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Robust Programmed Cell Death-1 Expression in a Subset of Ewing Sarcoma in Contrast to Ewing-like Sarcoma

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Abstract

Background: Programmed cell death-1 (PD-1) and programmed cell death ligand-1 (PD-L1) expression may be surrogate markers for a response to immune checkpoint inhibitors. However, PD-1 and PD-L1 expressions in Ewing and Ewing-like sarcoma are poorly defined.

Methods: Immunohistochemistry analysis was performed on formalin-fixed, paraffin-embedded pre-therapeutic tumor biopsies from patients with Ewing (n=11) and Ewing-like sarcoma (n=6). For the assessment of staining for PD-1/PD-L1 in tumor cells, rates forPD-1/PD-L1 positive tumor cells within total tumor cells: 0% as (–), >0% to <10% as (+), ≥ 10% to <50% as (++), ≥ 50% to <90% as (+++), and ≥ 90% as (++++), were used.

Results: None of Ewing (n=11) and Ewing-like (n=6) sarcoma cells expressed PD-L1. PD-1 expressing tumor infiltrating lymphocytes were not prevalent both in Ewing and Ewing-like sarcoma. However, PD-1 was robustly expressed in 4 of 11 (36.4%) Ewing sarcoma tumor cells with ≥ 50% to <90% (+++, n=3) or ≥ 10% to <50% (++, n=1). In contrast, none of 6 Ewing-like sarcoma tumor cells expressed PD-1.

Conclusion: In this study, PD-L1 expression was not observed in any of Ewing and Ewing-like sarcoma tumor cells. PD-1 was overexpressed in a subset of Ewing sarcoma, whereas not in Ewing-like sarcoma.

Keywords: Programmed cell death-1; Ewing sarcoma; Ewing-like sarcoma

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Introduction

Ewing sarcoma is a classic, malignant, small round cell sarcoma characterized by chromosomal translocations between *EWSR1* and the *ETS* family of transcription factors [1]. A subset of small round cell tumors lacks the specific *EWSR1-ETS* transcript while morphologically and immunohistochemically resembling Ewing sarcoma. These *EWSR1-ETS* negative small round cell sarcomas are referred to as Ewing-like sarcoma for practical purposes and managed similarly to Ewing sarcoma. Recently, *CIC-DUX4*, *BCOR-CCNB3,* and other less common fusions have been found among these *EWSR1-ETS* negative (Ewing-like) small round cell sarcomas [2-4]. Patients with disseminated Ewing and Ewing-like sarcoma often have a dismal outcome [5-8]. Therefore, novel therapeutic strategies are clearly needed for these patients.

Immune checkpoint inhibitors have become emerging therapeutic strategies for various types of adult cancer including, melanoma, non-small cell lung cancer, and renal cell carcinoma [9]. However, PD-1 and PD-L1 expression patterns in Ewing and Ewing-like sarcoma are poorly defined. Therefore, the present study aimed to investigate the expression patterns of PD-1 and PD-L1 in Ewing and Ewing-like sarcoma by immunohistochemistry analysis along with its clinical characteristics.

Materials and Methods

Immunohistochemistry analysis was performed on formalin-fixed, paraffin-embedded (FFPE) pre-therapeutic tumor biopsies from patients with Ewing (n=11) and Ewing-like sarcoma (n=6) diagnosed at Kobe Children's Hospital (Kobe, Japan) from 2003 to 2017. Diagnosis of Ewing sarcoma was confirmed by reverse transcription polymerase chain reaction (RT-PCR) for *EWSR1-FLI1*/*ERG*, *EWSR1* split FISH analysis, and/or immunohistochemical reactivity for combination of NKX2.2 and CD99. Ewing-like sarcoma was defined as round cell sarcoma without *EWSR1*rearrangementsthat

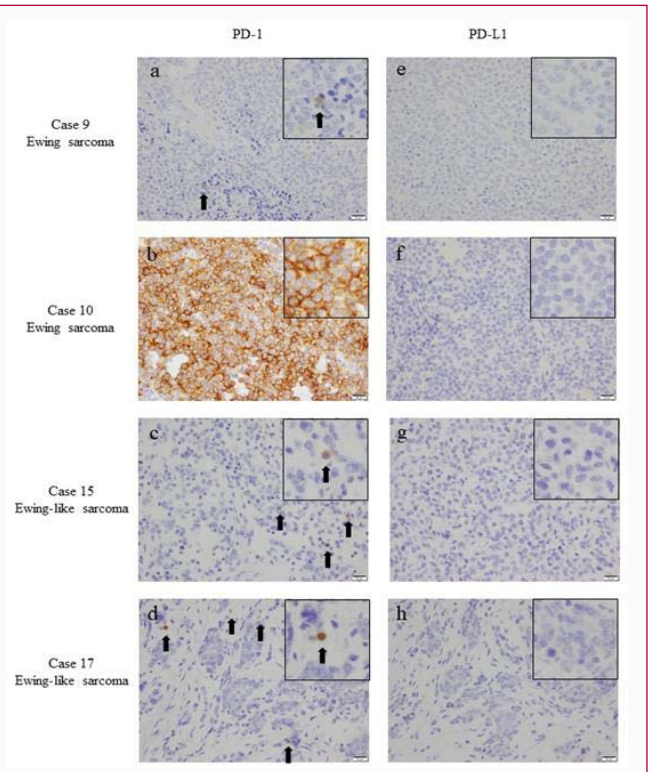

Figure 1: Representative images of immunostaining for PD-1 (a-d) and PD-L1 (e-h). Boxed images in the right upper corner are the magnification of each image. Ewing sarcoma tumor cells were negative for PD-1 in Case 9 (a), but strongly positive for PD-1 in Case10 (b). Ewing-like sarcoma tumor cells were negative for PD-1 in Case 15 and 17 (c, d). Arrowheads indicate "rare" PD-1 positive tumor-infiltrating lymphocytes in Case 9, 15, and 17 (a, c, d). Ewing and Ewing-like sarcoma tumor cells were negative for PD-L1 (e-h).

cannot be classified into any of other categories (undifferentiated/unclassified sarcoma based on the 2013 World Health Organization classification). Immunostaining was performed with antibody against PD-1 (NAT105; Abcam, Cambridge, UK) and PD-L1 (E1L3N; Cell Signaling Technology, Danvers, MA, USA) using Leica Bond-Max Automated Immunohistochemical Stainer and Bond Polymer Refine Detection Kit (Leica Bios stems, Wetzler, Germany) according to manufacturer's instructions. PD-1 immunostaining was performed using heat-induced epitope retrieval for 20 min with pH 6.0 buffer and 1:50 antibody dilution. PD-L1 immunostaining was performed using heat-induced epitope retrieval for 20 min with pH 9.0 buffers and 1:200 antibody dilutions. For the assessment of staining for PD-1/PD-L1 in tumor cells, rates for PD-1/PD-L1 positive tumor cells within total tumor cells: 0% as (–), >0% to <10% as (+), ≥ 10% to <50% as (++), ≥ 50% to <90% as (+++), and ≥ 90% as (++++), were used. PD-1 positive tumor infiltrating lymphocytes (TILs) per tissue section were semi-quantified as, "none", "rare", "many", and "massive". This study was approved by the institutional review board of the Kobe Children's Hospital. This study was conducted in compliance with the guidelines in the Declaration of Helsinki.

Results

Ewing sarcoma

The age at diagnosis ranged from 0 to 19 years (mean 8.5 years) with 5 males and 6 females. The primary disease site was skeletal in 7 of 11 (64%) patients and extra-skeletal in 4 of 11 (36%) patients (Table 1). PD-1 positive TILs were "rare" in 5 of 9 (56%), and "none" in 4 of 9 Ewing sarcoma samples (Figure 1a, Table 1). PD-1 was strongly expressed in 4 of 11 (36%) Ewing sarcoma tumor cells, with ≥ 50% to <90% (+++, n=3) (Case 4, 10, and 11) or ≥ 10% to <50% (++, n=1) (Case 3) (Figure 1b, Table 1). PD-1 expression in TILs was not evaluable in Case 4 and 10 because of strong PD-1 expression in tumor cells (Figure 1b). PD-L1 expression was not observed in tumor cells in any of the 11 samples (0%) with Ewing sarcoma (Figure 1e, 1f, Table 1). Two of 4 patients with PD-1 expressing Ewing sarcoma presented with disseminated disease (Case 4 and 10), while 2 of 7 patients with Ewing sarcoma without PD-1 expression in tumor cells presented with disseminated disease (Case 1 and 9). Overall, 7 of 11 patients (64%) are alive at the last follow-up.

Ewing-like sarcoma

The age at diagnosis ranged from 0 to 20 years (mean 8.5 years) with 4 males and 2 females. The primary disease site was extra-skeletal in all 6 patients (100%), while one patient (Case 17) presented with multiple bone metastases. BCOR was overexpressed in tumor cells in 4 patients (Case 13, 14, 15, and 17), suggesting sarcoma with *BCOR* genetic abnormalities [10]. In one patient (Case 17), primary disease site was in the pancreas tail. Other primary disease sites were intraperitoneal dissemination (Case 13) and retroperitoneal dissemination (Case 14), respectively. *BCOR*-ITD was confirmed by RT-PCR in Case 15. The primary disease site was pharynx in the infant (Case 15) with *BCOR*-ITD sarcoma. *CIC-DUX4* fusion was confirmed by RT-PCR in Case 16. The primary disease site was intrathoracic dissemination (Case 12) and the vulva (Case 16), respectively.

PD-1 positive TILs were "rare" in 4 of 6 (67%) Ewing-like sarcoma samples (Figure 1c, 1d, Table 1), and "none" in 2 of 6 samples. PD-1 was not expressed in Ewing-like sarcoma tumor cells in any of the 6 samples (0%) (Figure 1c, 1d). In addition, PD-L1 was not expressed in tumor cells in any of the 6 samples (0%) with Ewing-like sarcoma (Figure 1g, 1h). Overall, 4 of 6 patients (67%) were alive at the last follow-up.

Discussion

PD-L1 is expressed in multiple soft tissue sarcomas, whereas PD-1 is mainly expressed in TILs [11]. The reported PD-L1 expression in Ewing sarcoma cells is highly variable between studies, ranging from 0% to 67% (Table 2) [11-20]. The variation among studies may be due to different clones used to detect PD-L1, different cut-off values to determine positivity, and heterogeneity within tumors. We chose the PD-L1 cytoplasmic domain-specific monoclonal antibodies (E1L3N) that provide clear membranous staining and lower backgrounds [21]. In this study, PD-L1 expression was not observed in any of Ewing sarcoma tumor cells.

BCOR-ITD has recently been identified in soft tissue round cell sarcoma of infancy, showing overlapping clinicopathologic features with clear cell sarcoma of the kidney [22]. *BCOR*-ITD, was confirmed in Case 15. Case 13, 14, 15, and 17 were surmised to be sarcoma with *BCOR* genetic abnormalities based on the BCOR overexpression. *CIC-DUX4* was confirmed by RT-PCR in Case 16. In our study, PD-L1 and PD-1 were not expressed in Ewing-like sarcoma tumor cells. Furthermore, PD-1 expressing TILs were not predominant in Ewing-like sarcoma. One previous study reported the lack of PD-L1 expression in Ewing-like sarcoma cells, while PD-1 expression was not assessed in that study [20] (Table 2).

In the present study, PD-1 overexpression was observed in 36% (4/11) of Ewing sarcoma cells. Previous studies have demonstrated the PD-1 expression in 19% to 60% of Ewing sarcoma tumor cells (Table

Table 1: Clinical characteristics and PD-1/PD-L1 staining results in Ewing and Ewing-like sarcoma (–); 0%, (++); ≥ 10% to <50% positive, (+++); ≥ 50% to <90% positive.
DOD: Died of Disease; ELS: Ewing-like Sarcoma; ES: Ewing Sarcoma; F: Female; M: Male; N/E: Not Evaluable; PD-1: Programmed cell Death-1, PD-L1: Programmed cell Death Ligand-1; TILs: Tumor Infiltrating Lymphocytes

Case	Age (years)	Gender	Diagnosis	Disease sites	PD-L1 in tumor cells	PD-1 in tumor cells	PD-1+ TILs	Status at the last follow-up
1	6	M	ES	scapula, lungs	(–)	(–)	none	DOD (12months)
2	19	M	ES	rib	(–)	(–)	none	DOD (110months)
3	0	F	ES	posterior neck	(–)	(++)	rare	DOD (35 months)
4	13	M	ES	tibia, lumber vertebrae, rib, occipital bone, lower limb, lungs	(–)	(+++)	N/E	DOD (19 months)
5	10	F	ES	paraspinal	(–)	(–)	none	Alive (90 months)
6	2	F	ES	cranial base	(–)	(–)	rare	Alive (83 months)
7	4	F	ES	posterior cranial fossa	(–)	(–)	rare	Alive (72 months)
8	6	F	ES	cervical vertebrae	(–)	(–)	none	Alive (70 months)
9	4	M	ES	gluteal region, lungs	(–)	(–)	rare	Alive (26 months)
10	14	M	ES	pubis, ilium, ischium, femur, rib, cranial bone, thoracic vertebra, sacrum, obturator muscle, adductor magnus muscle, testis cord, lungs	(–)	(+++)	N/E	Alive (31 months)
11	16	F	ES	abdominal wall	(–)	(+++)	rare	Alive (22 months)
12	16	M	ELS	intrathoracic dissemination	(–)	(–)	rare	DOD (23 months)
13	0	M	ELS	intraperitoneal dissemination	(–)	(–)	none	DOD (14 months)
14	0	F	ELS	retroperitoneal dissemination	(–)	(–)	none	Alive (44 months)
15	0	M	ELS	pharynx	(–)	(–)	rare	Alive (24 months)
16	15	F	ELS	vulva	(–)	(–)	rare	Alive (23 months)
17	20	M	ELS	pancreas tail, intraperitoneal dissemination, supraclavicular node, rib, lumber vertebrae, ilium, ischium	(–)	(–)	rare	Alive (18 months)

Table 2: Summary of published studies on PD-1/PD-L1 immunohistochemistry in Ewing and Ewing-like sarcoma. N/A: Not Assessed.

Ewing sarcoma			Ewing-like sarcoma			
PD-L1 in tumor cells	PD-1 in tumor cells	PD-1+ TILs	PD-L1 in tumor cells	PD-1 in tumor cells	PD-1+ TILs	References
4/6 (67%)	N/A	4/6 (67%)	N/A	N/A	N/A	11
0/32 (0%)	6/32 (19%)	4/32 (12%)	N/A	N/A	N/A	12
71/370 (19%)	95/370 (26%)	53/347 (15%)	N/A	N/A	N/A	13
0/60 (0%)	27/47 (57%)	4/52 (8%)	N/A	N/A	N/A	14
13/20 (65%)	12/20 (60%)	12/12 (100%)	N/A	N/A	N/A	15
6/18 (33%)	N/A	N/A	N/A	N/A	N/A	16
8/14 (57%)	N/A	N/A	N/A	N/A	N/A	17
1/8 (13%)	2/8 (25%)	2/8 (25%)	N/A	N/A	N/A	18
0/25 (0%)	N/A	N/A	N/A	N/A	N/A	19
1/8 (13%)	N/A	N/A	0/6 (0%)	N/A	N/A	20
0/11 (0%)	4/11 (36%)	5/9 (56%)	0/6 (0%)	0/6 (0%)	4/6 (67%)	Our study

2) [12-15,18]. Encouraging response by PD-1 blockade was reported in a single patient with recurrent disseminated Ewing sarcoma with unknown PD-1 expression [23]. However, recent clinical trials on checkpoint inhibitors for soft-tissue sarcoma showed no objective response in a cumulative 15 patients with Ewing sarcoma [24,25]. The PD-L1 expression on neoplastic cells is associated with better clinical response to PD-1 blockade in many clinical trials [26-28]. The PD-1 expression in Ewing sarcoma cells might not necessarily correlate with or predict the efficacy of checkpoint inhibitors. However, the use of checkpoint inhibitors in patients with sarcoma deserves further evaluation. As our data is limited by the small number of patients and retrospective design, we cannot make definitive conclusions. Larger studies are needed to assess the expression patterns of PD-1 and PD-L1 in Ewing and Ewing-like sarcoma and the clinical efficacy of PD-1 blockade for these diseases.

Conclusion

In this study, PD-L1 expression was not observed in both Ewing and Ewing-like sarcoma cells. PD-1 expressing tumor infiltrating lymphocytes were not prevalent in Ewing and Ewing-like sarcoma. PD-1 was overexpressed in a subset of Ewing sarcoma, whereas not in Ewing-like sarcoma.

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