Potential role of the PD-L1 expression and tumor-infiltrating lymphocytes on neuroblastoma

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Abstract

Purpose: The programmed death 1 (PD-1)/programmed death ligand 1(PD-L1) pathway has garnered much attention for its roles in clinical oncology. The aim of this study was to examine the clinical impact of the PD-L1 expression and tumor-infiltrating lymphocytes (TILs) on neuroblastoma.

Methods: We evaluated the PD-L1 expression and TIL status in 31 patients with neuroblastoma who underwent a biopsy or resection by an immunohistochemical analysis. Furthermore, we performed the serial analysis of the PD-L1 status before and after chemotherapy in 15 patients.

Results: Among the 31 cases, 11 (35%) showed a positive PD-L1 expression. The survival analysis showed a trend toward an association between PD-L1 positivity and a decreased overall survival. PD-L1 positivity tended to be associated with higher levels of tumor markers. In the serial analysis of the PD-L1 status, positivity was noted in 8 of 15 patients before chemotherapy and 6 after chemotherapy. Notably, all four patients with a positive PD-L1 status both before and after chemotherapy had recurrence, and three of them died during the follow-up period.

Conclusion: Our findings suggest that the PD-L1 tumor expression might be a good biomarker for the treatment of neuroblastoma patients, especially for advanced neuroblastoma.

Keywords: Neuroblastoma, PD-L1, Tumor-infiltrating lymphocytes, Recurrence

Introduction

Neuroblastoma is the most common extra-cranial solid tumor of childhood and arises from the developing sympathetic nervous system. The tumor is known for its clinical and biological heterogeneity. Metastatic neuroblastoma diagnosed in patients over 18 months of age has a poor prognosis and is classified as a high-risk lesion. These patients have a 5-year survival rate <50%, even if they receive aggressive combination therapies including intensive chemotherapy, surgery, radiotherapy, autologous stem cell transplantation, and the administration of retinoids (13-cis-retinoic acid) [1-3]. Therefore, new treatment modalities, especially for high-risk neuroblastoma, are urgently needed.

The programmed death 1 (PD-1) receptor is known to be a major negative immune regulator in various tumor microenvironments in humans. The interaction between PD-1 on tumor-infiltrating T lymphocytes (TILs) and programmed death ligand-1 (PD-L1) allows cancer cells to evade the host immune response. It is hypothesized that the inhibition of PD-L1/PD-1 interaction by anti-PD-1 or anti-PD-L1 antibodies can restore T lymphocyte activity and kill cancer cells [4-9]. In fact, the effectiveness of blocking the PD-1/PD-L1 pathway of immune checkpoint inhibitors has been widely demonstrated in various types of PD-L1-expressing cancer in adult patients in the last decade [10, 11]. The introduction of such novel immunotherapy has resulted in a paradigm shift in human cancer treatment. Opdivo (nivolumab) and Keytruda (pembrolizumab) are PD-1 inhibitors approved by FDA for

advanced melanoma and non-small cell lung cancer. However, the efficacy of PD-1 inhibitors for neuroblastoma patients remains to be examined [12].

The tumor PD-L1 expression is considered the most important biomarker for predicting a response to checkpoint blockade. Furthermore, the density of TILs may also predict the response to anti-PD-1/PD-L1 therapies [13, 14]. Despite the fact that the density of T cells was shown to be associated with a favorable clinical outcome in neuroblastoma more than 40 years ago, the current knowledge concerning the types of immune cells infiltrating neuroblastomas is limited to a few studies conducted on a small number of specimens [15-17].

In the present study, we evaluated the PD-L1 expression and TIL status by immunohistochemistry and examined the prognostic role of PD-L1 and TILs on neuroblastoma.

Methods

Patients and specimens

We examined 31 patients with neuroblastoma who underwent a biopsy or resection and were treated at Nara Medical University and Kobe Children's Hospital from 2003 to 2018. All 31 patients underwent a block biopsy or resection only once before anticancer treatment, in 15 out of 31 patients underwent residual tumor resection after chemotherapy. Corresponding data, including the survival and recurrence, as well as the gender, age at the diagnosis, tumor location (adrenal gland or not), stage (International Neuroblastoma Staging System: 1, 2, 3, 4, 4s), n-

MYC amplification and levels of serum neuron specific γ-enolase (NSE), urine-vanillylmandelic acid (VMA) and homovanillic acid (HVA), were collected.

Immunohistochemistry

After de-paraffinization of tissue blocks, antigen retrieval was performed in antigen retrieval solution using a steamer autoclave at 121 °C for 15minutes (pH 9). To block endogenous peroxidase, sections were immersed in 0.3% solution of hydrogen peroxide in absolute methanol for 5 minutes at room temperature and washed in fresh phosphate-buffered saline 3 times, for 5 minutes each. The slides were incubated with primary antibody against PD-L1 (1:100; clone E1L3N, 13684S; Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C. Sections were washed in fresh phosphate-buffered saline three times, for 5 minutes each, and then secondary antibody (Anti-Mouse/Rabbit IgG Reagent; ImmPRESS, VECTOR LABORATORIES, INC, USA) was used according to the instructions of the manufacturer. Resection products were visualized with 3,3'-diaminobenzidine tetrahydrochloride, and the sections were counterstained with hematoxylin.

The evaluation of immunostaining

Complete circumferential or partial linear plasma membrane staining above a 1% threshold was regarded as indicating a positive PD-L1 expression (Fig. 1A-D) [18-20]. We evaluated

only viable tumor cells in post-chemotherapy samples. Membranous/cytoplasmic staining of lymphocytes was regarded indicating positivity for CD8 and CD45RO. $CD8^+$ and $CD45RO^+$ TILs per field at ×200 magnification were counted manually in 5 fields (Fig. 2E, F).

Statistical analyses

The SPSS Statistics software program (IBM, USA) was used for the statistical analyses. Correlations between the PD-L1 expression and $CD8^+$, $CD45RO^+$ T cell density with clinicopathological variables were performed using Person's chi-squared and Fisher's exact test. For the survival analysis, the Kaplan-Meier survival curves were analyzed using the log-rank test. p-Values less than 0.05 were considered to be statistically significant.

Results

Patients' characteristics

A total of 31 patients (19 males, 12 females) suffering from neuroblastoma were included in this study (Table 1). The median follow-up time of all patients for the survival analysis was 75 months. The median age at the diagnosis was 24 months. Thirteen children (41.9%) were younger than 18 months old at the diagnosis. N-MYC amplification was positive in 7 cases (22.5%). According to the INSS, 9 patients (29%) showed stage 1 disease, 4 patients (13%) stage 2, 2 patients (6%) stage 3, 15 patients (48%) stage 4 and 1 patient (3%) stage 4S. All

stage 4 patients were designated as the high-risk group. There were 10 cases of recurrence (32.2%) and 6 deaths (19.3%) during the follow-up period.

The relationship of the PD-L1 expression with clinicopathological characteristics

Among the 31 cases of neuroblastoma, 11 (35%) showed a positive PD-L1 expression. There were no significant differences in the age, gender, tumor stage, n-MYC amplification or recurrence between the PD-L1-positive and PD-L1-negative groups (Table 1). PD-L1-positive tumors were located in the adrenal gland more frequently than PD-L1-negative tumors. Although the precise mechanism is unclear, there may be organ specificity in the PD-L1 expression. Tumor markers (NSE, VMA and HVA) tended to be higher in the PD-L1-positive group than the PD-L1-negative group, although the difference did not reach statistical significance (Table 2).

The survival and recurrence according to the PD-L1 status

The Kaplan-Meier analysis showed a trend toward an association between a positive expression of PD-L1 and a decreased overall survival among all patients (P=0.074) and stage 4/4S patients (P=0.130) (Fig. 2). However, these trends did not reach statistical significance, possibly due to the small number of patients enrolled in this study.

Correlation of the PD-L1 status with recurrence and mortality

To clarify the clinical significance of the PD-L1 tumor expression, we further assessed the serial changes in the PD-L1 status during chemotherapy in association with postoperative recurrence and mortality. The pre-chemotherapy samples were obtained from a tumor biopsy before anticancer treatment, while the post-chemotherapy samples were obtained from surgical specimens after chemotherapy (Table 3). On comparing pre- and post-chemotherapy samples, the PD-L1 expression was positive in 8 out of 15 pre-chemotherapy samples and 6 out of 15 post-chemotherapy samples. Furthermore, 5 patients showed a negative PD-L1 status in both pre- and post-chemotherapy samples. Among them, only 1 patient (20%) had recurrence and died thereafter. Four patients showed a positive PD-L1 expression in pre-chemotherapy samples but not in post-chemotherapy ones. Two of them had recurrence, and one died. Two patients with a negative PD-L1 expression in pre-chemotherapy samples showed a positive status in post-chemotherapy samples. Both patients had recurrence, and one died. Ultimately, four patients showed a positive PD-L1 status in both pre- and post-chemotherapy samples. Notably, all such patients (100%) had recurrence, and 3 of them (75%) died during the follow-up period (Table 3).

The survival analysis suggested that the PD-L1 status during anticancer treatment might correlate with the patient survival (Fig. 3). There was a trend toward patients with a positive PD-L1 expression both before and after chemotherapy having a better prognosis than those

with a negative PD-L1 expression at both points.

Association of tumor-infiltrating $CD8^+$ and $CD45RO^+$ T-cells with the PD-L1 expression

We further examined the association of tumor-infiltrating T cells in neuroblastoma tissues with the PD-L1 expression. The average numbers of tumor-infiltrating $CD8^+$ and $CD45RO^+$ T-cells are shown in Table 2. Although the average numbers of tumor-infiltrating $CD8^+$ and $CD45RO^+$ T-cells were lower in PD-L1-positive tissues than those in PD-L1-negative tissues, there was no statistical significance between two groups (Table 2).

Discussion

In this study, we examined the prognostic impact of the PD-L1 expression in 31 patients with neuroblastoma. Although previous studies have investigated the PD-L1 expression in various adult tumors [21-25], only a few studies with small populations have evaluated the PD-L1 expression in pediatric tumors [26, 27]. Only one previous study reported a survival detriment for PD-L1 expression in neuroblastoma [18]. In that report, Majzner et al. examined the PD-L1 expression in a large number of biopsies for neuroblastoma and reported that 14% of patients showed positivity [18]. The patients with positive PD-L1 had an inferior survival to those with negative PD-L1. Our study showed a relatively high rate of PD-L1 positivity (35%) among pre-treatment samples. This discrepancy may be due to not only differences in staining

procedures and the evaluation of immunostaining but also a relatively high percentage of INSS stage 4 patients enrolled in our study. We also found a trend toward an association between the positive expression of PD-L1 and a decreased overall survival among all patients as well as stage 4 patients, which is consistent with a previous report [18].

Furthermore, we also evaluated both the $CD8^+$ and $CD45RO^+$ TIL status by immunohistochemistry. As a result, we found no significant association of tumor-infiltrating $CD8^+$ and $CD45RO^+$ T-cells with the PD-L1 expression. In general, high densities of $CD8^+$ TILs as cytotoxic T cells and $CD45RO^+$ TILs as memory T cells in several solid tumors have been associated with a favorable clinical outcome [28-30]. In neuroblastoma, the role of TILs remains to be fully elucidated. In fact, some previous studies have shown the relatively low immunogenicity of neuroblastoma [31, 32]. In contrast, the other study has suggested the important role of TILs in therapy-resistant neuroblastoma [33, 34]. Taken together, further larger scale studies are required to clarify the clinical importance of TILs especially in association with PD-L1 status.

We also performed the serial analysis of the PD-L1 status before and after chemotherapy in 15 patients. To our knowledge, this is the first report to have evaluated the serial changes in individual patients. Regarding our findings, first, the PD-L1 status did not clearly correlate with the performance of chemotherapy. This may be due to the sensitivity and resistance of each neuroblastoma to conventional standard chemotherapy. Second, patients

with a consistent PD-L1-positive status before and after chemotherapy had a poorer survival than those with a consistent PD-L1-negative status. In addition, all four patients with a positive PD-L1 status before and after chemotherapy showed recurrence, and three of them died during the follow-up period. Our data also suggest that the PD-L1 expression in neuroblastoma tissues may be associated with high malignancy and treatment resistance to conventional chemotherapy.

Immune checkpoint blockade with antibodies to PD-1 reportedly exhibits a durable favorable response in mismatch repair-deficient cancers, regardless of the cancers' tissue of origin [35]. Such clinical studies might lead to a paradigm shift in cancer treatment. Even though a few previous studies have described the low frequency of mismatch repair deficiency (microsatellite instability status) in neuroblastoma [36], PD-1 blockade holds great therapeutic promise, especially for advanced tumors. To our knowledge, there are no reports addressing the correlation between the PD-L1 expression, TILs and MSI status in neuroblastoma. Therefore, further studies are needed in order to assess the role of anti-PD-L1 therapies in the treatment of advanced and treatment-resistant neuroblastoma.

Several limitations associated with the present study warrant mention. First, the sample sizes were relatively small. This may be the main reason why most analyses failed to observe statistically significant differences. Second, the study was retrospectively conducted. Although this was a collaborative, multicenter study, there may be bias in some important clinical points,

including treatment options and strategies. Third, tumor biopsy for pathological diagnosis was taken once before treatment in each case, considering the risk in pediatric patient. However, since the heterogeneity of tumor cells is well documented with neuroblastoma, the data regarding PD-L1 might not completely represent the whole tumor. Finally, the various timing of obtaining tissue samples at surgery after chemotherapy might influence the results. The above limitations make it difficult to reach a definitive conclusion. However, our findings may provide new insight into the associated immunological mechanisms and future treatment strategies for neuroblastoma.

In conclusion, our data suggest that the PD-L1 tumor expression is a potentially useful biomarker for the treatment of neuroblastoma patients, especially for advanced neuroblastoma.

Compliance with ethical standards

Conflicts of interest

The authors declare that they have no conflicts of interest associated with this manuscript.

Ethical standards

This study was approved by the Ethics Committees of Nara Medical University Hospital (No. 1662), Kobe Children's Hospital (No. 30-33).

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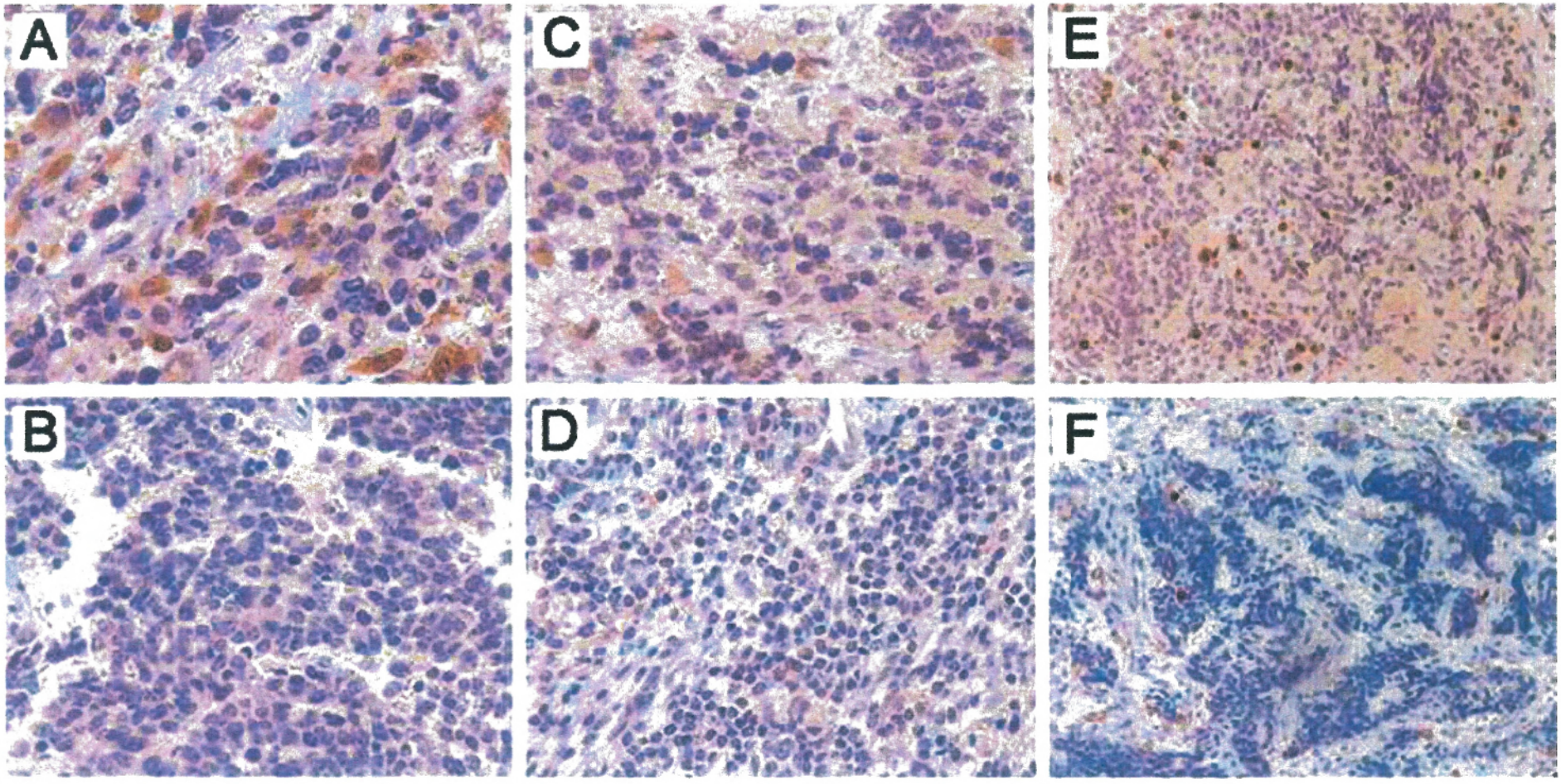
Figure legends

Fig. 1. The PD-L1 expression and TIL status in neuroblastoma. A: PD-L1 positive expression in Stage 4, ×400 magnification. B: PD-L1 negative expression in Stage 4, ×400 magnification. C: PD-L1 positive expression in Stage 1, ×400 magnification. D: PD-L1 negative expression in Stage 1, ×400 magnification. E: $CD8^+$ immunohistochemical staining, ×200 magnification. F: $CD45RO^+$ immunohistochemical staining, ×200 magnification.

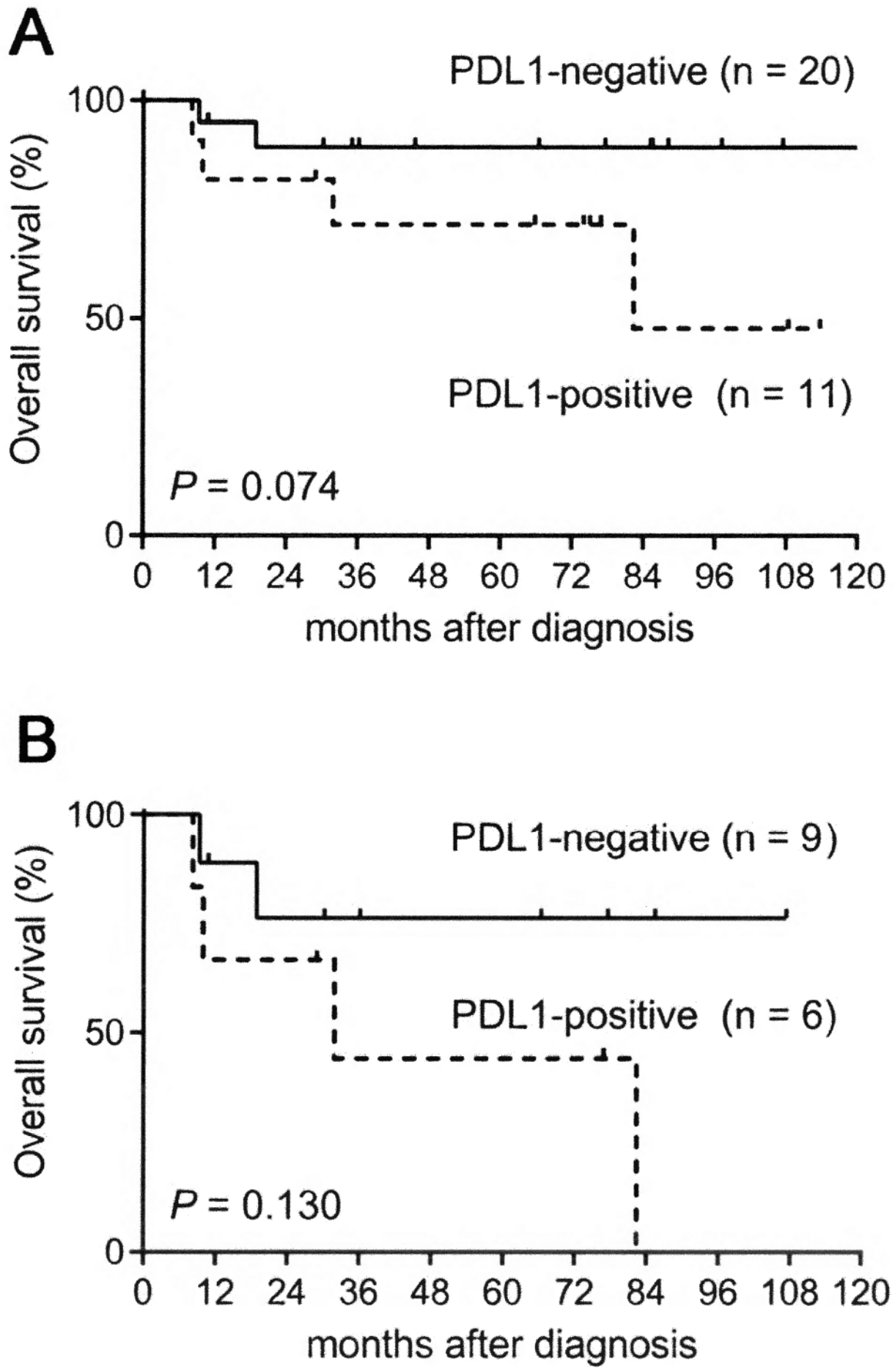
Fig. 2. The overall survival curves for all patients (A) and INSS stage 4 patients (B).

Fig. 3. The overall survival curves (A) and relapse-free survival curves (B) according to the PD-L1 status before and after chemotherapy in 15 patients.

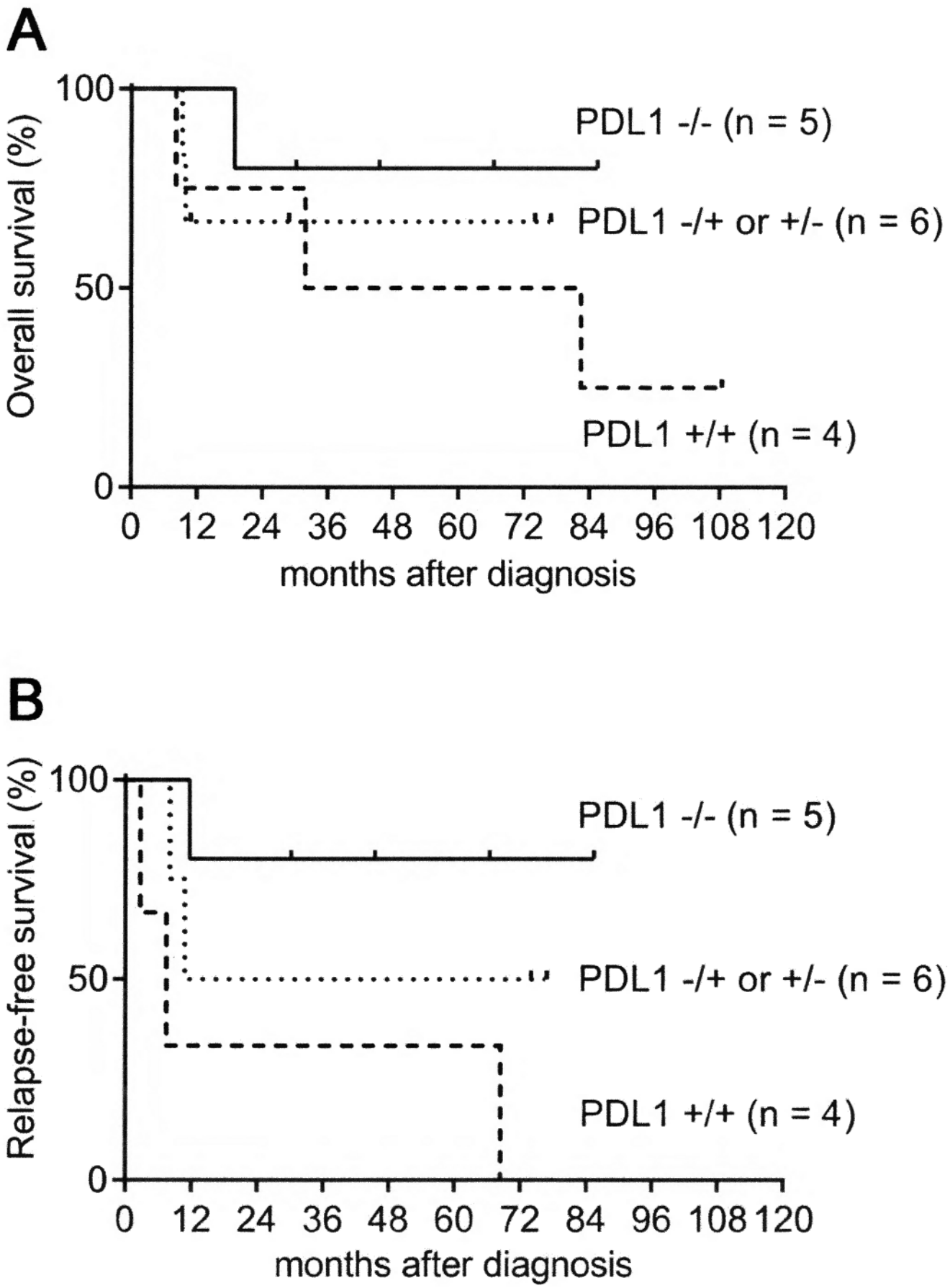
Figure



Figure


A
PDL1-negative (n = 20)
PDL1-positive (n = 11)
P = 0.074
Overall survival (%)
100
50
0
0 12 24 36 48 60 72 84 96 108 120
months after diagnosis
B
PDL1-negative (n = 9)
PDL1-positive (n = 6)
P = 0.130
Overall survival (%)
100
50
0
0 12 24 36 48 60 72 84 96 108 120
months after diagnosis

Figure


A
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Overall survival (%)
PDL1 -/- (n = 5)
PDL1 -/+ or +/- (n = 6)
PDL1 +/+ (n = 4)
0 12 24 36 48 60 72 84 96 108 120
months after diagnosis
B
100
50
0
Relapse-free survival (%)
PDL1 -/- (n = 5)
PDL1 -/+ or +/- (n = 6)
PDL1 +/+ (n = 4)
0 12 24 36 48 60 72 84 96 108 120
months after diagnosis

Table

Table 1 The relationship between clinicopathological characteristics and PD-L1 expression

Variables		Number of patients	Positive ($n = 11$, %)	Negative ($n = 20$, %)	p value
Age	≤18months	13	6(55)	7(35)	0.449
	>18months	18	5(45)	13(65)	
Gender	Male	19	8(73)	11(55)	0.452
	Female	12	3(27)	9(45)	
Tumor Location	Adrenal grand	20	10(91)	10(50)	0.047
	No-Adrenal grand	11	1(9)	10(50)	
INSS stage	1	9	2(18)	7(35)	
	2	4	2(18)	2(10)	
	3	2	0(0)	2(10)	
	4	15	6(55)	9(45)	
	4s	1	1(9)	0(0)	
N-myc amplification	Positive	7	4(36)	3(15)	0.210
	Negative	24	7(64)	17(85)	
Recurrence	Present	10	6(55)	4(20)	0.106
	Absent	21	5(45)	16(80)	

INSS International Neuroblastoma Staging System

[a]Indicates a value obtained from the *t*-test. [b]Indicates a value obtained from Welch test.

Table

Table 2 The tumor marker and tumor infiltrating T cells according to PD-L1 status

Variables	Positive ($n = 11$)	Negative ($n = 20$)	p value
NSE	493.8±543.9	213.9±295.5	0.137[c]
VMA	215.9±385.4	72.2±95.6	0.250[c]
HVA	177.5±269.1	95.2±97.0	0.348[c]
$CD8^+$	23.3±18.1[a]	31.7±39.1[a]	0.507[b]
$CD45RO^+$	16.7±10.0[a]	25.7±31.8[a]	0.371[b]

NSE neuron specific γ-enolase, VMA vanillylmandelic acid, HVA homovanillic acid

[a]$CD8^+$, $CD45RO^+$ T cell count average number: Menbranous/cytoplastic staining of lymphocytes was regarded as positive for CD8+, CD45RO+. $CD8^+$ and $CD45RO^+$ tumor-infiltrating T-lymphocytes (TILs) per field at ×200 magnification were counted manually in five fields. [b]Indicates a value obtained from the *t*-test. [c]Indicates a value obtained from Welch test.

Table

Table 3 Correlation of Pre- and Post-chemotherapy PD-L1 expression with recurrence and mortality

Case	INSS stage	PD-L1 expression		Recurrence	Mortality
		Pre-chemotherapy	Post-chemotherapy		
1	2	-	-	-	-
2	2	+	-	-	-
3	4	-	-	-	-
4	4	-	-	+[a]	+[b]
5	4	-	-	-	-
6	4	-	-	-	-
7	4	+	-	+[a]	+[b]
8	4	+	-	-	-
9	4	+	-	+[a]	-
10	4	-	+	+[a]	+[b]
11	4	-	+	+[a]	-
12	4	+	+	+[a]	+[b]
13	4	+	+	+[a]	-
14	4	+	+	+[a]	+[b]
15	4	+	+	+[a]	+[b]

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Pre-chemotherapy samples were obtained at biopsy and post-chemotherapy at surgery.

[a]recurrence+ recurrence present. [b]mortality+ patient was dead.