker for future combined dual-targeting immunotherapy.

There are some limitations of this study. First of all, this was a retrospective study which was conducted in a single institution, and there were no patients receiving immunotherapy otherwise the prognostic and predictive value of PD-L1 and CD47 co-expression would be more reliable. Secondly, we used TMAs instead of sections from surgical samples, several results might be under-estimated due to the limited tumor region we selected, and several detailed pathological characteristics could not be obtained, such as vascular and lymphatic emboli status. Thirdly, there were no definitive guidelines for antibody selection, interpretation criteria, or cut-off value of CD47 expression, which should

be further determined in future investigations. However, these results still exhibited instructive meanings and might give a clue for designing future prospective clinical trials.

Conclusion

We summarized the clinicopathological characteristics and described the PD-L1/CD47 expression status in a large cohort of PSC patients. PD-L1/CD47 co-expression was an independent prognostic factor and was associated with high CD8 + TILs and macrophages densities. PD-L1/CD47 co-expression may serve as a predictive biomarker for combined dual-targeting immunotherapy. Future multicentered prospective trials are needed for further exploration.

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Compliance with ethical standards

Conflict of interest No conflict of interest was declared.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the ethics committee of National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College (18-224/1782) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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