Integrative assessment of clinicopathological parameters and the expression of PD-L1, PD-L2 and PD-1 in tumor cells of retroperitoneal sarcoma

MAKITO MIYAKE[1], YUKI ODA[1], NOBUTAKA NISHIMURA[1], YOSUKE MORIZAWA[1],
SAYURI OHNISHI[1], KINTA HATAKEYAMA[2], TOMOMI FUJII[2], SHUNTA HORI[1], DAISUKE GOTOH[1],
YASUSHI NAKAI[1], SATOSHI ANAI[1], KAZUMASA TORIMOTO[1], SHINJI TSUKAMOTO[3],
HIROMASA FUJII[3], AKIRA KIDO[3], KANYA HONOKI[3], YOSHIAKI MATSUMURA[4],
EIJIRO OKAJIMA[5], NOBUMICHI TANAKA[1,6] and KIYOHIDE FUJIMOTO[1]

Departments of [1]Urology, [2]Diagnostic Pathology and [3]Orthopedic Surgery, Nara Medical University, Kashihara, Nara 634-8522;
[4]Department of Urology, Nara Prefecture General Medical Center, Nara 630-8581; [5]Department of Urology, Nara City
Hospital, Nara 630-8305; [6]Department of Prostate Brachytherapy, Nara Medical University,
Kashihara, Nara 634-8522, Japan

Abstract. Evidence is limited regarding the immunologic profile and immune microenvironment of soft tissue sarcoma subtypes. The aim of the present study was to describe the clinical significance and prognostic implications of PD-L1, PD-L2, and PD-1 in patients with retroperitoneal sarcoma (RSar). In this retrospective, multicenter, collaborative study, medical charts were reviewed and the immunohistochemical staining results of resected tissue specimens from 51 patients with RSar were examined. Immunohistochemical staining was performed with primary antibodies against PD-L1, PD-L2, PD-1, and Ki-67. The correlations between the baseline clinical parameters and expression levels of the four molecules in sarcoma cells were evaluated, and their prognostic values after tumor resection were assessed. Dedifferentiated liposarcoma (41%), leiomyosarcoma (20%), and undifferentiated pleomorphic sarcoma (16%) were the three major types identified. Dedifferentiated liposarcoma and leiomyosarcoma showed higher levels of PD-L1 expression than did other sarcomas. The Spearman correlation analysis revealed that baseline serum lactate dehydrogenase levels were moderately and positively correlated with PD-L1 (P=0.02, r=0.41) and PD-L2 (P=0.006, r=0.47) expression. The median recurrence-free and disease-specific survival was 58 and 16 months, respectively, during the 29-month median follow-up after surgery. On univariate analysis, a higher expression level of PD-1 was associated with a higher risk of recurrence, whereas multivariate analyses revealed that independent predictors of recurrence-free and disease-specific survival indicated a high expression of Ki-67 (P=0.03; hazard ratio, 2.29 vs. low expression) and prognostic stage IIIB (P=0.04; hazard ratio, 5.11 vs. stage I-II), respectively. Findings of the current study provide novel insights about the prognostic value of PD-L1, PD-L2, and PD-1 expression in RSar. Serum lactate dehydrogenase levels constitute a potential predictor of PD-L1 and PD-L2 expression levels in RSar. Further investigations are needed to determine the immunologic landscape of RSar and provide a foundation for therapeutic intervention using immune checkpoint inhibitors.

Correspondence to: Dr Makito Miyake, Department of Urology, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan
E-mail: makitomiyake@yahoo.co.jp

Abbreviations: RSar, retroperitoneal sarcoma; WDLPS, well-differentiated liposarcoma; DDLPS, de-differentiated liposarcoma; UPS, undifferentiated pleomorphic sarcoma; LMS, leiomyosarcoma; ICI, immune checkpoint inhibitors; PD-1, programmed cell death 1; PD-L1, program cell death ligand-1; PD-L, program cell death ligand-2; IHC, immunohistochemical; Hb, hemoglobin; LDH, lactate dehydrogenase; CRP, C-reactive protein; NLR, neutrophil-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; MLR, monocyte-to-lymphocyte ratio; CT, computed tomography; FNCLCC, French Federation Nationale des Centres de Lutte le Cancer; IQR, interquartile range; RFS, recurrence-free survival

Introduction

Soft tissue sarcomas can occur in any anatomic region. Those arising from the retroperitoneal cavity (retroperitoneal sarcoma; RSar) are rare, aggressive, heterogeneous malignancies,

accounting for approximately 15% of all soft tissue sarcomas (1). As RSar-induced symptoms are frequently nonspecific and painless, the median size at first diagnosis ranges from 15 to 18 cm (2). Enlarged RSars tend to invade the surrounding vital organs, including the kidneys, liver, pancreas, duodenum, small intestine, colon, inferior vena cava, and aorta, making complete surgical resection difficult in clinical practice (3). The poor prognosis of RSar has been driving the continual efforts for determining the prognostic factors (1,3-5), developing the molecular classification (6,7), and establishing perioperative interventions (8,9) and novel antitumor drugs and regimens (10-12). RSar is characterized by many different cell types, resulting in a wide variety of histological entities such as well-differentiated liposarcoma (WDLPS), dedifferentiated liposarcoma (DDLPS), undifferentiated pleomorphic sarcoma (UPS), and leiomyosarcoma (LMS). Considering the disease heterogeneity, it is very complicated to define and manage the treatment strategies.

As some patients with unresectable, treatment-refractory soft-tissue sarcoma respond to immune checkpoint inhibitors (ICIs) that target programmed cell death 1 (PD-1), programmed cell death ligand-1 (PD-L1), and cytotoxic T-lymphocyte antigen, the perioperative administration of ICIs would be beneficial for treatment-naive patients with less tumor burden (13). Several prospective clinical trials are ongoing to verify the efficacy of ICIs and/or radiotherapy in the neoadjuvant settings for patients with surgically resectable RSar (8,13). The immunologic profile, copy number alteration, and tumor mutation burden are useful predictive factors of the response to ICIs (14). Although the prognostic impact and biological role of PD-1 and PD-L1 in sarcomas have been investigated in previous studies including meta-analyses (15,16), only a few studies have focused on the immunologic profile of RSar.

In the current study, we evaluated the clinical significance and prognostic implications of PD-L1, PD-L2, PD-1, and Ki-67 expression in patients with RSar. It is well known that PD-L1 binding to PD-1 is a negative regulator of T-cell responses and contributes to immune escape in the tumor microenvironment (13). By contrast, the clinical and biological role of PD-L2 in tumor immunity remains unclear, especially in RSar (16). Thus, to better understand the role and prognostication of the PD-L1/PD-L2/PD-1 axis in RSar, we conducted this multicenter collaborative study of the Nara Urological Research and Treatment Group (NURTG) by performing immunohistochemical (IHC) staining analysis of surgically resected tissue specimens.

Materials and methods

Patient selection and data collection. This study was approved by the ethics committee of the Nara Medical University, and informed consent from the participants was obtained in the form of opt-out in the outpatient clinic and on the web-site (reference ID: 1256/1966). Between January 2000 and September 2019, a total of 64 patients were diagnosed with primary RSar at the Departments of Urology or Orthopedic Surgery of Nara Medical University Hospital, Urology of Nara Prefecture General Medical Center, and Urology of Nara City Hospital. Of the 64 patients, 9 were excluded owing to insufficient follow-up data. Four patients were excluded because only tissue biopsy, not surgical tumor resection, was

performed; finally, 51 patients with RSar who underwent surgical tumor resection were included in the analysis. The baseline clinicopathological information and follow-up data were collected by retrospectively reviewing the medical charts. The blood parameters included the levels of hemoglobin (Hb), albumin, C-reactive protein (CRP), and lactate dehydrogenase (LDH), calcium adjusted by albumin level (hereafter referred to as adjusted Ca), the neutrophil-to-lymphocyte ratio (NLR), the platelet-to-lymphocyte ratio (PLR), and the monocyte-to-lymphocyte ratio (MLR). All patients underwent contrast-enhanced computed tomography and/or magnetic resonance imaging prior to surgery to determine the tumor location and size.

Surgical resection of RSar. Complete surgical resection is among the principal treatment modalities for RSar. The resection of contiguous organs, major blood vessels, and major muscles is associated with a higher rate of complete resection, thereby resulting in improved disease-free survival (3,17). A previous study using a nationwide database revealed that contiguous organ resection was not associated with increased postoperative morbidity or severe morbidity (17). The extent of surgical resection depended on the attending physicians and surgeons in our cohort. We reviewed the contiguous organs, vessels, and muscles that were resected concomitantly. The resection of a tumor with at least one organ, major vessel, or major muscle was defined as 'extended resection'.

Pathological evaluation and tumor staging. All hematoxylin and eosin-stained (H&E) specimens obtained via surgical resection or biopsy (image-guided core needle biopsy or open/laparoscopic excision biopsy) were re-assessed independently by two experienced pathologists (K.H. and T.F.) considering the tumor histology, tumor-cell-free surgical margins, and tumor grade according to the French Federation Nationale des Centres de Lutte le Cancer (FNCLCC) system (18). The resection margins were coded by using the residual tumor (R) classification on the basis of the macroscopic and microscopic evaluation (19), and they were categorized into one of the following three categories: Microscopically negative (R0), microscopically positive (R1), or grossly positive (R2). TNM categories were classified and the prognostic stage (from stage IA to IV) was defined according to the American Joint Committee on Cancer (AJCC) eighth edition for the retroperitoneum-specific criteria (20).

Immunohistochemical staining. IHC staining was performed using paraffin-embedded, formalin-fixed tissue blocks (fixed in 10% buffered formalin at room temperature for 24-48 h), as previously described (21). The details about the primary and secondary antibodies and conditions are available in Table SI. Histofine Simple Stain™ MAX PO (MULTI) kit (catalogue no. 414151; Nichirei Corporation) was used for peroxidase color development according to the manufacturer direction. The membranous expression of PD-L1, PD-L2, and PD-1 and the nuclear expression of Ki-67 were evaluated in at least 3 independent high-power microscopic fields (x400, 0.0625 μm^2) of sarcoma cells, which were determined on the basis of the cell morphology. The percentage of positive sarcoma cells divided by the total counted sarcoma cells was calculated (0-100%).

The staining intensity of these markers was not taken into account. The cut-off values for defining low or high expression on IHC staining were based on the median-positive scores. The IHC staining results were evaluated by two investigators (Y.O. and S.H.) who were blinded to any clinicopathological data.

Postoperative management and follow-up. Additional chemotherapy and/or radiotherapy were performed on a case-by-case basis, especially for patients presenting with R1 or R2 disease. Doxorubicin plus ifosfamide or doxorubicin alone was administered for most cases (22). A chest/abdomen/pelvis CT scan was obtained approximately every 3 months for 3 years after surgery, every 6 months from years 4 to 5, and annually thereafter. Recurrence was defined as radiographically detectable local recurrence or distant metastases.

Statistical analysis. IBM SPSS version 23 (SPSS Inc.) and PRISM software version 5.00 (San Diego) were used for statistical analyses and data plotting, respectively. A P-value <0.05 was considered statistically significant. Continuous variables were expressed as the median and interquartile range (IQR) and compared using the Mann-Whitney U-test or the Kruskal-Wallis test, followed by the post hoc test (Dunn test). Data are expressed as box plots. Categorical variables were compared using a Chi-square or Fisher's exact test, as appropriate. The correlations among the studied parameters were examined using the Spearman correlation coefficient (ρ value) and linear regression analysis (Y-slope). The absolute values of Spearman ρ<0.2-0.4 were considered to indicate weak correlation; 0.4-0.7, moderate correlation; and >0.7, strong correlation.

Recurrence-free survival (RFS) and disease-specific survival (DSS) were the primary outcomes. Survival was estimated with the Kaplan-Meier method by calculating the RFS and DSS from the date of surgery to the date of events or the last follow-up, and were compared using log-rank tests. Multivariate analysis was used to identify independent prognostic variables by using a stepwise Cox proportional hazards regression model. Variables that potentially affected prognosis (P<0.1) on univariate analysis were included in the multivariate analysis.

Results

Patient characteristics. A total of 51 patients treated at three tertiary hospitals were included in this study. Table I summarizes the clinicopathological characteristics. Tumor biopsy prior to surgery was performed in 10 patients (20%) to make the diagnosis of sarcoma and determine its histologic subtype. The paired pathological results of biopsy tissues and surgically resected tissues are listed in Table SII. The pathological results of biopsy tissues and surgically resected tissues were the same or similar in 8 of the 10 patients (80%), while the pathological results were changed from LMS or rhabdomyosarcoma to DDLPS and from inflammatory tissue to DDLPS in 1 patient each. Simple tumor resection was performed in 20 patients (35%) and extended resection in 31 patients (65%). The number of resected organs and the concomitant procedure are shown in Table SIII. Eighteen (58%) of the 31 patients

Table I. Clinicopathologic characteristics of 51 cases with retroperitoneal sarcoma.

Variables	Value
Total	51 (100%)
Sex[a]	
Male	32 (63)
Female	19 (37)
Age at surgery, years[b]	65 (54-71)
Tumor size, cm[b]	7.6 (4.9-11.2)
Hemoglobin, g/dl[b]	12.8 (11.3-14.3)
Albumin, g/dl[b]	4.0 (3.7-4.3)
LDH, IU/l[b]	196 (169-226)
Adjusted calcium, mg/dl[b]	9.3 (9.1-9.5)
CRP, mg/dl[b]	0.2 (0.05-1.7)
NLR[b]	2.3 (1.7-3.4)
PLR[b]	147 (97-173)
MLR[b]	0.30 (0.21-0.49)
Biopsy prior to surgery[a,c]	
No	41 (80)
Yes	10 (20)
Extended resection[a,d]	
No	20 (39)
Yes	31 (61)
Tumor entity[a]	
WDLPS	5 (10)
DDLPS	21 (41)
Myxoid LPS	1 (2)
LMS	10 (20)
UPS	8 (16)
Epithelioid sarcoma	1 (2)
Fibrosarcoma	1 (2)
Myxofibrosarcoma	1 (2)
Synovial sarcoma	1 (2)
Sarcoma, NOS	2 (4)
FNCLCC grading[a]	
G1	10 (20)
G2	14 (27)
G3	27 (53)
Prognostic staging[a,e]	
IA	5 (10)
IB	5 (10)
II	9 (18)
IIIA	15 (29)
IIIB	15 (29)
NA	2 (4)
Resection margin[a,f]	
R0	23 (45)
R1	23 (45)
R2	5 (10)
Postoperative treatment[a]	

Table I. Continued.

Variables	Value
No	41 (80)
Chemotherapy	7 (14)
Chemotherapy + radiotherapy	3 (6)

[a]Presented as n (%). [b]Presented as median (interquartile range). [c]Paired pathology reports of biopsy tissues and surgically resected tissues are listed in Table SII. [d]Resected organs are listed in Table SIII. [e]AJCC 8th edition (20). [f]R classification based on the macroscopic and microscopic evaluation and categorized into three as follows: Microscopically negative (R0), microscopically positive (R1), and either as grossly positive (R2). WDLPS, well-differentiated liposarcoma; DDLPS, de-differentiated liposarcoma; LMS, leimyosarcoma; UPS, Undifferentiated pleomorphic sarcoma; LDH, lactate dehydrogenase; CRP, C-reactive protein; NLR, neutrophil-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; MLR, monocyte-to-lymphocyte ratio; NOS, not otherwise specified; FNCLCC, Federation Nationale des Centres de Lutte le Cancer; AJCC, American Joint Committee on Cancer.
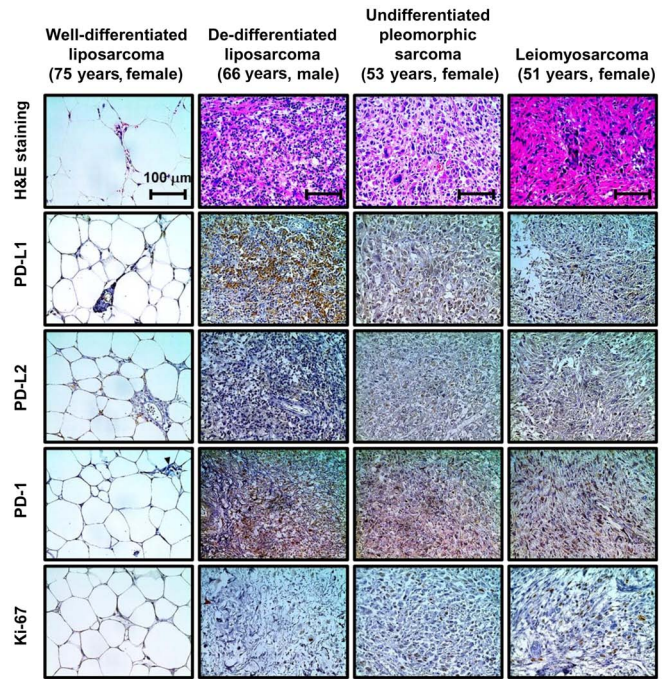

Figure 1. Representative images of immunohistochemical staining for PD-L1, PD-L2, PD-1 and Ki-67 in retroperitoneal sarcoma. The images show four major subtypes of retroperitoneal sarcoma: Well-differentiated liposarcoma, dedifferentiated liposarcoma, undifferentiated pleomorphic sarcoma, and leiomyosarcoma. All images were captured at x400 magnification. The scale bar indicates 100 μm. PD-L1, programmed death ligand-1; PD-L2, programmed death ligand-2; PD-1, programmed cell death protein 1.

underwent concomitant resection of one organ. Frequently resected targets included the kidneys (48%) and psoas muscle (35%). One patient (an 81-year-old man) with a large retroperitoneal DDLPS underwent multi-visceral resection of the left kidney, descending/sigmoid colon, psoas muscle, and common iliac artery via F-F bypass vascular reconstruction. Regarding the tumor histopathology, liposarcoma (WDLPS, DDLPS, and myxoid LPS) was the most frequent tumor entity, accounting for 53% of all cases. The three major types of RSar in our cohort were DDLPS (41%), LMS (20%) and UPS (16%) (Table I).

PD-L1, PD-L2, PD-1, and Ki-67 expression in RSar specimens. As no patients underwent neoadjuvant therapy in the studied cohort, all the specimens were treatment-naïve RSar. Representative images of IHC staining for PD-L1, PD-L2, PD-1, and Ki-67 in retroperitoneal sarcoma are shown in Fig. 1. The percentage of positive sarcoma cells divided by the total counted sarcoma cells was calculated (0-100%). The median scores and IQRs for PD-L1, PD-L2, PD-1, and Ki-67 expression in sarcoma cells were 7 (2 to 11), 2 (0 to 8), 15 (5 to 27), and 5 (2 to 19), respectively. These scores were compared considering the sarcoma subtypes and prognostic stage. There was a significant difference in the PD-L1-positive score among the tumor subtypes on the Kruskal-Wallis test, followed by multiple comparisons showing higher levels of PD-L1 expression in DDLPS and LMS than in other sarcomas (Fig. 2A). PD-L2, PD-1, and Ki-67 staining results were not associated with sarcoma subtypes. Although the prognostic stage was not correlated with the IHC staining results in our cohort, there seemed to be a tendency that PD-L1 and PD-L2 positive scores decreased from stage II to IIIA in that order (Fig. 2B).

Correlation analysis. To investigate the correlations among the baseline parameters and the expression of immune checkpoint proteins in the RSar specimens, we selected continuous variables for analysis using the Spearman correlation coefficient.

Fig. 3A summarizes the P-values and Spearman ρ values from the correlation coefficient analyses and the Y-slopes from the linear regression analyses. The tumor size was inversely correlated with Hb and albumin levels. Three systemic inflammation markers, i.e., the NLR, PLR, and MLR, were moderately to strongly correlated (all P<0.001; NLR vs. PLR ρ=0.56; PLR vs. MLR ρ=0.64: NLR vs. MLR ρ=0.78, respectively). Serum LDH levels were moderately and positively correlated with expression of PD-L1 (P=0.018, ρ=0.41) and PD-L2 (P=0.006, ρ=0.47) (Fig. 3B). A PD-L1 positive score was positively correlated with a PD-1 positive score (Fig. 3B; P=0.039, ρ=0.41), while the Ki-67 labeling index was positively correlated with PD-L2 (P=0.03, ρ=0.48) and PD-1 expression (P=0.01, ρ=0.52). Age and adjusted calcium levels were not significantly correlated with any parameters (Fig. 3A).

Prognostic values of prognostic staging and high expression of PD-L1 and Ki-67. The median follow-up period after surgery was 29 months (IQR, 10-50 months). During the follow-up period, 33 patients (65%) experienced tumor recurrence and 19 patients (37%) succumbed to RSar. As no patients died of other cause during the follow-up period, overall survival was not evaluated in this study. The median DSS and RFS were 58 and 16 months, respectively (Fig. 4A). The 5-year RFS and DSS rates were 27 and 49%, respectively. Univariate analyses were performed to determine the prognostic values of the studied parameters. The cut-off values on IHC staining were defined on the basis of the median positive scores as follows: 7% for PD-L1, 2% for PD-L2, 15% for PD-1, and 5% for Ki-67. On univariate analysis, a short RFS was associated with high
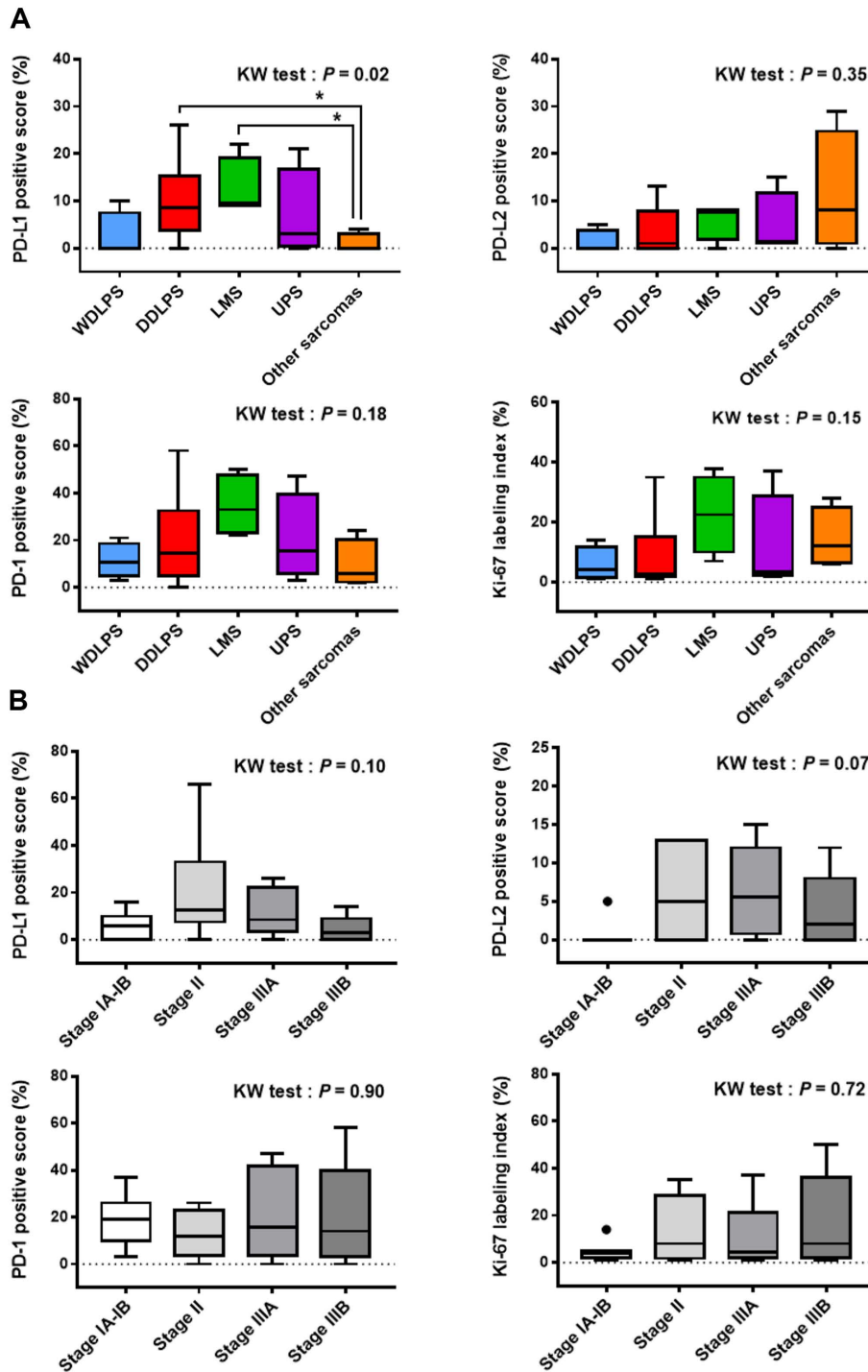
Figure 2. Association of PD-L1, PD-L2, PD-1, and Ki-67 expression with the (A) sarcoma subtypes and (B) prognostic stage. The prognostic stage (from stage IA to IIIB) was determined according to the TNM categories and tumor grade (the American Joint Committee on Cancer eighth edition for the retroperitoneum-specific criteria). All data are expressed in box-and-whisker plots. The black circles indicate outliers. Multiple data were compared using the Kruskal-Wallis test. *P<0.05 (post hoc Dunn test). PD-L1, programmed death ligand-1; PD-L2, programmed death ligand-2; PD-1, programmed cell death protein 1; WDLPS, well-differentiated liposarcoma; DDLPS, dedifferentiated liposarcoma; UPS, undifferentiated pleomorphic sarcoma; LMS, leiomyosarcoma.

expression levels of PD-1 and Ki-67, while DSS was significantly associated with prognostic stage IIIB disease and a high baseline level of CRP (Table II). The Kaplan-Meier curves for RFS and DSS are shown in Figs. 4B-D and S1. After controlling for possibly confounding variables, multivariate analyses revealed that independent predictors of RFS and DSS were a high expression of Ki-67 (P=0.03; hazard ratio, 2.29 vs. low expression) and prognostic stage IIIB (P, 0.04; hazard ratio,

5.11 vs. stage I-II), respectively (Table II). PD-L1, PD-L2, or PD-1 expression was not an independent prognostic factor in our study cohort of RSar.

Discussion

The current study investigated the clinical relevance of the expression of three pivotal immune checkpoint proteins,

A

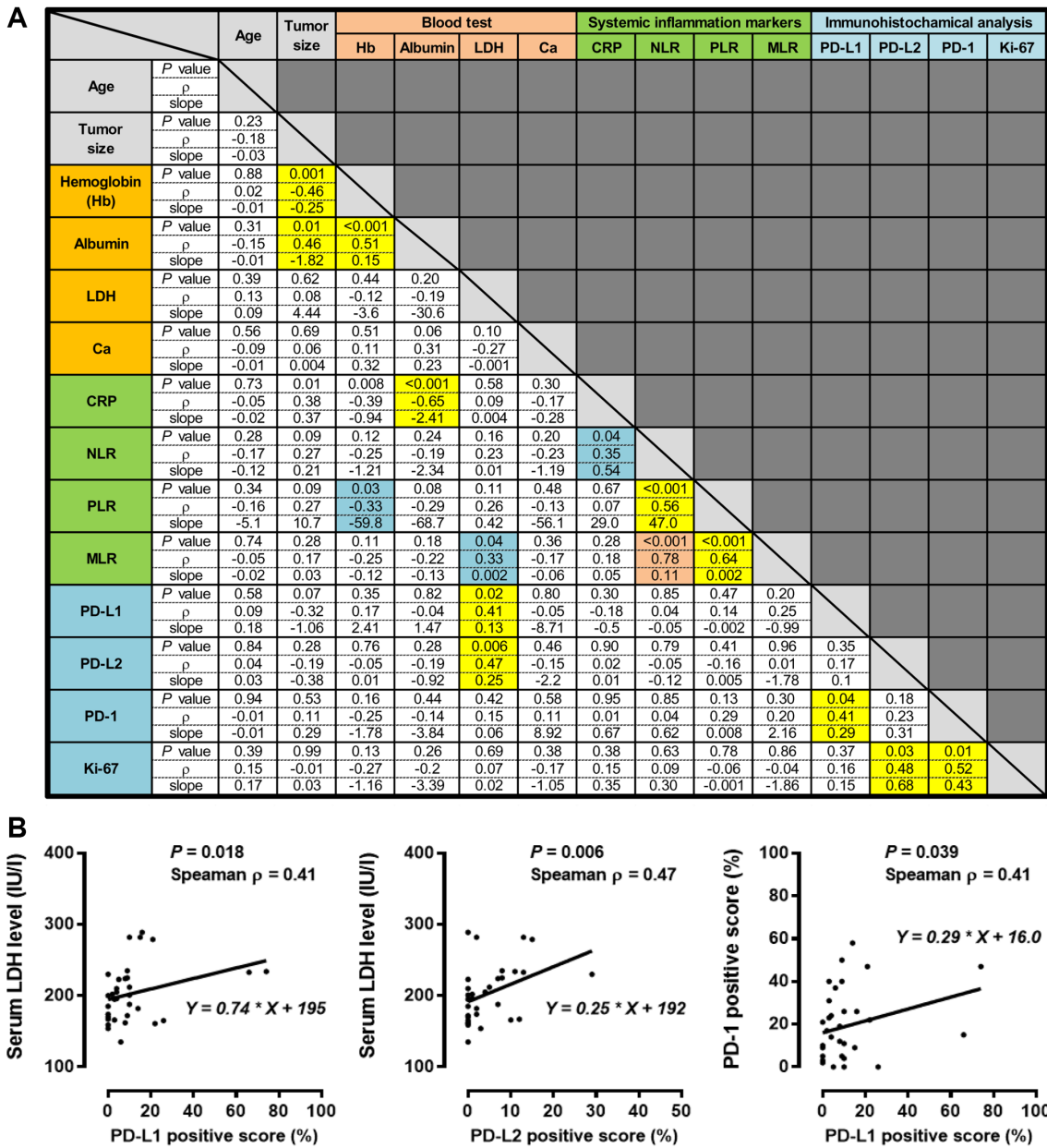|		Age	Tumor size	Hb	Albumin	LDH	Ca	CRP	NLR	PLR	MLR	PD-L1	PD-L2	PD-1	Ki-67
Age	P value														
	ρ														
	slope														
Tumor size	P value	0.23													
	ρ	-0.18													
	slope	-0.03													
Hemoglobin (Hb)	P value	0.88	0.001												
	ρ	0.02	-0.46												
	slope	-0.01	-0.25												
Albumin	P value	0.31	0.01	<0.001											
	ρ	-0.15	0.46	0.51											
	slope	-0.01	-1.82	0.15											
LDH	P value	0.39	0.62	0.44	0.20										
	ρ	0.13	0.08	-0.12	-0.19										
	slope	0.09	4.44	-3.6	-30.6										
Ca	P value	0.56	0.69	0.51	0.06	0.10									
	ρ	-0.09	0.06	0.11	0.31	-0.27									
	slope	-0.01	0.004	0.32	0.23	-0.001									
CRP	P value	0.73	0.01	0.008	<0.001	0.58	0.30								
	ρ	-0.05	0.38	-0.39	-0.65	0.09	-0.17								
	slope	-0.02	0.37	-0.94	-2.41	0.004	-0.28								
NLR	P value	0.28	0.09	0.12	0.24	0.16	0.20	0.04							
	ρ	-0.17	0.27	-0.25	-0.19	0.23	-0.23	0.35							
	slope	-0.12	0.21	-1.21	-2.34	0.01	-1.19	0.54							
PLR	P value	0.34	0.09	0.03	0.08	0.11	0.48	0.67	<0.001						
	ρ	-0.16	0.27	-0.33	-0.29	0.26	-0.13	0.07	0.56						
	slope	-5.1	10.7	-59.8	-68.7	0.42	-56.1	29.0	47.0						
MLR	P value	0.74	0.28	0.11	0.18	0.04	0.36	0.28	<0.001	<0.001					
	ρ	-0.05	0.17	-0.25	-0.22	0.33	-0.17	0.18	0.78	0.64					
	slope	-0.02	0.03	-0.12	-0.13	0.002	-0.06	0.05	0.11	0.002					
PD-L1	P value	0.58	0.07	0.35	0.82	0.02	0.80	0.30	0.85	0.47	0.20				
	ρ	0.09	-0.32	0.17	-0.04	0.41	-0.05	-0.18	0.04	0.14	0.25				
	slope	0.18	-1.06	2.41	1.47	0.13	-8.71	-0.5	-0.05	-0.002	-0.99				
PD-L2	P value	0.84	0.28	0.76	0.28	0.006	0.46	0.90	0.79	0.41	0.96	0.35			
	ρ	0.04	-0.19	-0.05	-0.19	0.47	-0.15	0.02	-0.05	-0.16	0.01	0.17			
	slope	0.03	-0.38	0.01	-0.92	0.25	-2.2	0.01	-0.12	0.005	-1.78	0.1			
PD-1	P value	0.94	0.53	0.16	0.44	0.42	0.58	0.95	0.85	0.13	0.30	0.04	0.18		
	ρ	-0.01	0.11	-0.25	-0.14	0.15	0.11	0.01	0.04	0.29	0.20	0.41	0.23		
	slope	-0.01	0.29	-1.78	-3.84	0.06	8.92	0.67	0.62	0.008	2.16	0.29	0.31		
Ki-67	P value	0.39	0.99	0.13	0.26	0.69	0.38	0.38	0.63	0.78	0.86	0.37	0.03	0.01	
	ρ	0.15	-0.01	-0.27	-0.2	0.07	-0.17	0.15	0.09	-0.06	-0.04	0.16	0.48	0.52	
	slope	0.17	0.03	-1.16	-3.39	0.02	-1.05	0.35	0.30	-0.001	-1.86	0.15	0.68	0.43	

B

Figure 3. Correlation analysis for the baseline clinical parameters and immune checkpoint proteins in 51 patients with retroperitoneal sarcoma. (A) Summary results from the correlation analysis for the preoperative clinical parameters and immunohistochemical staining analysis in the sarcoma specimens. The P-value and Spearman ρ from the correlation coefficient analysis and the Y-slope from the linear regression analysis are shown in each cell. Blue, yellow, and orange cells indicate weak, moderate, and strong correlations, respectively. (B) The relationships between the baseline LDH and the PD-L1 and PD-L2 positive scores were examined using the Spearman correlation coefficient and linear regression analysis. PD-L1, programmed cell death ligand-1; PD-L2, programmed death ligand-2; PD-1, programmed cell death protein 1; Hb, hemoglobin; LDH, lactate dehydrogenase; CRP, C-reactive protein; NLR, neutrophil-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; MLR, monocyte-to-lymphocyte ratio.

PD-L1, PD-L2, and PD-1, and Ki-67 expression in surgically resected RSar. Although we evaluated the prognostic value of PD-L1, PD-L2, and PD-1 expression, only high expression of PD-1 was a possible predictor of postoperative recurrence. By contrast, a high expression of Ki-67 was associated with a high risk of recurrence and disease-specific death. Several molecular markers, such as Ki-67, p27/kip1, α-SMA, and CD133, have been identified as prognostic factors (3,23-25). PD-L1 and PD-1 expression in sarcoma cells and tumor-infiltrating lymphocytes (TILs) as well as TIL density have been well investigated as potential prognostic biomarkers in various malignancies, including sarcomas. However, the

results were inconsistent (15). A recent study demonstrated that preoperative ^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography was able to predict preoperatively FNCLCC grade 3 of retroperitoneal LPS and estimate the short survival after surgery (cutoff: Maximum standardized uptake value >4.5) (26). However, to date, only a few molecular and radiographic markers have been used in clinical practice.

Some advanced/metastatic soft tissue sarcomas are amenable to immunotherapies (13), although there is a significant lack of evidence regarding the immunologic profile and immune microenvironment of soft tissue sarcoma subtypes.
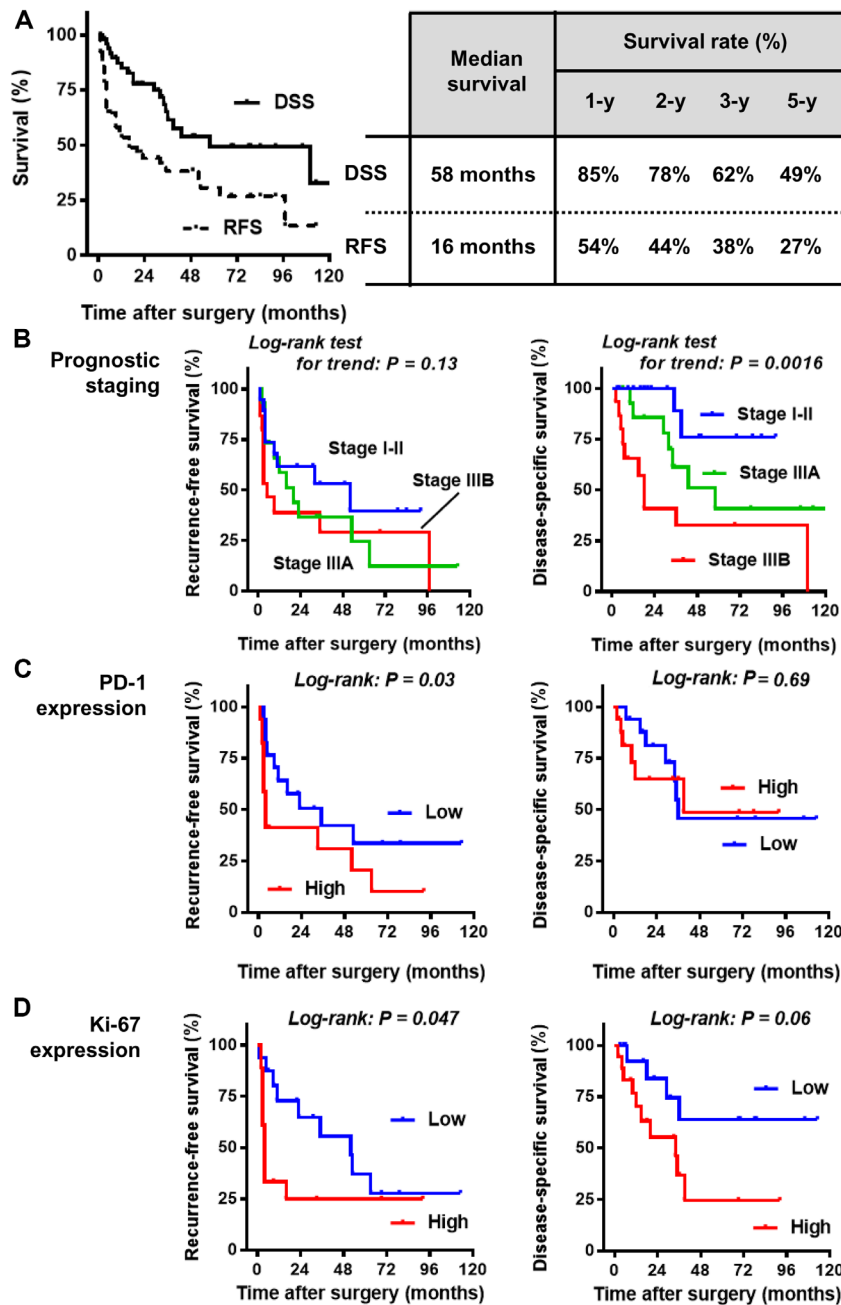
Figure 4. Recurrence-free survival (RFS) curves and disease-specific survival (DSS) curves after surgical resection of retroperitoneal sarcoma. (A) RFS and DSS curves of all cases. Survival rates were estimated by using the Kaplan-Meier method. The log-rank test was used for the comparison. The left and right panels show the RFS and DSS curves according to the (B) prognostic staging, (C) PD-1 expression in sarcoma tissue, and (D) Ki-67 expression in sarcoma tissue.

Pollack *et al* compared the expression levels of genes associated with antigen presentation, T-cell infiltration, and immune checkpoint proteins among common sarcomas including WDLPS, DDLPS, UPS, and LMS (16). UPS is a highly mutated sarcoma and shows high levels of PD-L1 and PD-1 on IHC analysis. By contrast, LPS was less mutated but highly expressed immunogenic self-antigens, which may support the need for immunotherapy with PD-1/PD-L1 blockade in this subset of common sarcomas. The results of the current study showed significantly higher levels of PD-L1 expression in DDLPS and LMS, but not in UPS, than in other sarcomas (Fig. 2A), while PD-1 and PD-L2 expression did not show any significant difference among the sarcoma subtypes. PD-1 is

the most intensively researched immune checkpoint molecule in all oncological fields, including soft tissue sarcoma. A meta-analysis of the prognostic value of PD-L1 in sarcomas showed that the positive rate of PD-L1 expression varied from 8.5 to 75.0% (15). This striking variability in the PD-L1 expression rate in sarcomas can be due to multiple factors, such as differences in the cut-off values for defining PD-L1 positivity, differences in IHC assays, antibodies used for PD-L1 expression, and differences in patient background characteristics, including the sarcoma subtypes (15). In the current study, we determined the absolute percentages of positivity in sarcoma cells and used those cut-off values for prognostic assessment. Nevertheless, further comprehensive evaluation

Table II. Prognostic variables for recurrence-free survival and disease-specific in 51 patients with retroperitoneal sarcoma undergoing resection surgery.

| | Recurrence-free survival | | | | | | Disease-specific survival | | | | | |
| | Univariate | | | Multivariate | | | Univariate | | | Multivariate | | |
Variables	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value
Age at surgery, years												
<65	1						1					
≥65	0.73	0.37-1.44	0.34				0.65	0.26-1.63	0.36			
Sex												
Male	1						1					
Female	0.69	0.34-1.37	0.29				0.56	0.22-1.35	0.21			
Prognostic staging[a]												
Stage I-II	1						1			1		
Stage IIIA	1.54	0.64-3.70	0.32				3.68	0.99-13.6	0.08	2.95	0.53-16.5	0.22
Stage IIIB	1.77	0.73-4.31	0.16				6.70	1.96-22.9	0.002	5.11	1.06-24.7	0.04
Resection margin[b]												
R0	1						1					
R1	1.09	0.51-2.31	0.81				0.87	0.33-2.31	0.89			
R2	1.79	0.53-6.08	0.25				0.96	0.20-4.58	0.97			
Tumor entity[c]												
DDLPS	1						1					
LMS	1.26	0.51-3.08	0.58				0.85	0.26-2.74	0.79			
UPS	0.83	0.33-2.07	0.69				1.32	0.41-4.22	0.62			
Other sarcomas	0.94	0.32-2.78	0.91				1.09	0.22-5.37	0.91			
LDH												
Low	1						1					
High	1.22	0.59-2.55	0.58				1.12	0.44-2.82	0.81			
CRP												
Low	1						1			1		
High	1.83	0.87-3.88	0.10				2.37	1.09-5.99	0.03	1.39	0.43-4.47	0.58
NLR												
Low	1						1					
High	1.50	0.67-3.36	0.30				1.49	0.50-4.46	0.47			
PLR												
Low	1						1					
High	0.57	0.26-1.27	0.57				0.88	0.30-2.62	0.82			
MLR												
Low	1						1					
High	1.11	0.50-2.46	0.79				1.30	0.44-3.86	0.64			
PD-L1 expression												
Low	1						1					
High	0.94	0.41-2.17	0.88				0.64	0.21-1.92	0.41			
PD-L2 expression												
Low	1						1					
High	1.37	0.59-3.17	0.44				1.53	0.51-4.59	0.43			
PD-1 expression												
Low	1			1			1					
High	2.15	1.27-5.32	0.03	1.61	0.66-3.94	0.29	1.25	0.41-3.78	0.69			

Table II. Continued.

Variables	Recurrence-free survival						Disease-specific survival					
	Univariate			Multivariate			Univariate			Multivariate		
	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value
Ki-67 expression												
Low	1			1			1			1		
High	2.19	1.03-5.13	0.047	2.29	1.16-5.50	0.03	2.81	0.97-8.08	0.06	2.80	0.84-9.29	0.09

[a]According to TNM categories and tumor grade (the American Joint Committee on Cancer eighth edition for the retroperitoneum-specific criteria). [b]R classification based on the macroscopic and microscopic evaluation and categorized into three as follows: Microscopically negative (R0), microscopically positive (R1), and either as grossly positive (R2). [c]Five cases with well-differentiated liposarcoma were excluded from the analysis because no recurrence and no disease-specific death was observed in this subset. HR, hazard ratio; CI, confidence interval; DDLPS, De-differentiated liposarcoma; LMS, leiomyosarcoma; UPS, undifferentiated pleomorphic sarcoma; LDH, lactate dehydrogenase; CRP, C-reactive protein; NLR, neutrophil-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; MLR, monocyte-to-lymphocyte ratio; PD-L1, programmed death ligand-1; PD-L2, programmed death ligand-2; PD-1, programmed cell death protein 1.

of multiple available antibodies (e.g., clones SP263, E1L3N, and 22C3) in various types of cells including sarcoma cells, tumor-infiltrating lymphocytes, and macrophages is likely to fill the gaps between the studies.

We provided a detailed overview of baseline clinical parameters and IHC analysis (Fig. 3). We selected the parameters expressed with continuous values on the basis of the previously reported possible prognostic factors. A total of 14 parameters were tested, and 15 correlations were evaluated as follows: 1 Strong, 11 moderate, and 3 weak correlations. A large tumor size, low Hb level, and low albumin level were moderately associated with each other. A high NLR, high PLR, and high MLR were moderately to strongly associated with each other. High levels of serum LDH were significantly correlated with high PD-L1 expression (Spearman $\rho=0.41$) and PD-L2 expression ($\rho=0.47$). LDH is an enzyme ubiquitously found in all cell types. In the last step of aerobic glycolysis, LDH catalyzes the conversion of pyruvate to lactate, leading to the accumulation of lactate and the production of an acidic tumor microenvironment (27). Eventually, this condition can cause immunosuppression in melanoma tumors (27). LDH-A mRNA expression was associated with an increased number of PD-L1- and PD-1-positive M2 macrophages in the tumor microenvironment of patients with extramammary Paget disease (28). A preclinical study revealed that the stimulation of melanoma cells with lactate upregulated the expression of PD-L1 and altered immunomodulation in the tumor microenvironment; also, blockade of LDH-A could improve the efficacy of anti-PD-1 treatment (27). A clinical study showed that an integrated algorithm involving baseline serum LDH levels in patients with metastatic solid tumors was able to provide a higher performance for response prediction to ICIs (29). A review regarding the biomarkers for predicting the efficacy of anti-PD-1 antibodies showed that elevated levels of serum LDH were associated with poor response to the treatment (30). Therefore, we believe that more evidence would clarify the potential benefit of serum and tumor LDH levels as clinically available biomarkers for RSar.

The present study has several limitations. First, the sample size was relatively small and there was a possible selection bias caused by the retrospective nature of the study. Second, we did not perform subgroup analysis for the different types of sarcomas considering the prognostic values of the immune checkpoint molecules because only limited patients with each major entity (DDLPS, LMS, and UPS) were included. Third, we excluded patients who were diagnosed with RSar and did not undergo surgical resection. Fourth, only a single antibody for each molecule was used in the IHC analysis, which could affect the staining result. Fifth, we did not evaluate the expression of cytotoxic T lymphocyte-associated antigen-4 (CTLA-4). Although immune checkpoint blockade by the combination of anti-CTLA-4 and anti-PD-1 antibodies have successfully been used for several malignancies (13), this treatment modality remains understudied in retroperitoneal soft tissue sarcoma. Future additional assessment of CTLA-4 expression in RSar is needed for better understanding the immune microenvironment that can affect tumor initiation and response to therapy.

In conclusion, the findings of the current study provide novel insights into the prognostic values of PD-L1, PD-L2, and PD-1 expression in RSar tissues as well as regarding the correlation between baseline clinical parameters and immune checkpoint molecules. However, the complex heterogeneity of RSar hinders the establishment of an ideal therapeutic strategy using immunotherapy. Nevertheless, further investigations are necessary to determine the immunologic landscape of RSar and provide a foundation for therapeutic intervention using ICIs.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Authors' contributions

MM, YM, DG, NT, and KF conceived the study. YO and SH designed the study. SO performed the immunohistochemical staining analysis. KH, TF, ST, HF, AK and KH collected and data. NN performed the statistical analysis. YN, SA, KT, YM and EO interpreted the data. All authors substantially contributed drafting the work and revising it critically for important intellectual content. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

This study was approved by the ethics committee of the Nara Medical University (reference ID: 1256 and 1966). Informed consent to participate in the study was obtained from all participants. The study was conducted in compliance with the study's protocol and in accordance with the provisions of the Declaration of Helsinki (2013).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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