

## Original Article

# Expression of programmed death-1 ligand (PD-L1) in tumor-infiltrating lymphocytes is associated with favorable spinal chordoma prognosis

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Received January 18, 2016; Accepted May 30, 2016; Epub July 15, 2016; Published July 30, 2016

**Abstract:** Aberrant expression of programmed death-1 (PD-1) receptor/PD-1 ligand (PD-L1) proteins alters human immunoresponse and promotes tumor development and progression. We assessed the expression status of PD-1 and PD-L1 in spinal chordoma tissue specimens and their association with clinicopathological characteristics of patients. Formalin-fixed paraffin-embedded tumor samples from 54 patients with spinal chordoma were collected for immunohistochemical analysis of PD-1 and PD-L1 expression. The association of the expression levels of PD-1 and PD-L1 with clinicopathological variables and survival data were statistically analyzed. Lymphocyte infiltrates were present in all 54 patient samples. Of 54 samples, 37 (68.5%) had both positive PD-1 and PD-L1 expression in tumor cell membrane. Moreover, 38 (70.4%) and 12 (22.2%) had positive PD-1 and PD-L1 expression in tumor-infiltrating lymphocytes (TILs), respectively. Tumors with positive PD-L1 expression were significantly associated with advanced stages of chordoma ( $p = 0.041$ ) and TIL infiltration ( $p = 0.005$ ), and had a borderline association with tumor grade ( $p = 0.051$ ). However, positive tumor PD-L1 expression was not significantly associated with local recurrence-free survival (LRFS) or overall survival (OS). PD-1 expression in TILs was associated with poor LRFS ( $\chi^2 = 10.051$ ,  $p = 0.002$ , log-rank test). Multivariate analysis showed that PD-L1 expression only in TILs was an independent predictor for LRFS (HR = 0.298, 95% CI: 0.098-0.907,  $p = 0.033$ ), and OS (HR = 0.188, 95% CI: 0.051-0.687,  $p = 0.011$ ) in spinal chordoma patients. In conclusion, PD-L1 expression in TILs was an independent predictor for both LRFS and OS in spinal chordoma patients. Our findings suggest that the PD-1/PD-L1 pathway may be a novel therapeutic target for the immunotherapy of chordoma.

**Keywords:** Spinal chordoma, tumor-infiltrating lymphocytes, PD-1, PD-L1, prognosis

## Introduction

Chordoma is a very rare mesenchymal tumor with a low to intermediate malignant grade [1], and accounts for 1-4% of all bone malignancies with an incidence rate of less than 1 case per million [2, 3]. Clinically, chordoma usually responds poorly to chemotherapeutic agents [4, 5] and conventional radiotherapy [6-8]. To date, treatment of chordoma with en bloc resection and wide resection margins offers the best chance of the long-term disease control [1, 5, 6, 8, 9]. However, to accomplish a radical surgical resection of chordoma lesions remains technically challenging, as the tumor is often adjacent to vital neurovascular structures

with poor margins and invasion into surrounding soft tissues [9]. Importantly, surgery has the potential to lead to a high risk of tumor recurrence [5, 10, 11] and 5-40% of patients develop metastases following surgery [12-16]. Patients with a metastatic disease will have only about 1-year median survival time [12, 17]. Thus, the development of novel therapeutic strategies is highly needed for these patients.

Programmed cell death 1 (PD-1), expressed in various immune cells, is a member of the B7-CD28 receptor family and can negatively regulate T-cell function, survival, and expansion [18-20]. This inhibitory signal is mediated by interaction with the co-stimulatory PD-1 ligands

## PD-L1 expression in TILs predicts prognosis of spinal chordoma

**Table 1.** Characterization of patients

Characteristic	Number of patients (%)
Age (range) in years	55.6 (23-79)
Gender	
Male	35 (64.8)
Female	19 (35.2)
Tumor size (cm)	6.3 (3-12)
Tumor location	
Sacral vertebra	42 (77.8)
Cervical vertebra	6 (11.1)
Thoracic vertebra	4 (7.4)
Lumbar vertebra	2 (3.7)
Surrounding muscle invasion	
Yes	35 (64.8)
No	19 (35.2)
Preoperative recurrence	
Yes	11 (20.4)
No	43 (79.6)
Tumor grade	
High	38 (70.4)
Low	16 (29.6)
Tumor stage	
IA	13 (24.1)
IB	6 (11.1)
IIA	4 (7.4)
IIB	27 (50)
III	4 (7.4)
Type of resection	
EI	17 (31.5)
EA	37 (68.5)
Tumor hemorrhage	
No	10 (18.5)
Yes	44 (81.5)
Tumor necrosis	
Absent	13 (24.1)
Mild	18 (33.3)
Moderate	15 (27.8)
Severe	8 (14.8)
Level of TILs	
Absent	0 (0)
Rare/few	23 (42.6)
Moderate	15 (27.8)
Prominent	16 (29.6)
Ki-67 staining index	
Low	25 (46.3)
High	29 (53.7)

EI, Enneking inappropriate; EA, Enneking appropriate; TILs, tumor-infiltrating lymphocytes.

(PD-L) [21], PD-L1 and PD-L2, which represents a mechanism allowing tumor cells to escape the host's immune response [22, 23]. Previous studies have shown that PD-L1 is expressed in different cancers [24-28] and its expression is associated with poor prognosis of cancer patients [29, 30]. Furthermore, tumor-infiltrating lymphocytes (TILs) express PD-1 and are associated with a high level of PD-L1 in various types of cancers [24, 31-34]. In addition, intratumoral infiltration of PD1-positive T-cells is positively associated with tumor progression [21]. Previous clinical trials showed promising results by targeting the PD-1/PD-L1 signaling pathway using monoclonal antibodies in several human cancers [35-37]. Emerging data indicate that the PD-1/PD-L1 pathway may be deregulated in chordoma [31, 38], suggesting that targeting the PD-1/PD-L1 signaling pathway is a promising strategy for the treatment of chordoma. However, to avoid bias [39], statistical analysis of the multivariate adjustment of the expression status of PD-1/PD-L1 with clinical outcome is essential. Furthermore, it is important to systematically investigate the level of intratumoral and TILs-associated PD-1 expression, and their association with clinicopathological features or outcome of chordoma patients.

In this study, we assessed PD-1 and PD-L1 expression in chordoma tissue specimens and in TILs as well as their association with clinicopathological data and local relapse-free survival (LRFS) and overall survival (OS) of chordoma patients.

### Material and methods

#### *Patients and tissue samples*

In this study, we collected 54 tissue specimens from spinal chordoma patients who were surgically treated in the Department of Spine Surgery, The Second Xiangya Hospital, Central South University (Changsha, China) between June 2002 and April 2015. We retrospectively reviewed the clinicopathological characteristics, including age, gender, tumor size, location, tumor grade, stage, surrounding muscle invasion, preoperative recurrence, type of resection, tumor hemorrhage and necrosis, from patients' medical records (**Table 1**). Regarding that previous treatment other than surgery

may influence the expression of the proteins of interest, we reviewed the complete patients' medical records, excluding those who had previously received any types of tumor-specific therapy, such as chemotherapy or radiotherapy. Tumor grade and stage were evaluated according to the Enneking staging system for the surgical staging of malignant bone and soft tissue tumors [1, 8, 40]. Resected tumor specimens were evaluated by anatomic pathologists and recorded as Enneking appropriate or Enneking inappropriate according to the Enneking principles [41]. Tumor-muscle invasion was confirmed by preoperative magnetic resonance images and histology [42]. Tumor recurrence was recorded in patients who had previously received tumor resection and relapsed on admission.

Immediately following surgery, tissue samples from these patients were immediately fixed in 10% buffered formalin and embedded in paraffin. Formalin-fixed paraffin-embedded blocks from these 54 chordoma patients were retrieved from the Department of Pathology and sectioned to 4- $\mu$ m thick tissue sections for immunohistochemistry. Tumor diagnosis was made on histological examination of hematoxylin and eosin-stained tissue sections according to criteria described in a previous study [43], and performed by two pathologists who confirmed the diagnosis of chordoma of the conventional subtype in all patients. This study was approved by the hospital ethical committee of The Second Xiangya Hospital, Central South University, and informed consent was obtained from each patient before participation in this study.

### *Follow-up of patients*

The patients received clinical and radiographical follow-up at three-month intervals over the first two years, then every six months for three years, and annually thereafter. Local recurrence of tumor was diagnosed from clinical manifestations and imaging finding during follow-up or histology of the second surgery [44]. Events were defined as the first evidence of local recurrence for LRFS or death related to any cause for OS. All patients were followed until September 2015 and observations were censored when a patient was tumor free (LRFS analysis) or was alive (OS analysis) at the time of last clinical follow-up (September 2015).

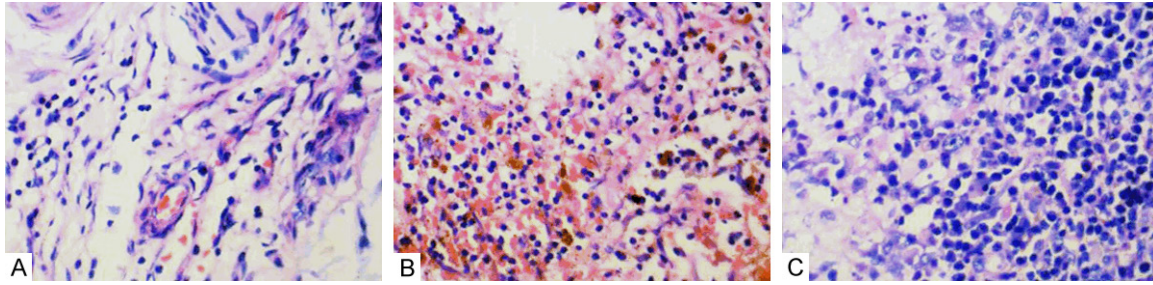
### *Immunohistochemistry*

Paraffin sections were deparaffinized twice in xylene and rehydrated in a series of ethanol and then subjected to antigen retrieval in microwave in 0.01 M citrate buffer, pH 6.0 at 121°C for 15 min. Next, the sections were incubated in 3% H<sub>2</sub>O<sub>2</sub> in methanol for 15 min to quench potential endogenous peroxidase activity. After being rinsed with phosphate buffer solution (PBS), the section were incubated with 10% normal goat serum to block non-specific binding at room temperature for 30 min and then with primary anti-PD-1 antibody (#ab137132, Abcam, Cambridge, MA, USA) at a dilution of 1:400, anti-PD-L1 antibody (#ab174838, Abcam) at a dilution of 1:50, and anti-Ki-67 (#ab16667, Abcam) at a dilution of 1:100 at 4°C overnight. On the next day, the sections were washed with PBS thrice and incubated with biotinylated goat anti-rabbit immunoglobulin and subsequently with a streptavidin-peroxidase conjugate (Auragene, Changsha, Hunan, China). Antibody binding was visualized using 3,3'-diaminobenzidine solution, counterstained briefly with hematoxylin and mounted. Negative control sections were incubated with PBS instead of the primary antibody, and positive control sections were from human tonsil tissues according to manufacturer instructions.

### *Evaluation of immunohistochemistry*

The immunostained sections were reviewed and scored under a microscope by two well-experienced pathologists (JY and SXL) who had no any prior information on clinical data. For PD-1 and PD-L1 expression in tumor tissues, positive staining was scored if tumor cells with membrane staining constituted  $\geq$  5% of all tumor cells, as previously described [27]. TILs were evaluated in hematoxylin and eosin-stained sections and scored as absent (0), rare/few (1), moderate (2), and prominent (3), similar to previous studies [27, 31]. The tissue specimens were classified as negative (score 0-1) and positive (score 2-3). PD-L1 and PD-1 expression was then scored in TILs using the same scoring scale (0-3) and samples with a score of 2-3 were considered PD-L1-positive or PD-1-positive according to the methods described in a previous study [27].

Ki-67 expression index in tumor tissues was scored as percentage of cells with cell nuclear



**Figure 1.** Representative illustration of tumor-infiltrating lymphocytes (TILs) in chordoma tissues (HE × 400). A. Rare/few TILs; B. Moderate TILs; C. Prominent TILs.

staining and divided into low (< 10% positivity) and high expression ( $\geq 10\%$ ) according to a previous study [42].

#### Statistical analysis

All statistical analyses were carried out using SPSS 17.0 (SPSS, Chicago, IL, USA). The  $\chi^2$  test or Wilcoxon rank sum test were used to analyze the association of PD-1 and PD-L1 expression in tumor cell membrane or TILs with clinicopathological characteristics from spinal chordoma patients, where appropriate. LRFS and OS were calculated by the Kaplan-Meier method, and univariate survival analysis was performed by the log-rank test. Multivariate analysis was performed with a Cox proportional hazard model to assess whether PD-1 or PD-L1 expression independently predicted the outcome with the inclusion of factors determined to be significant by univariate analysis. All tests were two-sided and a  $p$  value  $\leq 0.05$  was considered statistically significant.

## Results

### Patient characteristics

All clinicopathological data are summarized in **Table 1**. Briefly, 35 were males and 19 females with a mean age of 55.59 years old (ranging between 23 and 79 years old). There were 43 patients with primary chordoma and 11 with recurrent chordoma (**Table 1**). All patients were followed up until September 2015, with a mean follow-up period of 42.39 months (ranging between 5 and 158 months).

### All spinal chordoma tissue specimens have tumor-infiltrated lymphocytes

Tumor-infiltrated lymphocytes (TILs) in tumor lesions were assessed in tissue sections after

HE staining and showed positive in all of these 54 cases of patients (**Figure 1**). The levels of TILs were as follows: rarely or a few in 23 (42.6%), moderate in 15 (27.8%), and prominent in 16 (29.6%) cases (**Table 1**).

### Expression of PD-1 and PD-L1 in tumor cells and in TILs

PD-1 was not expressed in the tumor cells of 17 patients (31.5%) and expressed in 37 patients (68.5%) (**Table 2** and **Figure 2A, 2B**). In contrast, PD-1 was expressed in TILs in all 54 cases. Specifically, PD-1 level in TILs was scored as rare/few (1) in 15 patients (27.8%), moderate (2) in 20 patients (37.0%), and prominent (3) in 19 patients (35.2%) (**Figure 2C-E**). In summary, PD-1 expression level in TILs was considered negative (0 or 1) in 16 (29.6%) and positive (2 or 3) in 38 (70.4%) cases (**Table 2**).

PD-L1 protein was not expressed in tumor cells in 17 patients (31.5%), but was expressed in 37 patients (68.5%) (**Table 2** and **Figure 3A, 3B**). PD-L1 expression level in TILs was scored as absent (0) in 0 (0%), rare/few (1) in 24 (44.4%), moderate (2) in 18 (33.3%), and prominent (3) in 12 (22.2%) cases (**Figure 3C-E**). In summary, PD-L1 expression level in TILs was negative (0 or 1) in 42 (77.8%) and positive (2 or 3) in 12 (22.2%) cases (**Table 2**).

### Association of PD-1/PD-L1 expression with TILs in chordoma tissue specimens

Tumors with PD-L1 positive expression were more likely to have lymphocytes tumor infiltration (OR = 5.673, 95% CI: 1.611-19.981,  $p = 0.005$ ; **Table 2**). There was a borderline trend for patients with intratumoral PD-1-positive immune cells to have lymphocyte tumor infiltration (OR = 3.205, 95% CI: 0.952-10.790,  $p = 0.055$ ; **Table 2**). However, there was no statisti-

PD-L1 expression in TILs predicts prognosis of spinal chordoma

**Table 2.** Association between PD-1/PD-L1 expression and clinicopathological features of 54 spinal chordoma patients

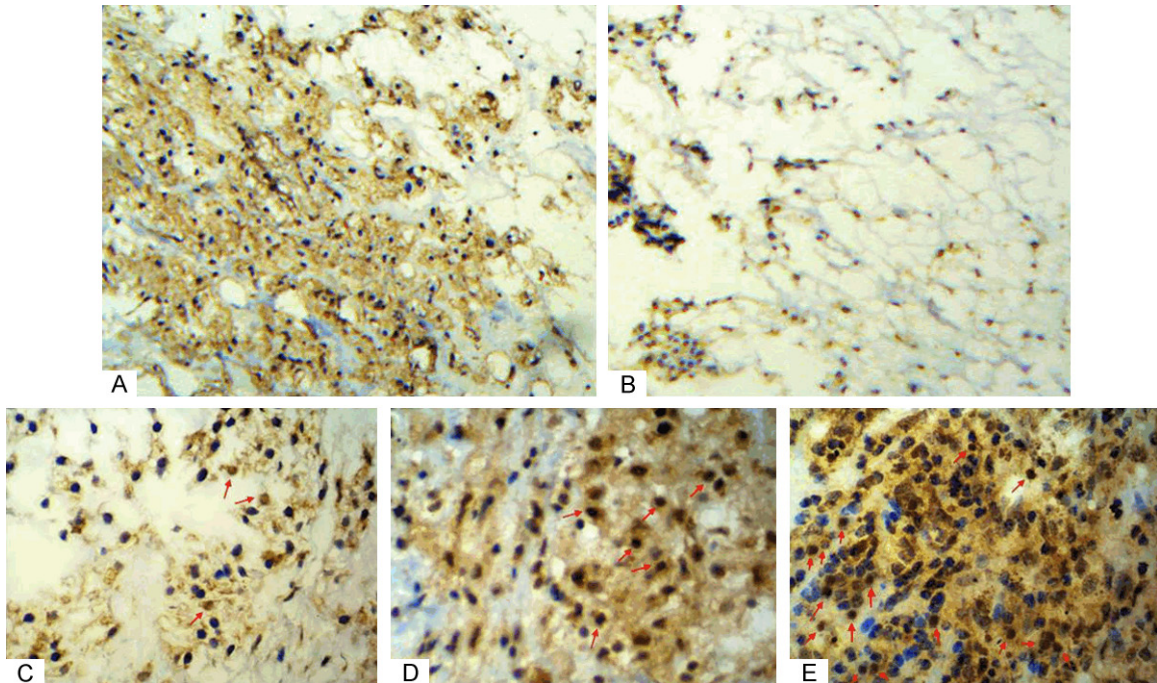
Clinicopathological factors	PD-1 in tumor			PD-1 in TIL			PD-L1 in tumor			PD-L1 in TIL		
	Negative (n = 17)	Positive (n = 37)	p- value	Negative (n = 16)	Positive (n = 38)	p- value	Negative (n = 17)	Positive (n = 37)	p- value	Negative (n = 42)	Positive (n = 12)	p- value
Age (years)												
≤ 50	9	14	0.29	9	11	0.058	7	16	0.88	15	8	0.056
> 50	8	23		7	27		10	21		27	4	
Sex												
Male	8	27	0.064	8	27	0.13	12	23	0.54	28	7	0.84
Female	9	10		8	11		5	14		14	5	
Tumor size												
≤ 5 cm	6	15	0.71	7	14	0.63	6	15	0.71	17	4	0.91
> 5 cm	11	22		9	24		11	22		25	8	
Tumor location												
Sacral vertebra	13	29	1.00	11	31	0.49	14	28	0.84	32	10	0.89
Cervical or thoracic or lumbar vertebra	4	8		5	7		3	9		10	2	
Surrounding muscle invasion												
Yes	9	27	0.14	8	28	0.092	11	25	0.83	29	7	0.72
No	8	10		8	10		6	12		13	5	
Preoperative recurrence												
Yes	5	6	0.45	4	7	0.85	6	5	0.13	6	5	0.095
No	12	31		12	31		11	32		36	7	
Grade												
High	12	26	0.98	12	26	0.87	15	23	0.051	27	11	0.14
Low	5	11		4	12		2	14		15	1	
Stage <sup>a</sup>												
IA	5	8	0.97	4	9	0.38	2	11	0.041	12	1	0.23
IB	2	4		1	5		0	6		6	0	
IIA	0	4		0	4		3	1		2	2	
IIB	8	19		9	18		9	18		18	9	
III	2	2		2	2		3	1		4	0	
Type of resection												
EI	4	14	0.30	3	15	0.14	5	13	0.67	17	1	0.083
EA	13	23		13	23		12	24		25	11	
Tumor hemorrhage												
No	4	6	0.79	4	6	0.68	3	7	1.00	7	3	0.81
Yes	13	31		12	32		14	30		35	9	

PD-L1 expression in TILs predicts prognosis of spinal chordoma

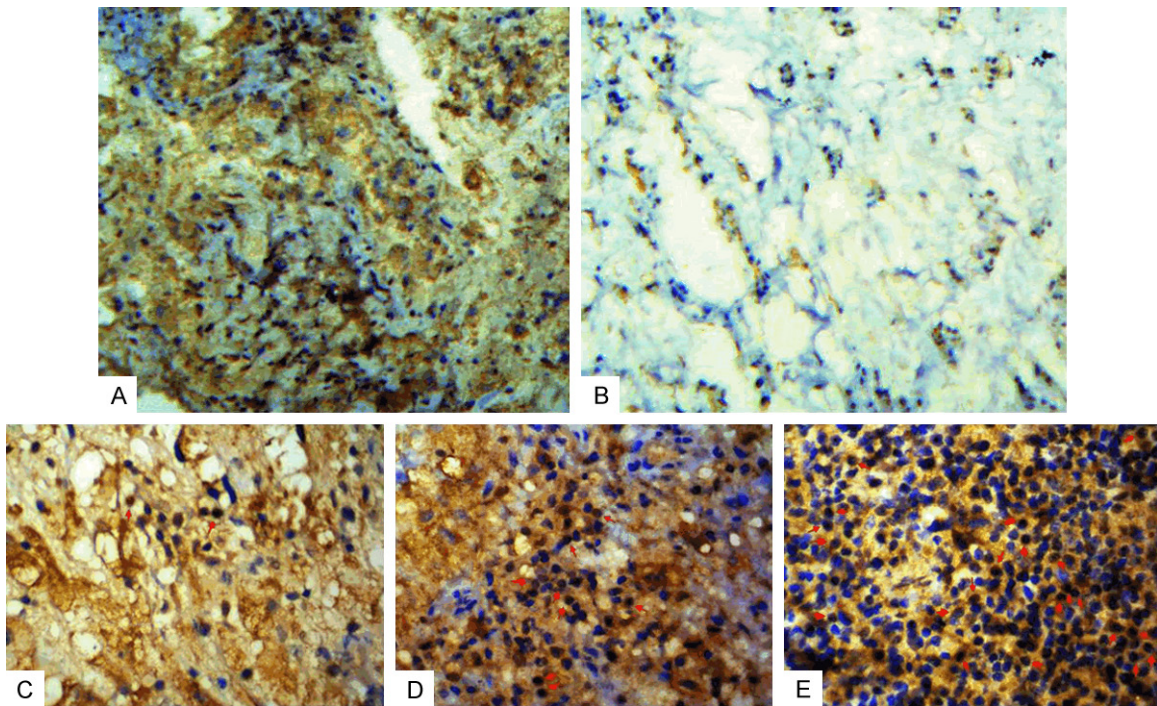
Tumor necrosis <sup>b</sup>												
Absent	4	9	0.42	3	10	0.64	4	9	0.41	10	3	0.34
Mild	8	10		6	12		5	13		16	2	
Moderate	3	12		4	11		3	12		11	4	
Severe	2	6		3	5		5	3		5	3	
Extent of TILs												
Negative	9	14	0.29	10	13	0.055	12	11	0.005	16	7	0.21
Positive	8	23		6	25		5	26		26	5	
Ki-67 index												
Low	10	15	0.21	8	17	0.72	8	17	0.93	19	6	0.77
High	7	22		8	21		9	20		23	6	

EI, Enneking inappropriate; EA, Enneking appropriate; TILs, tumor-infiltrating lymphocytes; PD-1, programmed cell death 1; PD-L1, programmed cell death-1 ligand 1. <sup>a,b</sup>Wilcoxon rank sum test.

## PD-L1 expression in TILs predicts prognosis of spinal chordoma



**Figure 2.** Immunohistochemical analysis of PD-1 expression in tumor cells and TILs of chordoma tissue specimens. A. PD-1 protein on tumor cell membrane; B. Lack of PD-1 expression on tumor cell membrane; C. Rare/few level of TILs that expressed PD-1 in chordoma tissues (*red arrow*); D. Moderate level of TILs that expressed PD-1 in chordoma tissues (*red arrow*); E. Prominent level of TILs that expressed PD-1 in chordoma tissues (*red arrow*) ( $\times 400$ ).



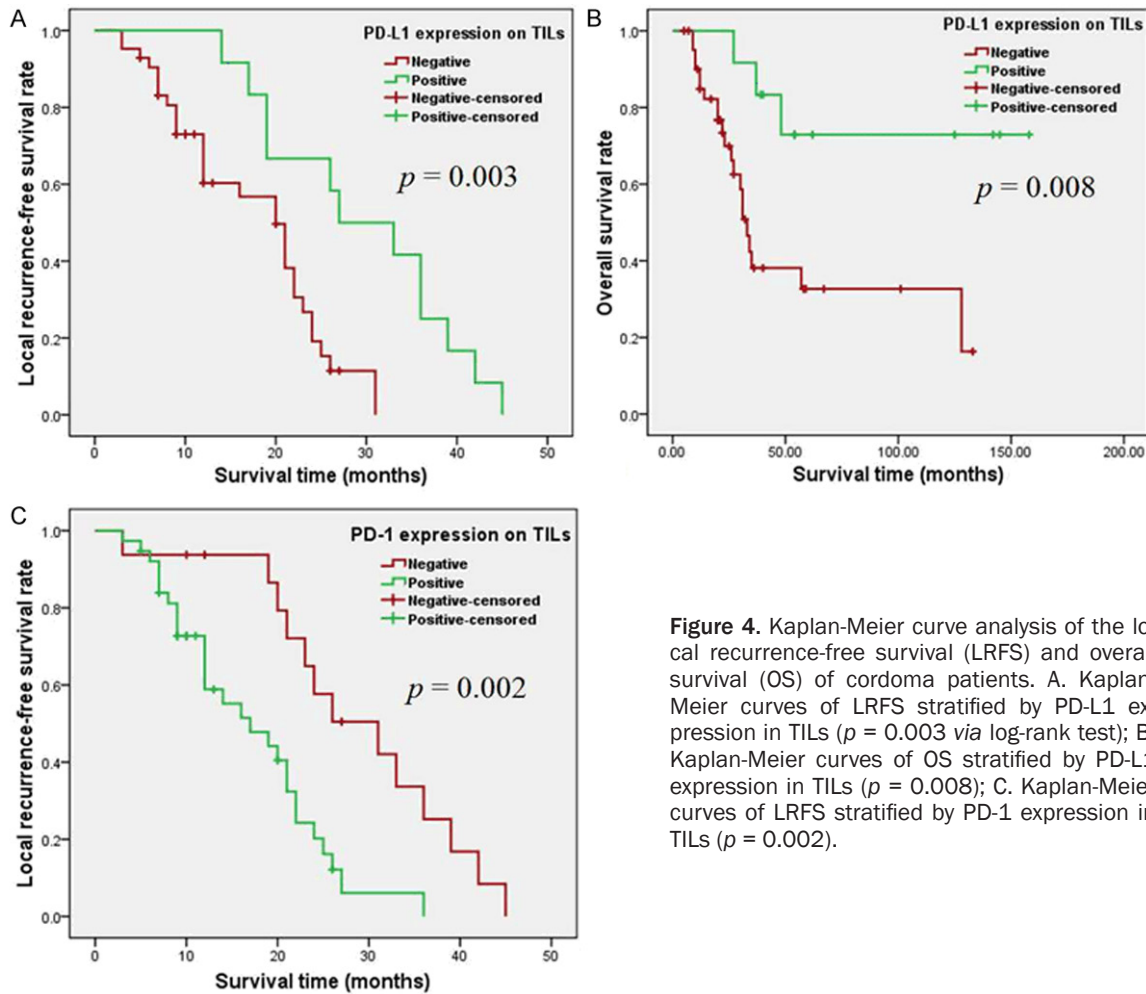
**Figure 3.** Immunohistochemical analysis of PD-L1 expression in tumor cells and TILs of chordoma tissue specimens. A. PD-L1 protein on tumor cell membrane; B. Lack of PD-L1 expression on tumor cell membrane; C. Rare/few level of TILs that expressed PD-L1 in chordoma tissues (*red arrow*); D. Moderate level of TILs that expressed PD-L1 in chordoma tissues (*red arrow*); E. Prominent level of TILs that expressed PD-L1 in chordoma tissues (*red arrow*) ( $\times 400$ ).

## PD-L1 expression in TILs predicts prognosis of spinal chordoma

**Table 3.** Univariate and multivariate Cox proportional hazard analyses of prognostic factors for local recurrence-free survival of spinal chordoma patients

Factors	Categories	Univariate analysis		Multivariate analysis	
		$\chi^2$	<i>p</i> -value	<i>p</i> -value	HR (95% CI)
Sex	Male/Female	2.949	0.086		
Age	≤ 50/> 50	6.560	0.010	0.25	1.693 (0.686-4.177)
Tumor size	≤ 5 cm/> 5 cm	0.278	0.59		
Tumor location	Sacral vertebra/Cervical or thoracic or lumbar vertebra	0.003	0.95		
Preoperative recurrence	Yes/No	0.954	0.32		
Surrounding muscle invasion	Yes/No	24.585	< 0.001	0.003	3.660 (1.544-8.675)
Grade	High/Low	0.231	0.631		
Stage	IA/IB/IIA/IIB/III	0.960	0.916		
Type of resection	EI/EA	16.472	< 0.001	0.30	1.607 (0.645-4.004)
Tumor hemorrhage	Yes/No	4.031	0.045	0.430	1.523 (0.536-4.329)
Tumor necrosis	Absent/mild/moderate/severe	0.189	0.97		
Extent of TILs	Negative/Positive	4.104	0.043	0.93	1.034 (0.472-2.263)
Ki-67 index	High/Low	14.163	< 0.001	0.002	3.806 (1.627-8.907)
PD-1 expression on tumor cells	Negative/Positive	3.406	0.065		
PD-L1 expression on tumor cells	Negative/Positive	1.035	0.30		
PD-1 expression in TILs	Negative/Positive	10.051	0.002	0.391	1.537 (0.576-4.102)
PD-L1 expression in TILs	Negative/Positive	8.792	0.003	0.033	0.298 (0.098-0.907)

EI, Enneking inappropriate; EA, Enneking appropriate; TILs, tumor-infiltrating lymphocytes; PD-1, programmed cell death 1; PD-L1, programmed cell death-1 ligand 1; HR, hazard ratio; CI, confidence interval.



**Figure 4.** Kaplan-Meier curve analysis of the local recurrence-free survival (LRFS) and overall survival (OS) of chordoma patients. A. Kaplan-Meier curves of LRFS stratified by PD-L1 expression in TILs ( $p = 0.003$  via log-rank test); B. Kaplan-Meier curves of OS stratified by PD-L1 expression in TILs ( $p = 0.008$ ); C. Kaplan-Meier curves of LRFS stratified by PD-1 expression in TILs ( $p = 0.002$ ).



## PD-L1 expression in TILs predicts prognosis of spinal chordoma

**Table 4.** Univariate and multivariate Cox proportional hazard analyses of prognostic factors for overall survival of spinal chordoma patients

Factors	Categories	Univariate analysis		Multivariate analysis	
		$\chi^2$	p-value	p-value	HR (95% CI)
Sex	Male/Female	1.540	0.21		
Age	$\leq 50 / > 50$	1.459	0.22		
Tumor size	$\leq 5 \text{ cm} / > 5 \text{ cm}$	0.127	0.72		
Tumor location	Sacral vertebra/Cervical or thoracic or lumbar vertebra	0.132	0.71		
Preoperative recurrence	Yes/No	0.546	0.46		
Surrounding muscle invasion	Yes/No	6.793	0.009	0.70	1.222 (0.434-3.439)
Grade	High/Low	0.641	0.42		
Stage	IA/IB/IIA/IIB/III	22.303	< 0.001	0.10	1.372 (0.941-1.999)
Type of resection	EI/EA	5.898	0.015	0.36	1.519 (0.619-3.724)
Tumor hemorrhage	Yes/No	0.319	0.57		
Tumor necrosis	Absent/mild/moderate/severe	4.032	0.25		
Extent of TILs	Negative/Positive	0.003	0.95		
Ki-67 index	High/Low	2.045	0.15		
PD-1 expression on tumor cells	Negative/Positive	1.047	0.30		
PD-L1 expression on tumor cells	Negative/Positive	0.005	0.94		
PD-1 expression in TILs	Negative/Positive	1.772	0.18		
PD-L1 expression in TILs	Negative/Positive	7.007	0.008	0.011	0.188 (0.051-0.687)

EI, Enneking inappropriate; EA, Enneking appropriate; TILs, tumor-infiltrating lymphocytes; PD-1, programmed cell death 1; PD-L1, programmed cell death-1 ligand 1; HR, hazard ratio; CI, confidence interval.

cally significant association between tumor PD-1 or PD-L1 expression in TILs and the level of TILs in chordoma samples (**Table 2**).

### *Association of PD-1/PD-L1 expression with clinicopathological data from chordoma patients*

Chordoma with positive PD-L1 expression was significantly associated with advanced stages ( $p = 0.041$ ). Moreover, patients with positive PD-L1 expression in tumor cells demonstrated a tendency of higher pathological grade at presentation, though such an association did not reach a statistical significance (OR = 1.015, 95% CI: 0.288-3.577,  $p = 0.051$ ; **Table 2**). However, there was no statistically significant association of PD-1/PD-L1 expression in tumor cells or TILs with other clinicopathological data (**Table 2**).

### *Association of PD-1/PD-L1 expression with LRFS and OS of spine chordoma patients*

During the follow-up period, 41 patients (75.93%) showed local recurrence and 24 patients (44.44%) were deceased. Kaplan-Meier analysis showed that TILs with positive PD-L1 expression was statistically associated with better LRFS ( $\chi^2 = 8.792$ ,  $p = 0.003$  by the log-rank test; **Table 3** and **Figure 4A**) and OS ( $\chi^2$

= 7.007,  $p = 0.008$  by the log-rank test; **Table 4** and **Figure 4B**) of spinal chordoma patients. LRFS was also statistically associated with Ki-67 staining, age, tumor muscle invasion, hemorrhage, and type of resection (**Table 3**). Furthermore, we found that PD-1 expression in TILs was statistically associated with LRFS ( $\chi^2 = 10.051$ ,  $p = 0.002$  by the log-rank test; **Table 3** and **Figure 4C**). In addition, tumor muscle invasion, Enneking inappropriate resection and tumor stage were significantly associated with poor OS (**Table 4**).

Then we performed univariate and multivariate analyses using a Cox proportional hazards model (**Tables 3** and **4**). The results showed that PD-L1 expression in TILs was an independent predictor for both LRFS (HR = 0.298, 95% CI: 0.098-0.907,  $p = 0.033$ , **Table 3**) and OS (HR = 0.188, 95% CI: 0.051-0.687,  $p = 0.011$ , **Table 4**). A high Ki-67 index (HR = 3.806, 95% CI: 1.627-8.907,  $p = 0.002$ , **Table 3**) and tumor muscle invasion (HR = 3.660, 95% CI: 1.544-8.675;  $p = 0.006$ , **Table 3**) were also independent predictors for poor LRFS.

## Discussion

In the current study, we determined the levels of PD-1 and PD-L1 proteins in tumor cells and TILs in spinal chordoma and the association of

their expression with the clinicopathological features and survival of the patients. We found that PD-1 and PD-L1 were differentially expressed in tumor cells and TILs of chordoma tissues. PD-L1 expression in tumor cells was significantly associated with advanced chordoma stage and increased TIL infiltration, and had a borderline association with higher tumor grades but was not associated with LRFS or OS. Moreover, PD-1 expression in TILs was associated with poor LRFS. Importantly, PD-L1 expression in TILs, but not in tumor cells, was an independent predictor for both LRFS and OS of spinal chordoma patients. This study revealed that PD-1 and PD-L1 were expressed in the microenvironment components of chordoma and associated with survival of patients, suggesting that targeting the PD-1/PD-L1 pathway might be a novel immunotherapeutic strategy to treat chordoma. However, further studies are needed to define the role of PD-L1 expression in TILs as a predictive and prognostic biomarker in spinal chordoma.

An increasing number of molecular biomarkers have been evaluated for their association with prognosis of spinal chordoma, such as aberrant expression of different proteins or epigenetic dysregulation in tumor tissues [45-48]. However, most of these investigations of prognostic biomarkers in spinal chordoma did not adjust for confounding factors using multivariate analysis, which may distort the usefulness of prognostic biomarkers [39, 45]. Thus, it is important to identify additional biologic markers as potential indicators of prognosis for this disease.

Recently, blockade of the PD-1/PD-L1 axis has resulted in substantive and durable clinical responses in patients with several advanced human cancers [36, 37]. In the current study, we aimed to profile the status of PD-1 and PD-L1 expression in both tumor cells and TILs in spinal chordoma tissues and to assess the association of immune checkpoint with spinal chordoma prognosis, after adjusting for other clinicopathological parameters by multivariate analysis. We found that PD-L1 was expressed in tumor cell membrane in most chordoma specimens (68.5%), which is consistent with a previous report of PD-L1 positive immunoreactivity in 94.9% of 78 chordoma samples [31]. Thus far, the precise mechanism of upregulation of PD-L1 in chordoma remains unclear.

Previous studies demonstrated that loss of tumor suppressor phosphatase and tensin homolog (PTEN) expression was able to upregulate PD-L1 expression in several human malignancies including chordoma [49-53]. A recent study showed that knockdown of PTEN expression using a short hairpin RNA in triple-negative breast cancer could induce expression of PD-L1 mRNA and protein [54]. It will be interesting to investigate whether PTEN suppress the expression of PD-L1 *via* PI3K-AKT dependent or independent mechanism in chordoma.

Furthermore, our current study showed that TILs expressing PD-L1 were widely present in chordoma tissues, which is consistent with a previous study [38]. Our findings are also supported by another recent study that showed PD-L1 expression in tumor-infiltrating mononuclear cells in 58 of 143 patients with urothelial carcinoma [27]. Actually, activated T-cells secrete cytokines to induce PD-L1 expression on surrounding immune and tumor cells [27]. The expression of PD-L1 on TILs as observed in this study suggests that these intratumoral lymphocytes are chordoma antigen-specific. Moreover, previous studies have shown that TILs express PD-1 in various cancers, including chordoma [24, 33, 38]. Similarly, we showed PD-1 expression in TILs in chordoma. Because the interaction of PD-1 with PD-L1 represents a major pathway that is often hijacked by tumors to suppress immune control, the correlated expression of PD-1 by TILs and PD-L1 in tumor cells may play a crucial role in chordoma evasion from host immunity. Indeed, for the first time, we unraveled PD-1 expression in chordoma tumor cells. This result is consistent with a recent study showing positive PD-1 expression in 43 of 122 non-small-cell lung cancer specimens [55]. Our finding suggests that the PD-1/PD-L1 pathway may be a novel therapeutic target for chordoma.

To characterize the association between the expression of PD-1 or PD-L1 proteins and clinical behavior, we correlated PD-1 or PD-L1 expression with patient survival rates and clinicopathological parameters and found a statistically significant association between PD-L1 expression on tumor cells and advanced chordoma stage or tumor grade. Previous studies reported that PD-L1 expression in tumor cells was associated with poor tumor differentiation, high grade, risk of recurrence and advanced

clinical or pathologic stage in various human cancer types [21, 56-59]. In chordoma, a recent study showed PD-L1 expression was associated with chordoma metastasis [31]. These data suggest that chordoma cells may escape from attack by immune cells through immune checkpoints, like PD-L1 [60]. Consistent with previous studies [24, 27, 31-34], our current data showed that tumor PD-L1 expression was associated with TILs infiltration in chordoma. This finding can be explained by the fact that TILs can induce PD-L1 expression by upregulation of cytokines [61]. It was reported that TIL infiltration was able to facilitate melanoma metastasis [33]; our results indicated the potential role of TILs in chordoma progression. In addition, we found that tumor-infiltrating PD-1-positive lymphocytes were statistically associated with survival, similar to the results of two previous studies [21, 62]. However, our current data provided the first evidence that only higher PD-L1 expression in TILs was associated with longer LRFS and OS. This association was also observed in urothelial carcinoma [27]. Moreover, a recent phase I clinical trial evaluating the efficacy of MPDL3280A, an anti-PD-L1 mAb, revealed that patients with PD-L1 expression in immune cells exhibited higher overall response rate compared to those without PD-L1 expression [63]. These data may support the rationale for use of PD-L1 expression in immune cells as a potential predictive biomarker for immunotherapy in spinal chordoma. However, it should be noted that patients with PD-L1-negative tumor also responded to anti-PD-L1 therapy, highlighting the need for better biomarkers to predict responses of agents targeting this pathway. Nevertheless, unlike studies of other types of cancers [27, 29, 30, 64], our analyses failed to show any association of tumor PD-L1 expression or the presence of TILs with the survival of spinal chordoma patients. This inconsistency may be attributed to the retrospective nature of the current study and our small number of patients, leading to a correspondingly low statistical power.

Our study does have some limitations: for example, it is retrospective, which may imply a potential selection bias. Moreover, it remains to be determined how PD-L1 expression in TILs of spinal chordoma tissues impacts survival of patients. Finally, the potential heterogeneity of PD-1 or PD-L1 expression within and between

tumor tissues may limit the ability to adequately assess the data as a previous study suggested [27].

In summary, we demonstrated that PD-L1 expression in tumor cells was significantly associated with advanced chordoma stage and increased TIL infiltration; PD-L1 expression in TILs was an independent predictor for both LRFS and OS in spinal chordoma patients; PD-1 expression in TILs was associated with poor LRFS. Our findings suggest that targeting the PD-1/PD-L1 pathway might be a novel immunotherapeutic strategy to treat chordoma.

### Acknowledgements

We would like to thank Auragene Bioscience (Changsha, China) for technical support and Medjaden Bioscience Limited (Hong Kong, China) for assistance in preparation of this manuscript.

### Disclosure of conflict of interest

None.

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