RESEARCH ARTICLE

Expression and Clinical Significance of Various Checkpoint Molecules in Advanced Osteosarcoma: Possibilities for Novel Immunotherapy

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Objectives: The fact that studies on anti-programmed cell death 1 (PD-1) or its relevant ligand 1 (PD-L1) have yielded such few responses greatly decreases the confidence in immunotherapy with checkpoint inhibitors for advanced osteosarcoma. We intended to characterize the expression of various checkpoint molecules with immunohistochemistry in osteosarcoma specimens and analyzed the relationship of the expression of these checkpoint molecules with patients' clinical courses.

Methods: This study was a retrospective non-intervention study from August 1st 2017 to March 1st 2020. Immunohistochemistry for B7-H3 (CD276, Cluster of Differentiation 276), CD47 (Cluster of Differentiation 47), PD-L1 (programmed cell death ligand 1), TIM3 (mucin-domain containing-3), TGF-β (TransformingGrowth Factor β), CXCR 4 (Chemokine Receptor 4), CD27 (Cluster of Differentiation 27), IDO1 (Indoleamine 2,3-dioxygenase 1), KIRs (Killer cell Immunoglobulin-like Receptors), and SDF-1 (Stromal cell-Derived Factor-1) was performed on 35 resected osteosarcoma specimens. Patients progressed upon first-line chemotherapy with evaluable lesions were qualified for this study, and their specimens previously stored in the pathological department repository would be retrieved for analysis. Associations between the immunohischemistry markers and clinicopathological variables and survival were evaluated by the χ^2 displayed by cross-table, Cox proportional hazards regression model, and Kaplan–Meier plots.

Results: The positive rates of B7-H3, CD47, PD-L1, TIM3, and TGF-β expression in this sample of 35 heavily treated osteosarcomas were 29% (10/35), 15% (5/35), 9% (3/35), 6% (2/35), and 6% (2/35), respectively, and diverse staining intensities were observed. Among these advanced patients, 15/35 (43%) had positive checkpoint expression, of which 33% (5/15) showed evidence of the co-expression of more than one checkpoint molecule. We did not find any obvious correlation with clinicopathological characteristics and the positive expression of these molecules.

Conclusions: The present study highlights that only a small subset of progressive osteosarcomas, which had been heavily-treated, expressed tumor immune-associated checkpoint molecules, of which B7-H3 was the most positively expressed checkpoint and might be a promising target for further osteosarcoma investigation.

Key words: Checkpoint Molecules; Co-expression; Immunotherapy; Osteosarcoma; Prognosis

Introduction

Osteosarcomas are bone-forming tumors characterized by the presence of an extracellular osteoid matrix produced by cancer cells and associated with a very complex local environment including bone cells, blood vessels, stromal cells, and immune infiltrates.[1] The peak incidence of osteosarcoma occurs during the adolescent growth spurt, which suggests that bone growth and pubertal hormones are important in the etiology of the disease.[2] Although modern multiagent, dose-intensive chemotherapy (in conjunction

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with surgery) achieves a 5-year event-free survival (EFS) of approximately 60%–71% in extremity localized, non-metastatic disease,[3,4] the prognosis for relapsed or refractory disease that progresses despite chemotherapy remains dismal after decades of clinical trials for new agents.[5] Recently, the remarkable results achieved with the advent of cancer immunotherapies and checkpoint inhibitors have revolutionized the field of oncology by putting the host immune response under the spotlight as a target for anticancer therapeutic interventions.[6] Nivolumab,[7] ipilimumab,[7] pembrolizumab,[8,9] and camrelizumab (SHR-1210)[10] have all been studied in patients with advanced disease either alone or in combination. However, only a limited number of patients have derived meaningful clinical benefits, with no statistical advantage for the whole population.[7,9–11] Challenges remaining on the path forward include the identification of the most suitable checkpoint and immunotherapy, the prevention of paradoxical or hyperprogressive disease, and the exploration of predictive biomarkers for more personalized immunotherapies for osteosarcoma patients.[12,13]

The tumor microenvironment (TME), where cancer cells can functionally sculpt their microenvironment through the secretion of various cytokines, chemokines, and other factors, is complex and continuously evolving.[14] The T cell receptor (TCR) starts the signaling cascade upon its interaction with peptide antigen in the context of the major histocompatibility complex (MHC), but optimal activation of naive T cells depends on a costimulatory signal through CD28.[15] Additional interactions between ligands and activating or inhibitory receptors are crucial for further regulating T cell activation and tolerance. Therapeutics targeting these and other pathways are in various stages of clinical development.[6] Osteosarcoma usually acts in an immune rheostat or "immunostat" condition.[16] It is suspected that upregulated immunoinhibitory pathways other than programmed cell death 1 or its relevant ligand 1 (PD-1/PD-L1) might dampen or arrest the antitumor immune response in osteosarcoma.[17] It was recently found in other tumors that combining immunological agents may improve response rates and the duration of response by stimulating antitumor immunological memory.[6,14]

However, literature on the expression of different checkpoint molecules in advanced osteosarcoma is scarce.[18–23] Mochizuki et al.[21] explored the expression of various checkpoint molecules and tumor-infiltrating lymphocytes (TILs) with immunohistochemistry in common pediatric solid tumors, and among 12 untreated osteosarcoma specimens, 100% expressed moderate to high levels of herpes virus entry mediator (HVEM) on the tumor. TILs were detected in all tumor samples except one osteosarcoma sample. Piperdi et al.[22] suggested that CD47 was expressed in 87.7% of specimens, with 28.4%, 27.2%, and 32.1% demonstrating high, intermediate, and low expression, respectively, in a tissue microarray (TMA) of 81 osteosarcoma specimens.

In this study, we evaluated the expression of B7-H3, CD47, PD-L1, TIM3, TGF-β, CXCR 4, CD27, IDO1, KIRs, and SDF-1 in 35 advanced osteosarcoma specimens with immunohistochemistry. Specifically, we tried to investigate: (i) the intensity and positivity of all these checkpoint molecules expressed on the osteosarcoma cells; (ii) the associations between these molecules and clinicopathological variables; and (iii) the correlation between the expression of these molecules and survival.

Methods

Patients and Samples

This study was approved by the Institutional Review Board of Peking University People's Hospital (No. 2018PHB059-01). The samples were collected from the Musculoskeletal Tumor Center of Peking University People's Hospital between August 2017 and March 2020. Written informed consent was obtained from all patients before using their specimens previously stored in the pathological department specimen repository. From June 1st, 2017, to March 26th, 2020, 373 consecutive patients initially treated at Peking University People's Hospital and histologically diagnosed with high-grade osteosarcoma were reviewed. We included: (i) patients who had progressed upon first-line chemotherapy; (ii) patients who had evaluable lesions; (iii) patients who had ever been operated in our hospital with enough paraffin-embedded tissue for this study. The exclusion criteria were: (i) clinical information was not complete; (ii) lost to follow-up. Table 1 summarizes the demographic information of the patients examined in this study. Each sample was pathologically diagnosed as high-grade osteosarcoma by using hematoxylin and eosin (HE) staining as well as several required immunohistochemical (IHC) stains. For the current study, two senior pathologists (SDH and SKK) further confirmed the results of the tissue samples with new HE staining.

Clinical follow-up was continuously performed every 2 months until the death of the patients. The hospital records, laboratory examination results, and imaging results of all patients were retrieved and reviewed.

Procedure for Immunohistochemistry

IHC analyses were performed using the avidin–biotin complex (ABC) method.[18] A significant proportion of specimens were decalcified: 87% (20/23) of resected musculoskeletal specimens and 28% (2/7) of metastasectomy specimens of the lung. Tumor tissue sections (5 μm thick) were deparaffinized in xylene and rehydrated in a graded series of ethanols. Sections were incubated for 15 min with 1.0% hydrogen peroxide (H_2O_2) to block endogenous peroxidases; rinsed in phosphate-buffered saline (PBS); incubated in 10 mM sodium citrate buffer, pH 6.0, for 10 min, with an interval of 1 min and another heating step of 5 min; incubated for 20 min in normal goat serum (Vector Laboratories, Burlingame, CA)[19] to block nonspecific binding of antibodies; and subjected to antigen retrieval according to the optimal protocol for each primary antibody (Table S1).

TABLE 1 Comparison of baseline clinicopathological characteristics based on B7-H3, CD47, and PD-L1 expression in advanced osteosarcoma

Clinical data	Total no.	%	B7-H3 expression				*p* value	CD47 expression				*p* value	PD-L1 expression				*p* value
			Negative		Positive			Negative		Positive			Negative		Positive		
			No.	%	No.	%		No.	%	No.	%		No.	%	No.	%	
Sex	35	100	25	71	10	29		30	86	5	14		32	91	3	9	
Male	20	57	14	70	6	30	0.945	16	80	4	20	0.443	19	95	1	5	0.383
Female	15	43	11	73	4	27		14	93	1	7		13	87	2	13	
Age group																	
<35 years	32	91	23	72	9	28	0.849	28	88	4	12	0.687	29	91	3	9	0.758
≥35 years	3	9	2	67	1	33		2	67	1	33		3	100	0	0	
Histopathology																	
Conventional	30	86	20	66	10	34	0.484	26	87	4	13	0.725	27	90	3	10	0.808
Small cell	2	6	2	100	0	0		2	100	0	0		2	100	0	0	
Telangiectatic	2	6	2	100	0	0		1	50	1	50		2	100	0	0	
Low grade maltransformation	1	3	1	100	0	0		1	100	0	0		1	100	0	0	
Specimen source																	
From bone	25	71	18	72	7	28	0.982	21	84	4	16	0.258	23	92	2	8	0.672
From lung	7	20	5	71	2	29		7	100	0	0		6	86	1	14	
From lymph nodes	3	9	2	67	1	33		2	67	1	33		3	100	0	0	
Primary tumor site																	
Distal femur	15	43	9	60	6	40	0.084	12	80	3	20	0.079	14	93	1	7	0.761
Proximal tibia	8	23	6	75	2	25		8	100	0	0		7	88	1	12	
Proximal humerus	4	11	4	100	0	0		4	100	0	0		3	75	1	25	
Proximal femur	1	3	0	0	1	100		0	0	1	100		1	100	0	0	
Axial skeleton	6	17	5	83	1	17		6	100	0	0		6	100	0	0	
Others	1	3	1	100	0	0		0	0	1	100		1	100	0	0	
Chemotherapy resistance																	
Yes	14	40	11	79	3	21	0.445	10	71	4	29	0.019	14	100	0	0	0.259
No	21	60	14	67	7	33		20	95	1	5		18	86	3	14	
Anti-angiogenesis TKI resistance																	
Yes	4	11	3	75	1	25	0.867	2	50	2	50	0.365	4	100	0	0	0.687
No	31	89	22	71	9	29		28	90	3	10		28	90	3	10	
Anti-PD-1 therapy efficacy																	
Effective	4	11	2	50	2	50	0.057	3	75	1	25	0.061	4	100	0	0	0.105
Invalid	9	26	9	100	0	0		8	89	1	11		9	100	0	0	
Unused	17	49	11	65	6	35		16	94	1	6		16	94	1	6	
Not determinable (combination therapy)	5	14	3	60	2	40		3	60	2	40		3	60	2	40	

Abbreviations: PD-1, programmed cell death 1; PD-L1, programmed cell death 1 ligand 1; TKI, tyrosine kinase inhibitor.

Sections were incubated overnight at 4°C with primary antibodies, rinsed in PBS, incubated for 30 min with biotinylated anti-rabbit or anti-mouse goat IgG (Vector Laboratories) as secondary antibodies, rinsed in PBS, incubated for 30 min with components from a VECTASTAINVR EliteVR ABC KIT (Vector Laboratories), and treated with 3.3′-diaminobenzidine tetrahydrochloride and H_2O_2 solution to allow color development. Finally, all slides were rinsed with water, counterstained with hematoxylin, dehydrated in a graded series of ethanols, and cleared in xylene; coverslips were mounted with Permount (Fisher Scientific, Pittsburgh, PA).

IHC Scoring

The immunostaining was interpreted by a pulmonary pathologist (SKK and DY) as negative (0–<1% tumor cell staining) or positive (<1%). First, the two pathologists individually assigned IHC scores for all sections in the present study. In this process, the two pathologists were blinded. Then, the two pathologists worked together to reexamine the sections that were assigned different scores and discussed the discrepancies. Then, the pathologists provided final values after reaching an agreement.

In this study, only cell surface expression was evaluated for each checkpoint molecule, and we evaluated the percentage of tumor cells with any expression regardless of the intensity of staining.[21] To be considered positive, staining had to be membranous and circumferential.

On the tumor cells: A semiquantitative scoring system (0–<1% = negative, recorded as N; 1–<5% = low, recorded as L; 5–<50% = moderate, recorded as M; and ≥ 50% = high, recorded as H) was applied for the evaluation of immune checkpoint ligand expression.[16]

On the tumor-infiltrating lymphocytes: Tumor-infiltrating lymphocytes (TILs) were lymphocytes that had infiltrated tumor tissues, and TILs were subjectively identified microscopically by two pathologists individually. A semiquantitative scoring system (0–<1% = negative, recorded as N; 1–<5% = low, recorded as L; 5–<50% = moderate, recorded as M; and ≥ 50% = high, recorded as H) was applied for the evaluation of immune checkpoint receptor expression on the surface of TILs.[15]

Statistical Analysis

All values are expressed as the mean ± standard deviation (SD). Statistical data analysis was performed using GraphPad Prism V.5.03 (GraphPad Software La Jolla, California, USA) statistical package and R language (V.3.6.1). Interdependence between staining and clinical data was calculated using the χ^2 displayed by cross-tables. Student's *t*-test was used for the comparison of data between the two groups. The Cox proportional hazards regression model and Kaplan–Meier plots were generated by the survival package, and the log-rank test was used to compare survival curves between different groups. Statistical significance was defined as a $p < 0.05$.

Results

Our investigation revealed low positive rates of immune checkpoint molecules for the whole advanced osteosarcoma population. However B7-H3 seemed to be of relatively high expression on tumor membranes, which should be further studied as a promising checkpoint target for osteosarcoma. We tried to correlate these molecules with patients' clinical pathologic characteristics as well as prognosis but no significant finding was observed. We did perceive some immune response by IHC classification during our past treatment courses, however secondary resistance finally developed after years of anti-PD-1 therapy.

Patient Clinicopathological Characteristics and Outcome

The clinicopathological characteristics of the patients included in the study are summarized in Table 1. All these patients were in a metastatic and refractory state, and their disease had progressed upon first-line chemotherapy.[24,25] The median age at diagnosis was 14 (interquartile range [IQR], 6–22) years. The male-to-female ratio was approximately 4:3. Among this population, 30/35 (85.7%) had conventional osteosarcoma, while 29/35 (82.9%) of the primary tumors were located at the extremities. Twenty-five of the 35 specimens (71.4%) were resected from musculoskeletal lesions, 7/35 (20.0%) were resected from pulmonary metastatic lesions, and 3/35 (8.6%) were resected from osteosarcoma lymph node metastases.

We usually conducted our first-line chemotherapy following the Peking University People's Hospital-Osteosarcoma (PKUPH-OS 02) regimen (Figure S1),[24,25] which included high-dose methotrexate, doxorubicin, cisplatin, and ifosfamide. Our second-line chemotherapy was usually ifosfamide (1.8 g/m^2/d d1-5) and etoposide (100 mg/m^2/d d1-5 Q_{3W}), while our third-line systemic therapy was usually antiangiogenic tyrosine kinase inhibitor (TKI)-based therapy, such as apatinib and regorafenib.[24,25] For this group of patients, 14/35 (40%) had been confirmed to be refractory to first- and second-line chemotherapy, while 4/35 (11.4%) had been progressive upon TKI therapy. We tried anti-PD-1 antibodies in combination therapy in some of our patients, but in clinical evaluations, we found confirmed efficacy of this treatment in only 4/35 (11.4%) patients, with progression-free survival (PFS) of more than 1 year, while this treatment was firmly believed to be ineffective in 9/35 (25.7%) of patients. A total of 35 patients (83.3%) were followed up for an average of 34.7 months, which ranged from 14.7 to 110.1 months. At the time of the last follow-up, four deaths had occurred among the 35 patients.

Checkpoint Ligands Expression on Advanced Osteosarcoma

First, we examined the expression of immune checkpoint ligands on tumor cells. As shown in Figure 1, the patterns of PD-L1 staining were predominantly membranous. B7-H3 was also detected to be the most commonly expressed

checkpoint and was mainly expressed on the cell membrane of tumor cells. In some studies, PD-L1 was also observed in vascular endothelial cells and tumor-infiltrating immune cells. However, in this study, according to the specifications of the B7-H3 antibody, pathologists agreed to judge the expression as negative if tumor cells and background were all stained positive. As shown in Tables 1 and 2, in this sample of tumors, only 9% (3/35) were PD-L1, 6% (2/35) were TGF-β, 15% (5/35) were CD47, and 29% (10/35) were B7-H3 positive; these results are inconsistent with those of a previous study. No CXCR-4 or KIR expression was observed in these heavily treated osteosarcoma samples. Twenty-six percent of osteosarcomas showed moderate to high expression levels of B7-H3 on their surface, while 3% had only low expression levels (Table 2). For CD47, only 9% of osteosarcomas expressed moderate to high levels, while 6% had low staining intensity. We noticed that 14% (5/35) of our patients showed evidence of the co-expression of several checkpoint molecules, and we compared the prognoses of these patients with those of the other patients; no significant difference was observed. We conducted a statistical analysis of the correlation between staining and baseline clinicopathological characteristics, but we found no significant correlation. However, for this small sample, a tendency for benefiting from anti-PD-1 therapy was observed for patients with positive B7-H3 expression ($p = 0.057$) (Table 1).

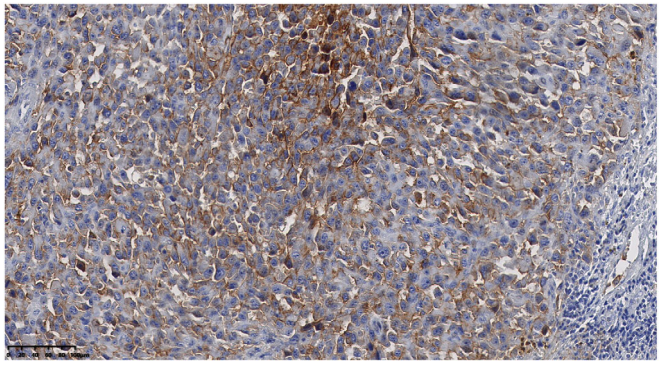

Fig. 1 Microscopical manifestation of programmed cell death 1 ligand-1 (PD-L1) staining for advanced osteosarcoma (200×, Abcam 28–8), expressing PD-L1 in a membranous pattern

Expression of B7 Family Proteins in Tumor-Associated Immune Cells

Next, we evaluated checkpoint molecule expression on tumor-associated immune cells. Only TIM3 was found to be 6% (2/35) expressed on the TILs with low staining intensity. No positive expression was found for the checkpoints CD27, IDO1, and SDF-1. Intriguingly, we noticed that a large portion of our samples (more than half) had background staining, which had then been identified as negative by our pathologists. TIM3, a receptor within the subfamily of TIM (T cell-immunoglobulin-mucin domain) proteins, is another T cell-expressed IgSF protein of significant interest. Multiple TIM3 ligands have been described, including phosphatidylserine, galectin 9 (GAL9), high mobility group protein B1 (HMGB1), and carcinoembryonic antigen-related cell adhesion molecule (CEACAM1). Because the blocking characteristics of therapeutic TIM3-specific antibodies have not been completely described, their mechanism of action is poorly understood, and we did not further explore ligand expression. Interestingly, both of these patients who had TIM3 expression also showed evidence of the co-expression of PD-L1 at low intensity levels, and one even had moderate-intensity expression of B7-H3.

Clinical Significance of B7 Family Protein Co-Expression in Selected Osteosarcoma Patients

We further analyzed the relationship between these high-risk osteosarcoma patients' clinical prognoses and their B7 family ligand/receptor expression. No obvious expression of these molecules and PFS (for chemotherapy resistance) and overall survival (OS) had an inverse association (p all >0.05) (shown in Table 3). However, in this small subset of the study

TABLE 2 Scoring of various checkpoint molecule immunochemistry staining intensitiesfor 35 advanced osteosarcomas*

Checkpoint molecules	Positivity	Staining intensity		
		Low	Moderate	High
B7-H3	10/35 (29%)	1/35 (3%)	6/35 (17%)	3/35 (9%)
CD47	5/35 (15%)	2/35 (6%)	2/35 (6%)	1/35 (3%)
PD-L1	3/35 (9%)	3/35 (9%)	0/35 (0%)	0/35 (0%)
TIM3	2/35 (6%)	2/35 (6%)	0/35 (0%)	0/35 (0%)
TGFβ	2/35 (6%)	0/35 (0%)	1/35 (3%)	1/35 (3%)

Abbreviations: PD-L1, programmed cell death 1 ligand 1; TILs, Tumor-infiltrating lymphocytes.; *If these checkpoint molecules were expressed on the tumor cells, a semiquantitative scoring system (0–<1% = negative/rare, 1–<10% = low, 10–<50% = moderate, and >50% = high) was applied for the evaluation of immune checkpoint ligand expression; If these checkpoint molecules were expressed on the tumor-infiltrating lymphocytes, a semiquantitative scoring system (0–<1% = negative/rare, 1–<10% = low, 10–<50% = moderate, and > 50% = high) was applied for the evaluation of immune checkpoint receptor expression on the surface of the CD3-positive TILs.

TABLE 3 Univariate cox proportional hazards analysis of PFS and OS for patients with advanced osteosarcoma

	PFS		OS	
	Univariate analysis, HR (95% CI)	*p* value	Univariate analysis, HR (95% CI)	*p* value
Sex				
Male	0.45 (0.20–1.02)	0.055*	1.00	0.437*
Female	1.00		2.22 (0.30–16.58)	
Age group				
≥35 years	0.043 (0.000, 196.2)	0.463	0.00 (0.00–.)	0.997*
<35 years	1.00		1.00	
Histopathology		0.980**		0.186**
Conventional	1.00		1.00	
Small cell	0.70 (0.09–5.26)	0.730*	0.00 (0.00–.)	0.998*
Telangiectatic	1.28 (0.16–9.99)	0.811*	22.36 (1.39–359.04)	0.028*
High-grade transformation of a previously low-grade osteosarcoma	0.00 (0.00–.)	0.991*	0.00 (0.00–.)	0.998*
Primary tumor site		0.907**		0.989**
Distal femur	1.00		1.00	
Proximal tibia	0.78 (0.28–2.13)	0.626*	0.00 (0.00–.)	0.998*
Proximal humerus	1.15 (0.32–4.19)	0.829*	0.00 (0.00–.)	0.998*
Proximal femur	1.45 (0.18–11.62)	0.725*	0.00 (0.00–.)	0.999*
Axial skeleton	0.52 (0.14–1.87)	0.314*	1.76 (0.25–12.58)	0.573*
Others	0.00 (0.00–.)	0.994*	0.00 (0.00–.)	0.999*
B7-H3		0.317*		0.760*
Positive	1.61 (0.63–4.08)		1.43 (0.14–14.21)	
Negative	1.00		1.00	
CD47		0.380*		0.997*
Positive	1.63 (0.55–4.83)		0.00 (0.00–.)	
Negative	1.00		1.00	
PD-L1		0.858*		0.217*
Positive	0.88 (0.20–3.78)		4.55 (0.41–50.46)	
Negative	1.00		1.00	
Multipoint positive expression		0.832**		0.988**
More than two checkpoints	0.86 (0.28–2.68)	0.797*	0.00 (0.00–.)	0.997*
One checkpoint	1.28 (0.45–3.60)	0.642*	1.20 (0.12–11.53)	0.877*
Negative for all	1.00		1.00	
Whether checkpoint positive		0.901*		0.773*
At least one positive	1.06 (0.45–2.49)		0.71 (0.07–6.97)	
All negative	1.00		1.00	

Abbreviations: CI, confidence interval; HR, hazard ratio; OS, overall survival; PD-L1, programmed cell death 1 ligand 1; PFS, progression-free survival.; * Covariate Wald *P*-value.; ** Type 3 Wald *P*-value.

population, we noticed that females and telangiectatic subtypes of osteosarcoma had a tendency for poorer PFS or OS, respectively, which was not in accordance with the long-term follow-up results of European and American Osteosarcoma Study-1 (EURAMOS-1).[4] One of the five patients who showed evidence of the co-expression of more than one checkpoint molecule had high levels of B7-H3, CD47, and TGFβ but was negative for PD-L1 (Figure 2A–D). He was diagnosed in November 2015 with proximal femoral osteosarcoma (Figure 2E), which progressed and became chemotherapy-resistant (including adriamycin, cisplatin, high-dose methotrexate, and ifosfamide) in August 2017. In 2017 (3 years ago), he had shown evidence of a high level of PD-L1 expression and had a tumor proportion score (TPS) of 50% (Figure 2F) in his amputation specimen. He had been in stable disease by combination therapy of pembrolizumab and cabozantinib for 1 year (2017–2018) and then single pembrolizumab for more than 1 year (from 2018 to 2020). However, the tumor progressed again in September 2019 in his ipsilateral inguinal lymph nodes, and we performed lesion resection again with IHC staining in the current study. The newly resected lesion was identified as undifferentiated pleomorphic sarcoma (UPS) (Figure 2G) by our pathologists.

Discussion

Our study was the first to investigate multiple checkpoint molecules for advanced osteosarcoma patients. However limited positive rates and intensity were observed, which indicated that single immune checkpoint blockade might not be an option for improving prognosis for the overall population. In the meantime, we still observed that B7-H3 seemed to be of relatively high expression on tumor membranes, which should be further studied as a promising checkpoint target for osteosarcoma. Selected patients might benefited

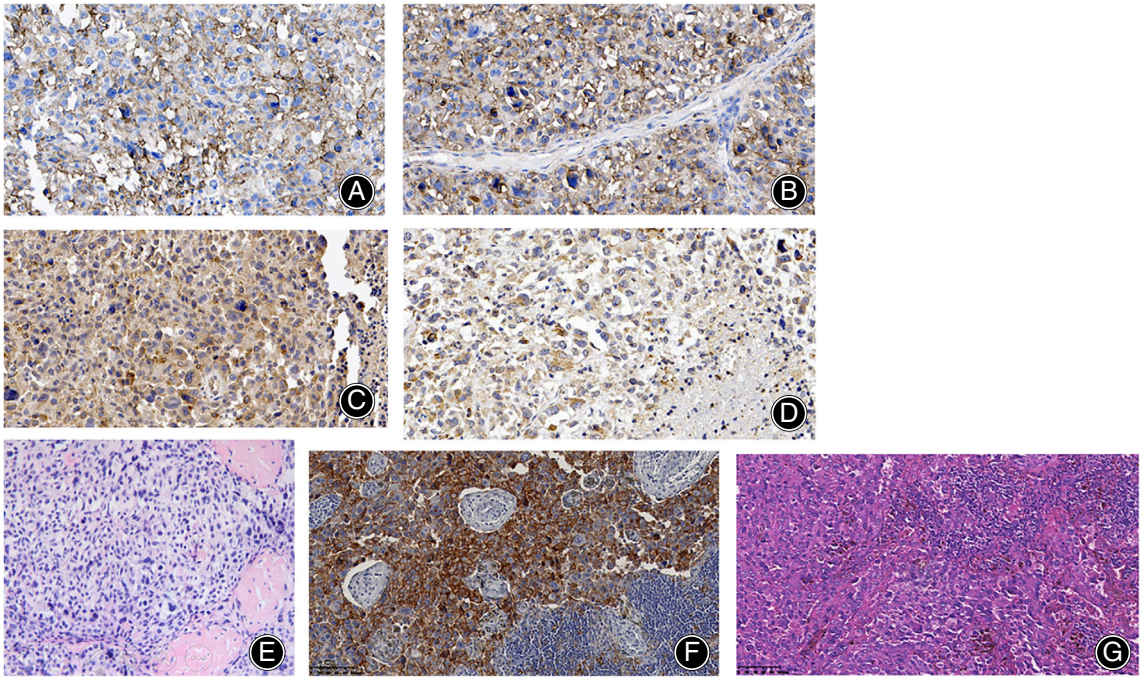

Fig. 2 Microscopical manifestation of one patient's sample with multiple checkpoint modules (200×). (A), High staining intensity of B7-H3 on tumor cells for his current sample of lymph nodes; (B), High staining intensity of CD47 on tumor cells for his current sample of lymph nodes; (C), High staining intensity of TGFβ in diffuse cytoplasmic tumor cells for his current sample of lymph nodes; (D), Negative expression of programmed cell death 1 ligand-1 (PD-L1) on tumor cells for his current sample of lymph nodes; (E), Previous diagnosis with high-grade osteosarcoma in proximal femur with hematoxylin and eosin (H&E) staining; (F), Formerly with high staining intensity of PD-L1 (DAKO 22C3) in his amputation specimen; (G), Current specimen of his metastatic lymph nodes was re-diagnosed with undifferentiated pleomorphic sarcoma (H&E)

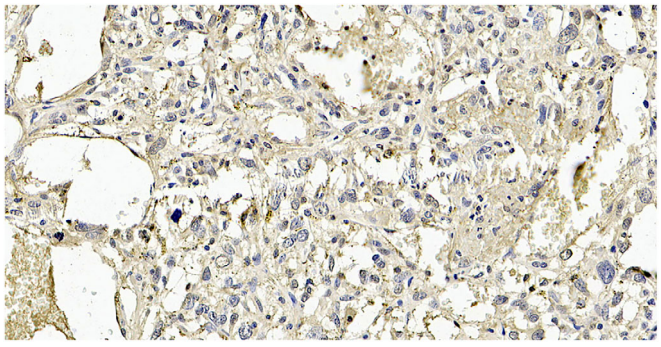

Fig. 3 Negative expression of TIM3 but with background staining in one sample (200×)

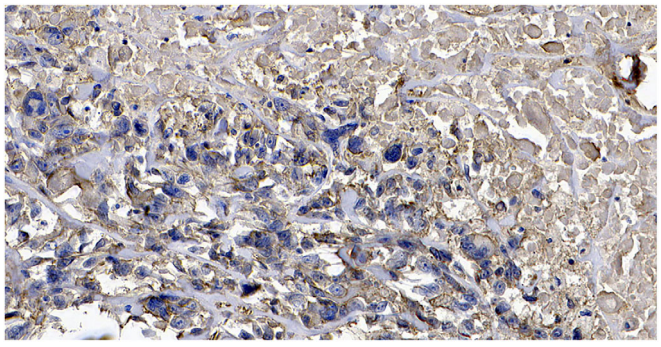

Fig. 4 Negative expression of B7-H3 but with background staining in one sample (200×)

from anti-PD-1 therapy by simple IHC; however, secondary resistance finally developed with long-term follow-up and new checkpoint molecules werethen found positive, which indicated the mechanism of drug resistance.

Multiple Checkpoint Molecules for Osteosarcoma

According to limited publications,[10,12,16,18–23] the current study reported the lowest rate of positive checkpoint molecule expression in advanced osteosarcoma, with B7-H3, CD47, PD-L1, TIM3, and TGFβ levels as low as 29%, 15%, 9%, 6%, and 6%, respectively. Pinto et al.[26] also described childhood cancers in which PD-L1 was detected in the majority of patients, including those with Ewing sarcoma (65%), neuroblastoma (77%), and osteosarcoma (80%). Koirala et al.[12] showed that patients with PD-L1 expression in osteosarcomas were significantly associated with worse 5-year EFS than patients without PD-L1 expression in osteosarcomas (25.0% vs. 69.4%, $p = 0.014$). We conducted a

phase II trial for apatinib plus camrelizumab in patients with advanced osteosarcoma, and those who were positive for PD-L1 (with a PD-L1-positive rate of 21.4%) seemed to benefit from combination therapy more and have a longer PFS.[10] The reported positivity of PD-L1 in osteosarcoma seems to be variable (Table S2). The different anti-PD-L1 antibodies used in each study differ in their targeted epitope, and binding affinity may be one of the major reasons for the differences.[8–10,12,13,18,20,26] For the other checkpoints, Mochizuki et al.[21] reported that 100% of osteosarcomas expressed HVEM, and moderate to high levels of GAL9, which was a ligand for TIM3 were observed in 36% of osteosarcomas, while Piperdi et al.[22] suggested that CD47 was expressed in the TMA of 87.7% of 81 osteosarcoma specimens; however, all these checkpoint molecules seemed to be only scarcely expressed on our specimens other than B7-H3 and we had tried further to explore the different expression on survival but without any positive results. This might just be the real situation for heavily treated advanced osteosarcomas based on the very few immune-responsive cases in the past decades of trials for advanced osteosarcoma.[7–10]

In this study, we demonstrated the co-expression of checkpoint molecules in 35.7% (5/14) of our patients, usually the co-expression of B7-H3 and CD47 (in four patients) or TIM3 and PD-L1 (in two patients). False-negative interpretation might still exist, although both of the pathologists strictly followed the protocols and if any costaining for tumor cells and TILs was present, they discussed the findings with each other or even a third pathologist to identify whether this was background staining. However, extensive background staining was eventually affirmed and concluded to be negative (shown in Figures 3 and 4). For TIM3, CD27, IDO1, and SDF-1, which are expressed on tumor-associated immune cells, we did not first examine TILs with CD3 staining, as some of the literature has reported. Microscopically, TILs sometimes could be difficult to identify morphologically compared with HE staining, which might also be one of the reasons for the low positive rates observed. Furthermore, a significant proportion of osteosarcoma specimens needed to be decalcified, which also might have resulted in the loss of low molecular expression levels observed in the current study. However, Chen et al.[18] reported that both decalcified and non decalcified specimens showed evidence of the expression of PD-1 and PD-L1, and decalcification did not cause much change in PD-L1 expression.

At the same time, we performed IHC analyses of the specimens after multiple lines of treatment, which contributed the most to this low positive rate. From our perspectives, chemotherapy for osteosarcoma was so intense that it might tremendously destroy tumor-associated immune cells in the tumor immune microenvironment. It is suspected that these checkpoint molecules might fade away after multiple lines of treatment. Thus, the positive rate might be much lower than that reported by Mochizuki et al.,[21] who reported that they investigated the initial treatment samples preserved at diagnosis.

Change of Checkpoint Molecules during Treatment Courses

On the basis of our experience, only sporadic patients could truly benefit from anti-PD-1 therapy, and these patients usually showed evidence of the co-expression of multiple checkpoint molecules. In clinical practice, with so few patients who are responsive to immunotherapy, we usually need to observe patients who are on combination therapy with checkpoint inhibitors, such as chemotherapy or targeted therapy, for longer than a year. In addition, PFS was a more appropriate indicator than the objective response rate for advanced osteosarcoma clinical evaluation because most of our target lesions had calcification and ossification.[5] Although tumor cells sometimes vanished pathologically after treatment, the remains of ossification were still there for clinical evaluation. One of the five patients who had evidence of the coexpression of more than one checkpoint molecule had high-intensity staining for B7-H3, CD47, and TGFβ but was negative for PD-L1 (shown in Figure 1); however, 2 years ago, he had high expression of PD-L1 on his tumor cells. The case of this patient demonstrates the evolutionary course of secondary drug resistance to anti-PD-1 antibodies. Later, anti-TGFβ, anti-B7-H3, or anti-CD47 therapy might be practicable for this patient.

B7-H3 for Osteosarcoma

Our study showed that B7-H3 was obviously overexpressed on tumor cell membrane among all the other immune checkpoint molecules as a member of the B7 ligand family, which was also in accordance with the recent publication by Wang et al.,[27] who used an integrated proteomic and transcriptomic surfaceome profiling approach to identify cell-surface proteins that are highly expressed in osteosarcoma. Nguyen et al.[28] and Wang et al.[29] had also tried to identify the positive rate and intensity of B7-H3 for osteosarcoma patients and found it as an attractive target, which encouraged Kendsersky et al.[30] to do B7-H3-targeting antibody-drug conjugates against preclinical osteosarcoma models with significant anti-tumor activity. However, the mechanism for why B7-H3 is so highly expressed on osteosarcoma membrane is still unkown. Due to the small size of this study, we did not find obvious survival difference for this cohort of patients. Nevertheless, we further investigated the expression of B7-H3 in RNA sequencing data in TARGET (Therapeutically Applicable Research To Generate Effective Treatments) database and observed obvious differences in PFS ($p = 0.043$, Figure S2). We had planned further to investigate the mechanism of how B7-H3 took part in the regulation of tumor microenvironment by experiments in vivo and in vitro and tried to identify this molecule with larger sample by paired specimens to observe its clinical significance.

Limitation and Strengths

The limitations of our analysis are that our study was performed at a single institution, utilized a retrospective cohort, and had a relatively small sample size, which precluded us

from clarifying whether subtypes with a poor prognosis express checkpoint molecules more frequently than others. Second, we did not collect patient paired samples from biopsy samples before treatment or progressive samples after multiple treatments to observe the evolution of the expression of these checkpoint molecules. Third, we did not examine the expression of tumor-associated checkpoint molecules together with their TILs expressing corresponding receptors for confirmation, which could have made the results more convincing. Fourth, TILs were subjectively identified microscopically by two pathologists individually. The pathologists did not use CD3 or CD8 staining to identify those TILs, which could make the interpretation difficult and the results unreliable.

Nevertheless our study was the first to investigate multiple checkpoint molecules, especially those new molecules, which all had drugs now being investigated in phase IA or IB clinical trials in China, for advanced osteosarcoma patients. Also, we had long-term follow-up time for all these patients that correlated their IHC results with clinical courses, which were all detailed and ample. Overall it made this study not just a basic pathological examination but provided 35 stories involving clinical treatment courses for these patients.

Conclusions

In summary, the present study highlights that only a small subset of progressive osteosarcomas, which had been heavily treated, expressed tumor immune-associated checkpoint molecules. Those osteosarcomas that had ever been responsive to anti-PD-1 therapy usually had evidence of the coexpression of multiple checkpoint molecules, which might also be the reason for secondary drug resistance.

Authorship Declaration

We acknowledge that all authors listed meet the authorship criteria according to the latest guidelines of the International Committee of Medical Journal Editors and that all authors are in agreement with the manuscript.

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Conflicts of Interest

The authors declare that there is no conflict of interest.

Authors' Contributions

Conception and design: LX, CC, and WG; Provision of study materials or patients: XL, JX, and XS; Collection and assembly of data: LX, CC, and XL; Pathological review of the slides of this study: KS; Clinical evaluation of the study: RY, XT, and WG; Laboratorial work and molecular biological analysis of this study: CC and XL; Data analysis and interpretation: LX, JX, and WG; Manuscript writing: LX; Final approval of the manuscript: LX, CC, XL, JX, XS, KS, RY, XT, and WG; Accountability for all aspects of the work: LX, CC, XL, JX, XS, KS, RY, XT, and WG.

Informed Consent

We promised to ensure patients' data confidentially. However, patient data will be made available in a disguised manner, including data dictionaries, for approved data sharing requests. Individual data will be shared that underlie the results reported in this article after the deidentification and normalization of information (text, tables, figures, and supplementaries). The statistical analysis plan will also be available upon request. Anonymized data will be available beginning 3 months later and ending 2 years after the publication of this article to researchers after methodological review of the proposed analysis plan by the directors of the Musculoskeletal Tumor Center of Peking University People's Hospital. Proposals should be directed to xie.lu@hotmail.com. To gain access, data requestors will need to sign a data access agreement.

Ethical Statement

Institutional review board approval (No. 2018PHB059-01) to perform this research and review the patients' medical records and radiographic materials was obtained from Peking University People's Hospital. The outcome data were then retrospectively combined. Written informed consent was obtained in Chinese. This study is registered in Clinicaltrials.gov with identifier of NCT03582527 and the first date of registration is July 11th, 2018 (11/07/2018). This study was complied with good clinical practice guidelines and the Declaration of Helsinki. Trial registration: This study is registered in Clinicaltrials.gov with identifier of NCT03582527 and the first date of registration is July 11th, 2018 (11/07/2018).

Disclosure Statement

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Supporting Information

Additional Supporting Information may be found in the online version of this article on the publisher's web-site:

Figure S1. The first line chemotherapy protocol for osteosarcoma patients in Peking University People's Hospital.

Figure S2. Survivals for patients in TARGET (the Therapeutically Applicable Research to Generate Effective Treatments) data with B7-H3 different expressions in RNA sequencing (98 patients). Figure A Progression-free survival for first-line chemotherapy; Figure B Overall survival.

Table S1. Details of the primary antibodies used for immunohistochemistry and antigen retrieval methods

Table S2. Summary of all previous studies for immunochemistry staining of various checkpoint molecules in osteosarcoma specimens

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