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Original Article

Expression of PD-L1 associated with Ki-67 and chemotherapy response but not p53 in osteosarcoma

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Abstract: Purpose: Osteosarcoma (OS) is highly aggressive and confers poor prognosis. Novel immune checkpoint inhibition holds promise for treatment yet the role of PD-L1 in OS remains undetermined. Methods: Paraffinized OS sections were collected and stained with immunohistochemistry (IHC) for PD-L1, Ki-67, and p53. Clinicopathological parameters of corresponding patients were gathered and analyzed statistically for association and correlation. PD-L1 positivity was evaluated using 1% and 5% cutoffs. Results: Ninety-two OS samples, excised between 1997 to 2017, were collected. Sixty-five samples were assessed as PD-L1 positive using 1% cutoff and 50 samples were positive using 5% cutoff. PD-L1 positivity (1%) was significantly associated with trunk location (P=.018), elevated serum lactate dehydrogenase (LDH) (P=.018), advanced clinical stage (P<.001), distant metastasis (P<.001), and poor response to chemotherapy (P<.001). PD-L1 positivity (1%) was not associated with age, gender, tumor size, or serum level of alkaline phosphatase. PD-L1 positivity (5%) was significantly associated with all significant parameters associated with 1% cutoff, with an additional association with larger tumor size (P=.022) and exception of elevated LDH (P=.017). Cutoff of 1% showed significant correlation with expression of Ki-67 (P<.001) and p53 (P=.024). Cutoff of 5%, however, was only correlated with expression of Ki-67 (P<.001). Conclusion: PD-L1 expression is positive in most OS and is associated with a more aggressive phenotype. PD-L1 expression is also associated with poor chemotherapy response and is correlated with Ki-67. The present results support the application of PD-L1 blockade in OS.

Keywords: Osteosarcoma, PD-L1, immunohistochemistry, Ki-67

Introduction

Osteosarcoma (OS), also termed as osteogenic sarcoma, is a bone malignancy. It is the most common histological form of primary bone cancer [1]. OS is an aggressive malignant neoplasm that arises from primitive transformed cells of mesenchymal origin. It exhibits osteoblastic differentiation and produces malignant osteoids. It is most prevalent in teenagers and young adults.

The etiology of OS, however, remains unclear. Some scholars have suggested that combined effects of cancer stem cells together with genetic dispositions contribute to the tumorigenesis of OS. Rarely, radiotherapy for unrelated conditions has been related to OS. Like other diseases that affect teenagers, familial cases where retinoblastoma (RB) genes are inactivated due to loss of chromosome 13q14 have been associated with high risk of osteosarcoma development [2]. Risk of OS can also be increased in cases of bone dysplasia, including Paget's disease of bone, fibrous dysplasia, enchondromatosis, and hereditary multiple exostoses [3]. Despite all those reports, the most comprehensive way to understand the genetic and genomic landscape of OS is next generation sequencing technique. However, due to its rarity, OS is often sequenced together with other soft tissue tumors, like other sarcomas. Whole-genome sequencing has recently been performed in a study of different cancer types, showing that a subset of osteosarcomas undergo chromothripsis, a single catastrophic genomic instability event, resulting in hundreds of genomic rearrangements, making OS a genomi-

Table 1. Correlation of PD-L1 expression with clinicopathological features of osteosarcoma

Clinicopathological features	No.	PD-L1 (1% cutoff)				P	PD-L1 (5% cutoff)				P
		Neg		Pos			Neg		Pos		
		N	%	N	%		N	%	N	%	
Age (years)											
<55	40	8	20.0%	32	80.0%	.108	14	35.0%	26	65.0%	.092
≥55	52	19	36.5%	33	63.5%		28	53.8%	24	46.2%	
Sex											
Male	53	19	35.8%	34	64.2%	.164	27	50.9%	26	49.1%	.291
Female	39	8	20.5%	31	79.5%		15	38.5%	24	61.5%	
Tumor size (cm)											
>8 cm	49	13	26.5%	36	73.5%	.647	28	57.1%	21	42.9%	.022
≤8 cm	43	14	32.6%	29	67.4%		14	32.6%	29	67.4%	
Anatomic location											
Extremity	57	22	38.6%	35	61.4%	.018	32	56.1%	25	43.9%	.017
Trunk	35	5	14.3%	30	85.7%		10	28.6%	25	71.4%	
Serum level of lactate dehydrogenase											
Elevated	49	14	28.6%	35	71.4%	.018	24	49.0%	25	51.0%	.017
Normal	43	13	30.2%	30	69.8%		18	41.9%	25	58.1%	
Serum level of alkaline phosphatase											
Elevated	55	19	34.5%	36	65.5%	.244	30	54.5%	25	45.5%	.054
Normal	37	8	21.6%	29	78.4%		12	32.4%	25	67.6%	
Clinical stage											
IIA	53	27	50.9%	26	49.1%	<.001	42	79.2%	11	20.8%	<.001
IIB/III	39	0	0.0%	39	100.0%		0	0.0%	39	100.0%	
Distant metastasis											
Absent	23	21	91.3%	2	8.7%	<.001	21	91.3%	2	8.7%	<.001
Present	69	6	8.7%	63	91.3%		21	30.4%	48	69.6%	
Response to chemotherapy											
Good	38	24	63.2%	14	36.8%	<.001	24	63.2%	14	36.8%	.006
Poor	54	3	5.6%	51	94.4%		18	33.3%	36	66.7%	

cally complex and unstable tumor [4]. However, this instability may also bring therapeutic targets, as unstable genomes often render cells more detectable to immunocytes where immunotherapy may play a role [5].

Programmed death-ligand 1 (PD-L1), also known as cluster of differentiation 274 (CD274) or B7 homolog 1 (B7-H1), is a protein that, in humans, is encoded by the CD274 gene upregulation of PD-L1. This may allow cancers to evade the host immune system. PD-L1 is a cell-surface protein that suppresses cytotoxic $CD8^+$ T-cell-mediated immune response. Multiple agents targeting the PD1/PDL1 system are currently at different stages of clinical development. Those agents have been proven surprisingly effective in a variety of cancers, including renal cell carcinoma, melanoma, and non-small cell lung cancer [6]. However, expression and the clinical relevance of PDL1 expression in sarcomas remain poorly understood.

Many studies have focused on immune check point profiles in OS. Therefore, the current study investigated expression of PD-L1, one of the most important biomarkers for PD-1/PD-L1 blockade therapy, in OS. This study examines its association with various clinicopathological parameters. The present study may hold promise in promoting novel immunotherapies for this fatal disease.

Materials and methods

General information

Osteosarcoma (OS) samples, surgically removed between January 1997 and January 2017, at Wuxi Second People's Hospital and Shanghai

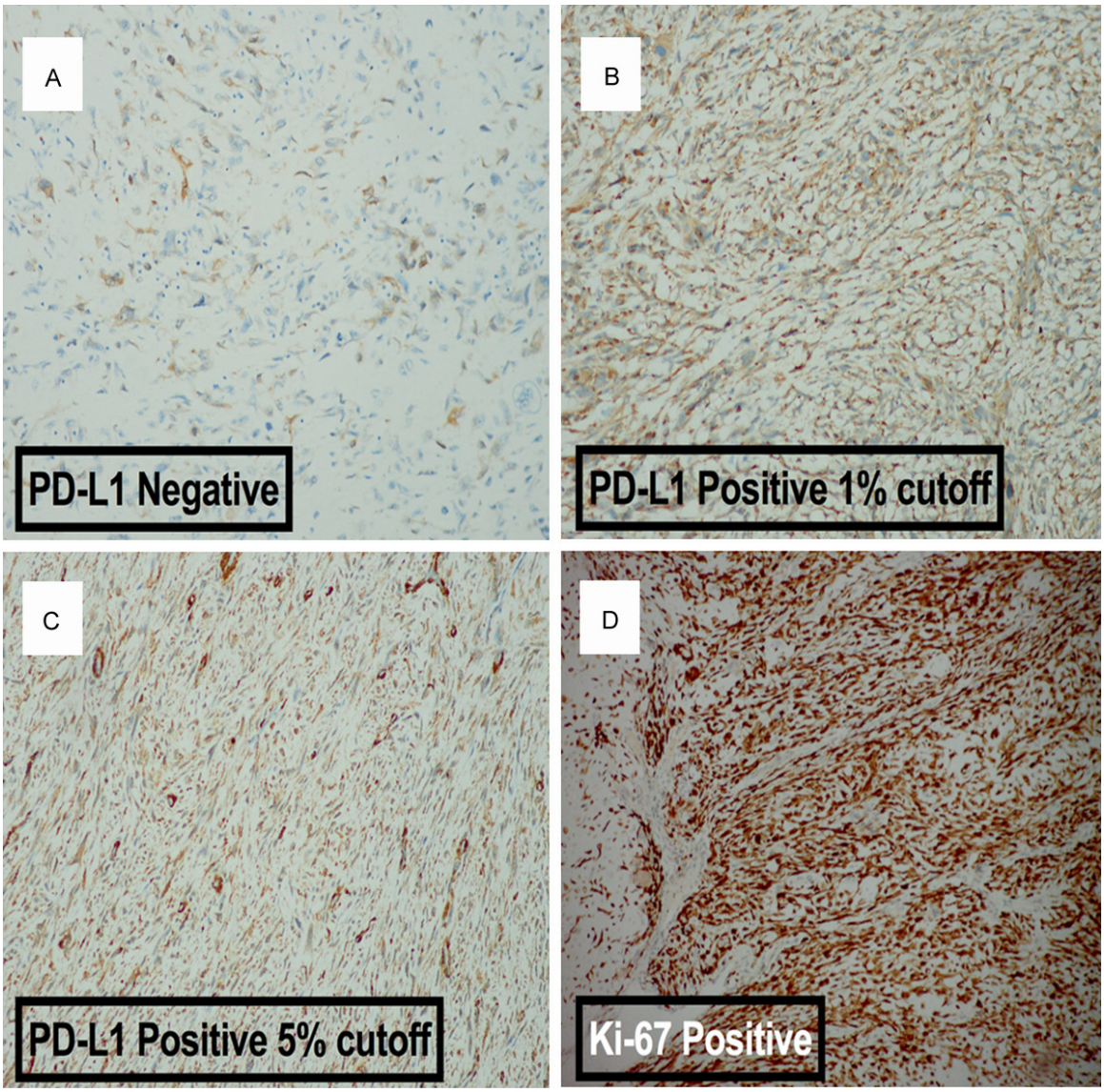

Figure 1. Immunohistochemical staining of PD-L1 and Ki-67 in osteosarcoma, showing (A) Negative staining for PD-L1; (B) Positive staining (1% cutoff) for PD-L1; (C) Positive staining (5% cutoff) for PD-L1; and (D) Positive staining (20% cutoff) of Ki-67 o (All captured at magnification of 100×).

Ninth People's Hospital, were collected for the current study. No patients received blood transfusions, radiotherapy, or chemotherapy before surgery. Clinical stage was classified according to the sixth edition of the tumor-node-metastases (TNM) classification of the International Union against Cancer (UICC). This study was approved by the Research Ethics Committee of Wuxi Second People's Hospital and Shanghai Ninth People's Hospital, PR China. Written informed consent was obtained from all patients.

Immunohistochemistry

Standard IHC protocol was followed [7]. All samples were formalin-fixed and subsequently paraffin-embedded. Slices were cut at 4 μm and endogenous peroxidase was blocked through incubation with 3% hydrogen peroxide for 15 minutes. Heat-mediated antigen retrieval was performed by boiling the slides in 0.01 M citrate buffer, pH 6.0, for 20 minutes in a microwave oven. PD-L1 IHC analysis was performed using clone 28-8 (dilution 1:450; Abcam, Cambridge, UK) commercially available rabbit monoclonal antibodies. TP53 (Novocastra, Newcastle, UK) was diluted at 1:50 and Ki67 (Novocastra, Newcastle, UK) was diluted at 1:100. The immune complex was detected with DAKO EnVision Detection System (Dako). All slides were then counterstained with Mayer's

Table 2. Correlation between PD-L1 expression with Ki-67 and p53

		PD-L1 (1% cutoff)				P	PD-L1 (5% cutoff)				P
		Neg		Pos			Neg		Pos		
Ki-67	Pos	N	0	N	56	<.001	N	6	N	50	<.001
		%	0.00%	%	100.00%		%	10.70%	%	89.30%	
	Neg	N	27	N	9		N	36	N	0	
		%	75.00%	%	25.00%		%	100.00%	%	0.00%	
P53	Pos	N	11	N	43	.024	N	21	N	33	.123
		%	20.40%	%	79.60%		%	38.90%	%	61.10%	
	Neg	N	16	N	22		N	21	N	17	
		%	42.10%	%	57.90%		%	55.30%	%	44.70%	

hematoxylin blue in 0.3% ammonia. Slides were subsequently dehydrated through graded alcohols to xylene and mounted in mounting medium. For positive controls, this study used placenta for PD-L1, colon carcinoma for TP53, and bladder cancer for Ki-67. For negative controls, the primary antibodies were omitted.

Assessment of PD-L1, Ki67, and TP53 staining

Proportions of PD-L1-positive tumor cells and tumor-infiltrating lymphocytes were estimated as the number of stained cells divided by the total numbers, respectively. Each slide was reviewed by 3 independent pathologists. The final reading was valid only when a consensus was reached. Only membrane positive cells were accepted. All IHC analyses were evaluated according to two different criteria. First, cases with fewer than 1% stained cells were considered negative (1% cutoff). Second, cases with fewer than 5% stained cells were considered negative (5% cutoff). For Ki-67, at least 1,000 tumor cells (×400 magnification) from the most immunopositive regions of the noninvasive part of each neoplasm were visually counted. A cutoff of <20% was designated as negative. For TP53, however, a cutoff of <8% was designated as negative [8].

Statistics

Stata version 12.0 for Macintosh was used for statistical analyses. All data were binary. Fisher's exact test was used to evaluate association between clinicopathological parameters and IHC results. Spearman's test was used to evaluate correlation between expression of factors. *P* values <0.05 are considered statistically significant.

Results

A total of 92 OS samples were collected. Demographic data are summarized in **Table 1**. Sixty-five samples were assessed as PD-L1 positive using 1% cutoff and 50 samples were positive using 5% cutoff (**Figure 1**). PD-L1 positivity (1% cutoff) was significantly associated with trunk location (**Table 1**), elevated serum lactate dehydrogenase (LDH) (**Table 1**), advanced clinical stage (**Table 1**), distant metastasis (**Table 1**), and poor response to chemotherapy (**Table 1**). PD-L1 positivity (1% cutoff) was not associated with age, gender, tumor size, or serum level of alkaline phosphatase (ALP) (**Table 1**). PD-L1 positivity (5% cutoff) was significantly associated extremity location (**Table 1**), advanced clinical stage (**Table 1**), distant metastasis (**Table 1**), and poor response to chemotherapy (**Table 1**). Additionally, PD-L1 positivity (5% cutoff) was associated with larger tumor size (P=.022). Unlike 1% cutoff, 5% cutoff was significantly associated with normal LDH (P=.017). PD-L1 positivity (1% cutoff) showed sigxnificant correlation with expression of Ki-67 (**Table 2**) and p53 (**Table 2**), respectively. PD-L1 positivity (5% cutoff), however, was only correlated with exprression of Ki-67 (P<.001).

Discussion

The current study demonstrated that PD-L1 was positive in more than half of OS cases with 1% cutoff. This finding is in accord with a few other reports on PD-L1 expression in OS [9-12]. There is growing evidence that immune check point biomarker PD-L1 plays important roles in OS. Liao et al. [13] revealed that overall and five-year survivals in patients with high levels of PD-L1 expression are significantly shorter than in patients with low levels. High levels of PD-L1

expression have also been associated with metastasis in osteosarcoma patients. They also showed that expression of PD-L1, knocked out by CRISPR/Cas9 system, can increase drug sensitivities of doxorubicin and paclitaxel. Those results are in line with the present observation that positive PD-L1 expression is associated with poor chemotherapy response. Koirala et al. [12] reported that expression of PD-L1 was significantly associated with the presence of T-cells, dendritic cells, and natural killer cells, yet only infiltration by dendritic cells and macrophages was associated with worse five-year survival. Shen et al. [10] demonstrated that the pulmonary origin of metastases correlated with high PD-L1 expression and prominent TILs. Likewise, the present study also demonstrates that positive PD-L1 expression is associated with distant metastasis. Given that most metastatic patients in the present study had pulmonary metastasis, those results well represent the biology of OS. Zhang et al. [11], on the other hand, showed that patients with metastasis had significantly higher levels of PD-1 on $CD4^+$ T-cells than those without metastasis. PD-1 expression on $CD4^+$ T-cells starts to increase in stage III, whereas PD-1 expression on $CD8^+$ T-cells begins to increase in stage II. These results indicate that PD-1 demonstrates a similar pattern as PD-L1 in OS. Moreover, Shen et al. [9] demonstrated that, in human osteosarcoma samples, PD-L1 mRNA gene expression ranged over 5,000-fold difference. The presence of TILs is associated with high PDL1 expression.

The abovementioned studies, together with the present findings, provide strong evidence that OS patients may be benefitted by the PD-1/PD-L1 blockade. It is now used in a variety of cancers with satisfactory effects. PD-1 inhibitor pembrolizumab has been associated with significantly longer overall survival (by approximately 3 months), with a lower rate of treatment-related adverse events than chemotherapy, as a second-line therapy for platinum-refractory advanced urothelial carcinoma [14, 15]. Pembrolizumab also shows an acceptable side-effect profile and antitumor activity in patients with advanced non-small-cell lung cancer (NSCLC). PD-L1 expression in at least 50% of tumor cells correlated with improved efficacy of pembrolizumab [16]. Combined immunotherapy also plays a role in cancer treatment. Among previously untreated patients with metastatic melanoma, PD-1 inhibitor nivolumab, alone or combined with CTLA-4 inhibitor ipilimumab, has resulted in significantly longer progression-free survival than ipilimumab alone. In patients with PD-L1-negative tumors, the combination of PD-1 and CTLA-4 blockade is more effective than either agent alone [17]. Nivolumab also has substantial therapeutic activity and an acceptable safety profile in patients with previously heavily treated relapsed or refractory Hodgkin's lymphoma [18]. Immunotherapy has even overtaken the effects of some long-established first-line chemotherapies. In patients with advanced NSCLC and PD-L1 expression on at least 50% of tumor cells, pembrolizumab has been associated with significantly longer progression-free and overall survival, along with fewer adverse events than platinum-based chemotherapy [19]. Moreover, PD-L1 antibodies are clinically potent for cancer therapy. Atezolizumab has shown durable activity and good tolerability in this patient population. Increased levels of PD-L1 expression on immune cells has been associated with increased response [20]. Another randomized phase 3 study reported that atezolizumab treatment resulted in a clinically relevant improvement of overall survival, versus docetaxel, in previously treated non-small-cell lung cancer, regardless of PD-L1 expression or histology, with a favorable safety profile [21].

In conclusion, OS has an aggressive disease course and occurs at an early age. OS lacks an effective treatment to substantially prolong survival. The present findings, together with similar reports, support extending the indication of immune check point inhibition into OS. Various clinically available agents with proven efficacy and tolerable adverse effects makes this implication even more pragmatic.

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Disclosure of conflict of interest

None.

Abbreviations

OS, Osteosarcoma; IHC, immunohistochemically; LDH, lactate dehydrogenase.

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