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Original Article

Assessment of PD-L1 expression in patients with neuroblastoma and renal tumors

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ABSTRACT

Background. Programmed death 1 (PD-1) is a co-receptor which is located at the surface of cells like natural killer, monocytes, T and B cells. It has two ligands including programmed death ligand-1 (PD-L1) and ligand-2 (PD-L2). T cell functions are inhibited by activation of PD-1/PD-L1 pathway and this pathway is used by viruses and some tumor cells in order to escape from immune eradication. In our study we evaluated PD-L1 expression in the tissue specimens of patients with Wilms tumor, neuroblastoma and other renal tumors.

Methods. Totally 60 patients who were followed up at Gazi University Hospital with the diagnosis of neuroblastoma, Wilms tumor and other renal tumors were included. PD-L1 expression was examined in tumor samples of the patients.

Results. Positive staining with PD-L1 was detected only in two male patients. Both of them had neuroblastoma and advanced stage disease. None of the patients with Wilms tumor and other renal tumors had positive PD-L1 staining.

Conclusions. Unlike adult tumors, PD-L1 expression is not common in childhood tumors due to differences in immune system between children and adults. Further studies are needed to establish the importance and effects of PD-1/PD-L1 pathway in pediatric tumors.

Key words: programmed death-1, programmed death ligand-1, programmed death ligand-2, neuroblastoma, childhood renal tumors.

The Programmed death 1 (PD-1) receptor is located on the surface of natural killer (NK) cells, activated monocytes and some subgroups of dendritic cells as well as T cells and B cells.[1] The PD-1 receptor has two ligands; "programmed death ligand 1 (PD-L1)" and "programmed death ligand 2 (PD-L2)".[2,3] Although PD-L1 expression is easily inducible in many different cell types, PD-L2 expression is limited to only antigen presenting cells. This finding suggests that PD-L1 may play a more general and more specific role in inhibiting T cell activation than PD-L2. The PD-1/PD-L1 pathway is used by some tumors and viruses to escape from immune eradication. Activation of PD-1/PD-L1 pathway leads inhibition of T cell functions in secondary lymphoid tissues.[4]

PD-L1 is a transmembrane surface glycoprotein expressed in many solid tumors. There are many studies about the role of PD-1/PD-L1 pathway in adult malignancies and PD-1 and PD-L1 inhibiting agents have an important place in the treatment of adult cancers. However, the number of studies on the expression of PD-L1 in childhood solid tumors like neuroblastoma (NB), Wilms tumor (WT) and other primary renal tumors are not enough and results of these studies are conflicting. PD-1 and PD-L1 inhibiting agents have also begun to be used in the treatment of some childhood cancers.[5,6] Although PD-L1 blockade may be an appropriate

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treatment option especially in patients with unfavorable prognosis and refractory disease despite treatment with current therapies, there is insufficient data about the effects of PD-L1 blockade on immune functions in pediatric patients.[7] Therefore, we aimed to investigate whether PD-L1 expression is increased in tumor tissues of NB, WT and other primary renal tumors [congenital mesoblastic nephroma (CMN), rhabdoid tumor of the kidney (RTK), renal cell carcinoma (RCC), clear cell sarcoma (CCS)] and whether there is a relationship between the degree of PD-L1 expression and the stage and prognosis of diseases.

Material and Methods

A total of 60 patients under 18 years of age who were followed between 2006 and 2017 with the diagnoses of NB (n=34), WT (n=17) and other renal tumors (n=9) at Department of Pediatric Oncology in Gazi University Faculty of Medicine were included in the study. We evaluated NB patients' characteristics including age, Turkish Pediatric Oncology Group (TPOG) risk group of tumor, Shimada histological classification, metastasis status, mitosis karyorrhexis index (MKI), neuron-specific enolase (NSE), vanillylmandelic acid (VMA)/homovanillic acid (HVA) ratio, ferritin and lactate dehydrogenase (LDH) level, maximum standardized uptake value (SUVmax) on PET/CT, MYCN gene amplification and other mutations.[8,9] We also assessed prognostic factors in patients with WT such as stage, tumor weight, tumor histology (presence of anaplasia) and metastasis status. In addition, stage and metastasis status in patients with other primary renal tumors were evaluated. Overall survival (OS) and event-free survival (EFS) were estimated in all patients. The time from diagnosis to death was defined as OS, and the time from diagnosis to relapse or treatment failure was defined as EFS.

PD-L1 expression was evaluated in tumor tissues of all cases. Hematoxylin & eosin stained slides of each case were examined and two separate slides of each case with dense tumor and the least necrotic ones were selected. Immunohistochemical staining of PD-L1 was performed on slides prepared by modified tissue microarray method. Formalin fixed sections (4 μm thickness) were exposed to anti-PD-L1 (clone SP142 rabbit monoclonal primary antibody) antibody and they were stained via OptiView DAB IHC Detection Set and OptiView Amplification kit on VENTANA BenchMark ultra instrument. Tonsil tissue was used as positive control. PD-L1 stained slides were evaluated by two different observers who were unaware of the clinical features of the cases. PD-L1 expression was evaluated in detail, assuming significant membranous staining in tumor cells, similar to previous studies. Staining of the tumor cells were scored according to percentage of stained cells as shown in Table I.[6,10] Tumor cells scored +1, +2 and +3 were considered positive for PD-L1 expression.

The study protocol was approved by the Institutional Ethics Committee of Gazi University Faculty of Medicine with the decision number 11/2017-546 of December 01, 2017. This study was supported by Gazi University Scientific Research Projects Unit. Informed consent was taken from all families.

Statistical Analysis

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) for Windows 20 (IBM SPSS Inc., Chicago, IL). Continuous variables were summarized as mean ± standard deviation (SD) while discrete data were presented as median and minimum-maximum values. Survival analysis was performed using Kaplan-Meier curves and log-rank tests.

Table I. Assessment of staining score and percentage of stained tumor cells.

Staining score in tumor cells	Percentage of stained tumor cells (%)
0	<1
+1	≥1 and <5
+2	≥5 and <50
+3	≥50

Results

A total of 60 cases enrolled in the study. Mean age was 41.6 ± 38.8 months and 53.3% (n=32) of the patients were male. Most of the patients were diagnosed NB (n=34) while other patients were diagnosed WT (n=17) and other renal tumors (n=9). The median follow-up was 79 (6-143) months.

Patients with neuroblastoma

Thirty-four patients with NB were included in the study. Mean age was 30.7 ± 27.9 months and 20 patients (58.8%) were male. The demographic characteristics of the cases are showed in Table II. Mean OS was 110.1 ± 10.1 months and EFS was 92.8 ± 11.6 months (Fig. 1).

Patients with Wilms Tumor

Seventeen patients with WT were included in the study. Mean age was 42.5 ± 39.7 months and 6 (35.2%) patients were male. The demographic characteristics of the cases are discussed in detail in Table III. Mean OS was 104.2 ± 9.3 months and EFS was 103.0 ± 8.8 months (Fig. 2).

Patients with Other Renal Tumors

Four patients with RCC were included in the study. The mean age of the patients with RCC was 138.6 ± 59.4 months and three of them were male. Two patients of them had stage II and others had stage IV disease. Tumor tissues of three patients with RCC were obtained before chemotherapy. Two patients of them had metastatic disease and they died at 4th and 21th months of treatment. Another two patients with RCC are still alive with complete remission for 103 months.

There were two patients with CMN. Ages of the patients with CMN were 10 and 20 months and one of them was female. Tumor tissues were obtained before chemotherapy in both of the patients with CMN and they did not have metastatic disease and no relapse or death was observed during the follow-up period. They are still alive with remission for 56 months.

Table II. Characteristics of patients with neuroblastoma.

Number of patients (n)	34
Age (months), mean (± SD)	30.7 ± 27.9
<18 months	15 (44%)
≥18 months	19 (56%)
Sex, male, n (%)	20 (58.8%)
Stage	
I	3 (%8.8)
II	1 (%2.9)
III	9 (%26.5)
IV	21 (%61.8)
Biopsy	
Before chemotherapy	32 (94%)
After chemotherapy	2 (6%)
SUVmax	
<2.5	6 (23.1%)
≥2.5	20 (76.9%)
Metastasis	20 (59%)
Metastasis site	
Bone marrow	13 (28.9%)
Bone	10 (22.3%)
Liver	6 (13.4%)
Lymph node	5 (11.1%)
Others	11 (32.3%)
TPOG risk group	
Low risk	5 (14.7)
Intermediate risk	13 (38.2%)
High risk	16 (47.1%)
Shimada classification	
Favorable	26 (76.5%)
Unfavorable	8 (23.5%)
MKI	
<2%	21 (61.8%)
2-4%	7 (20.6%)
>4%	6 (17.6%)
Primary region	
Abdomen	26 (76.5%)
Posterior mediastinum	6 (17.6%)
Pelvic	2 (5.8%)
NSE >100 ng/mL	30 (88%)
Ferritin >150 ng/mL	11 (32.4%)
VMA/HVA ratio >1	27 (79.4%)
LDH >1500U/L	12 (35.3%)
Mutations	
MYCN gene amplification	8 (23.5%)
1p deletion	18 (75%)
11q deletion	9 (37.5%)
Gain of 17q	17 (70.8%)
Hyperdiploid	8 (33.3%)
Relapse/resistant disease	9 (26.4%)
Death	7 (20.6%)

SUVmax: maximum standardized uptake value, TPOG: Turkish Pediatric Oncology Group, MKI: mitosis karyorrhexis index, NSE: neuron-specific enolase, HVA: homovanillic acid, VMA: vanillylmandelic acid, LDH: lactate dehydrogenase.

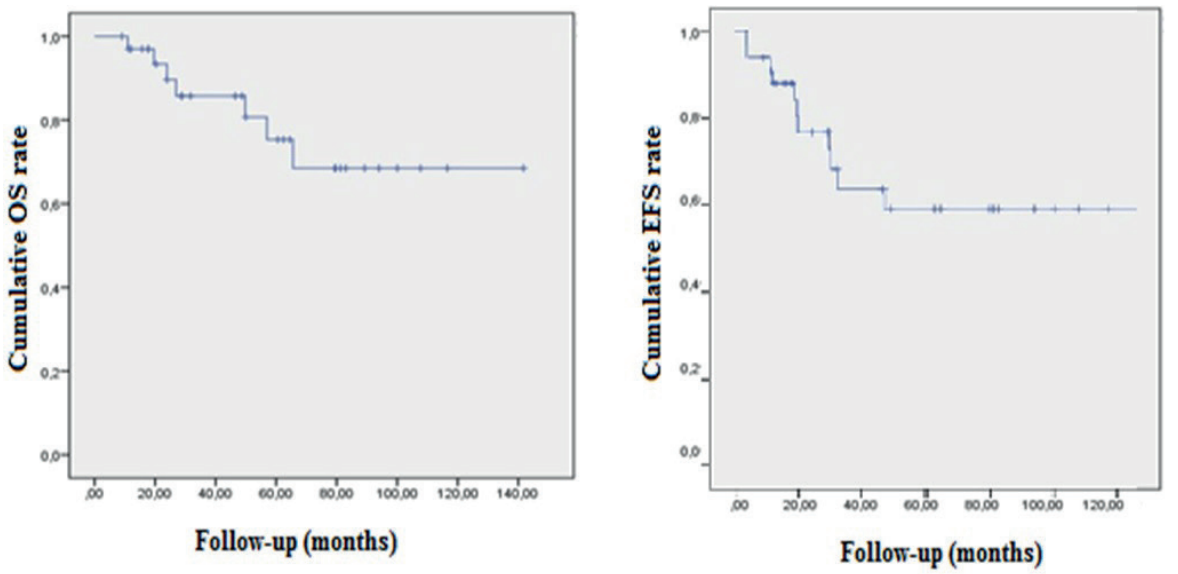

Fig. 1. OS and EFS curve of patients with neuroblastoma.

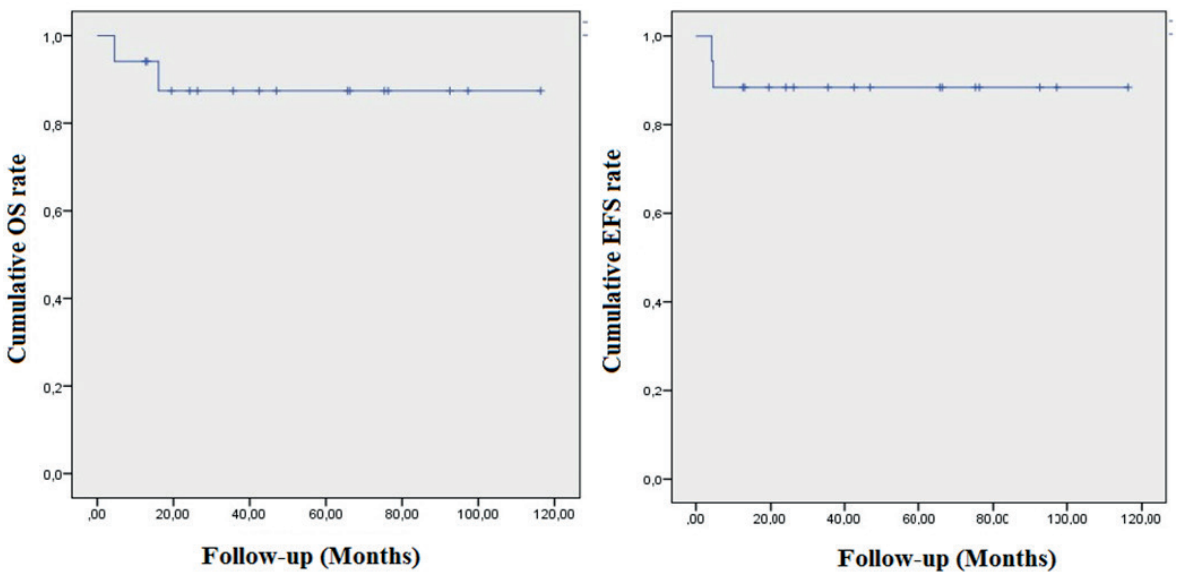

Fig. 2. OS and EFS curve of patients with Wilms tumor.

There were two patients with RTK. Ages of the patients with RTK were 10 and 102 months and one of them was male. Tumor specimen was obtained before chemotherapy in one patient and after chemotherapy in the other case. Both of the patients with RTK had metastatic disease and they died at 7th and 11th months of treatment.

The only patient with CCS diagnosis was male and he was 30 months of age. His tumor tissue was obtained after chemotherapy and he did not have metastatic disease. No relapse occurred and he is alive with remission for 83 months.

PD-L1 Expression in Patients with neuroblastoma

PD-L1 positive staining (membranous staining of 1% or more) (Fig. 3) was detected in two of 34 patients with NB (Table IV). In one patient with NB, PD-L1 staining was observed below 1% and

it was accepted negative for PD-L1 expression. Table V shows detailed clinical features of PD-L1 positive staining cases.

Table III. Characteristics of patients with Wilms tumor.

Number of patients (n)	17
Age (months), mean (± SD)	42.5 ± 39.7
Sex, male, n (%)	6 (35.2%)
Stage	
I	3 (17.6%)
II	3 (17.6%)
III	5 (29.4%)
IV	6 (35.4%)
Biopsy	
Before chemotherapy	6 (35.3%)
After chemotherapy	11 (64.7%)
Metastasis	5 (29.4%)
Metastasis site	
Lung	5 (100%)
Pathology	
Favorable	15 (88.2%)
Unfavorable/Anaplasia (+)	2 (11.8%)
Tumor weight	
<550 gr	11 (64.7%)
≥550 gr	6 (35.3%)
Relapse/resistant disease	1 (5.9%)
Death	2 (11.8%)

PD-L1 Expression in Renal Tumors

None of the patients with WT, CMN, RTK, RCC and CCS had positive staining for PD-L1. Therefore, the relationship between PD-L1 expression and prognostic factors, stages and prognosis of patients could not be evaluated in these group of patients.

Discussion

Significant advances occurred in targeted therapy thanks to the definition of various genetic mutations in childhood cancers. Immunotherapy which detects tumor cells as foreign bodies and activates the host immune response has become prominent in the treatment of some cancers, due to the strong relationship between tumor microenvironment and host immune system. Antibodies targeting the PD-1/PD-L1 pathway which was developed to induce immune system against tumor cells might also increase patients' survival with less toxicity than conventional chemotherapeutic regimens.[11] Therefore; the clinical importance of PD-L1 expression and its relationship with prognosis and treatment response should be highlighted in childhood cancers. So we evaluated PD-L1 expression in NB and childhood renal tumors.

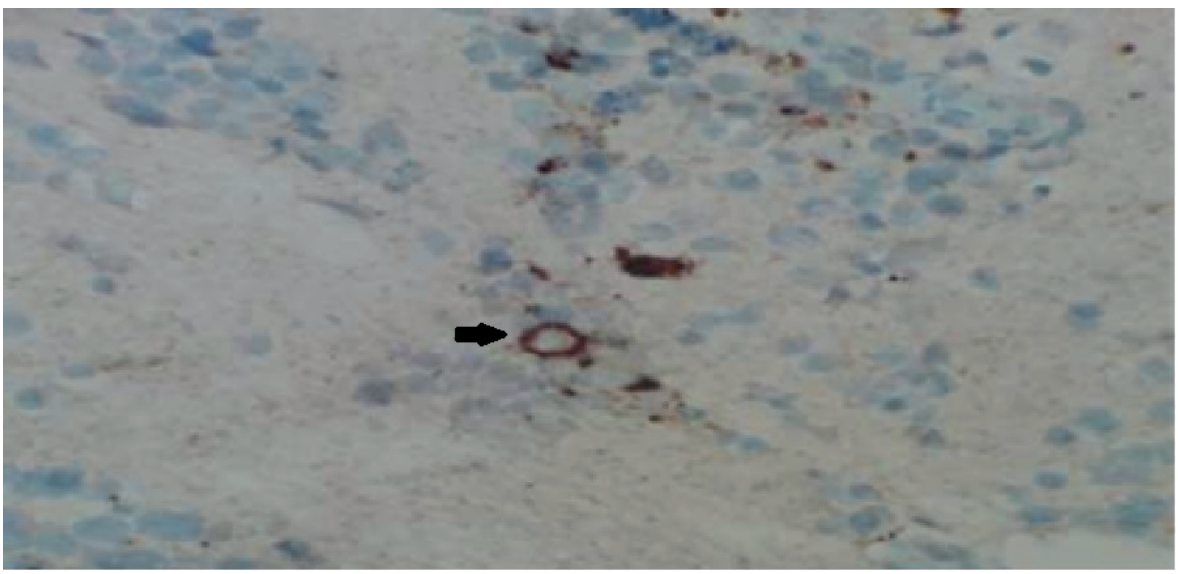

Fig. 3. PD-L1 staining between 1% and 5% of tumor cells in neuroblastoma (X100).

Table IV. PD-L1 expression and clinicopathologic features in patients with neuroblastoma.

	PD-L1 (-)	PD-L1 (+)
Sex (n=34)		
Female	14 (100%)	0
Male	18 (90%)	2 (10%)
Age (months)		
<18	14 (93.3%)	1 (6.7%)
≥18	18 (94.7%)	1 (5.3%)
Stage		
I, II, III, IVS	13 (100%)	0
IV	19 (90.5%)	2 (9.5%)
Biopsy		
Before chemotherapy	30 (93.7%)	2 (6.3%)
After chemotherapy	2 (100%)	0
TPOG risk group		
Low risk	5 (100%)	0
Medium risk	13 (100%)	0
High risk	14 (87.5%)	2 (12.5%)
Shimada classification		
Favorable	26 (100%)	0
Unfavorable	6 (75%)	2 (25%)
MKI		
<2%	20 (95.2%)	1 (4.8%)
2-4%	7 (100%)	0
>4%	5 (83.3%)	1 (16.7%)
MYCN gene amplification		
Negative	24 (92.3%)	2 (7.7%)
Positive	8 (100%)	0

TPOG: Turkish Pediatric Oncology Group, MKI: mitosis karyorrhexis index.

Table V. Demographic characteristics of two PD-L1 positive patients with neuroblastoma.

	Case 1	Case 2
Age (months)	24	14
Sex	Male	Male
Stage	IV	IV
Biopsy	Before chemotherapy	Before chemotherapy
SUVmax	4.9 (↑)	5.3 (↑)
Primary region	Posterior mediastinum	Abdomen
Metastasis	Yes	Yes
Metastasis site	Bone marrow	Bone, bone marrow, lymph node
TPOG risk group	High risk	High risk
Shimada classification	Unfavorable	Unfavorable
MKI	<2% (↓)	>4% (↑)
NSE	159 ng/mL (↑)	187 ng/mL (↑)
VMA/HVA ratio	1.2 (↑)	0.8
Ferritin	54 ng/Ml	197 ng/Ml (↑)
LDH	2300 U/L (↑)	1873 U/L (↑)
MYCN gene amplification	Negative	Negative
Hyperdiploid	Not assessed	No
1p deletion	Not assessed	Positive
11q deletion	Not assessed	Negative
Gain of 17q	Not assessed	Negative
Relapse/resistant disease	No	No
Death	No	Yes
Outcome	Exitus (28^{th} months)	Exitus (11^{th} months)

SUVmax: maximum standardized uptake value, TPOG: Turkish Pediatric Oncology Group, MKI: mitosis karyorrhexis index, NSE: neuron-specific enolase, HVA: homovanillic acid, VMA: vanillylmandelic acid, LDH: lactate dehydrogenase.

In our study, positive PD-L1 expression was observed in only two of 34 cases with NB. Due to the small number of PD-L1 positive cases (n=2), it was not suitable to perform statistical analysis and evaluate the relationship between PD-L1 expression and prognostic factors. Both of the PD-L1 positive cases were stage IV NB and high risk NB. Therefore; PD-L1 positivity was detected in 9.5% of the stage IV NB patients and in 12.5% of the high-risk NB cases. Both of the patients were within 8 cases with unfavorable prognosis according to Shimada classification (25%) and they were within 26 cases with negative MYCN gene amplification (7.7%). The two cases with positive PD-L1 expression died at 11^{th} and 28^{th} months of follow-up. Although there are many studies in the literature about PD-L1 expression in adult cancers, there are limited and small studies about PD-L1 expression in pediatric malignant tumors.[11-13] Moreover, contradictory results have been obtained in the current studies.[14-19] It

is reported that PD-L1 expression is increased in patients with unfavorable prognosis according to Shimada classification and advanced stage tumors in NB. PD-L1 expression correlates with poor overall survival, but there are also studies showing no relationship between PD-L1 expression and OS rate, tumor stage and histologic type.[14-16,20] In the study published by Uehara et al.[15] that includes 41 pediatric patients with NB high PD-L1 expression was detected in 5 patients (12%) with advanced stage tumors, and PD-L1 expression was associated with poor OS. In another study including 43 patients with NB, positive PD-L1 expression was shown in 31 cases (72%), and tumors with high PD-L1 expression were found to have a better OS rates than those without expression.[16] Majzner et al.[20] expressed that positive PD-L1 expression was observed in 17 (14%) of 118 patients with NB and no significant relationship was found between positive PD-L1 expression and OS rates at any stage or in any risk group. Dondero et al.[21] showed positive PD-L1 expression in 3 (15.7%) of 19 patients with metastatic NB. There is only one study which compares the prognostic factors and PD-L1 expression in patients with NB in the literature. In this study by Saletta et al.[19], positive PD-L1 expression was found in 48 (19%) of 254 patients with NB. Positive PD-L1 expression was detected in 19 (20.9%) of 91 cases with advanced stage NB and in 13 (17.6%) of 74 cases with high risk NB. Positive PD-L1 expression was reported in 38 (31.9%) of 119 cases with negative MYCN gene amplification and only two out of 34 patients with positive MYCN gene amplification (5.9%). Positive PD-L1 expression was significantly higher in patients with negative MYCN gene amplification than counterparts with positive MYCN gene amplification, and there was no statistically significant relationship between PD-L1 expression and other prognostic factors. Additionally, unlike other studies, it has been observed that NB patients with positive PD-L1 expression have better OS rates.[19]

PD-L1 positive staining was not detected in any of the patients with WT, CMN, RTK, RCC and CSS in our study. In the literature; Routh et al.[14] found that positive PD-L1 expression was detected in 11 (14%) of 81 patients with WT and PD-L1 expression has been reported to be a prognostic marker. In another study by Pinto et al.[18], no positive PD-L1 expression was detected in any cases with WT as in our study.

In our study, chemotherapy was not administered to 44 cases (73.3%) before biopsy. Thirty-two patients with NB (94%) and 6 patients with WT (35.3%) did not receive chemotherapy before biopsy, including the two patients with positive PD-L1 expression. Although biopsy samples of our patients were taken before chemotherapy (73.3%), PD-L1 expression rate was found to be low. Other studies in the literature also included patients with biopsies taken both before and after chemotherapy as similar with our study.[15,16,19,20] In the study by Saletta et al.[19] reported that 64 patients with NB (34.4%) received chemotherapy while 122 patients with NB (65.6%) did not receive chemotherapy before biopsy. In the same study, PD-L1 expression positivity was found to be higher in patients who did not receive chemotherapy prior to biopsy [27.9% (34/122)] than those who received chemotherapy [15.6% (10/64)].

Variations in PD-L1 expression rates in studies may also be associated with heterogeneity of the patient population and differences in the use of different scoring systems, antibody staining kits, staining procedures and antigen uptake techniques. For example, while evaluating PD-L1 staining positivity in tumor tissue, the median H-score was used as a cut-off value in some studies; while the median value of the staining percentage of 5% and above was considered as the cut-off value in other studies.[14-17] In our study, positive PD-L1 expression was defined as the presence of ≥1% staining in tumor cells. In previous studies, staining in ≥1% of tumor cells were also accepted as positive for expression of cytokines, IFN-ϒ and other immune markers.[6,19,22-24] We preferred the most commonly used scoring system; however, different results might have been attained with separate scoring systems.

The use of different antibodies (recombinant/polyclonal antibodies) in the immunohistochemical evaluation of PD-L1 expression in the studies may also play an important role in conflicting results. Different clone anti PD-L1 antibodies may lead to different results in the same tumor.[25] Majzner et al.[20] used clone 28-8 as PD-L1 antibody in their study while clone 5H1 was used as the PD-L1 antibody in another study published by Routh et al.[14] in patients with WT. The anti-PD-L1 antibody in these studies is different from the clone (SP 142) which we used in our study. However, a lower rate of response to tumor-associated antigens may occur in children, since burden of mutations is low but immunogenicity is higher in childhood cancers than adult counterparts. Therefore, PD-L1 expression in childhood tumors is lower than in adult cases in the literature.[26]

Modified tissue microarray method has been used the most in the literature for evaluating PD-L1 expression in childhood tumors as in our study.[19,20] When PD-L1 expression is evaluated in sections using modified tissue microarray method, it is observed that PD-L1 shows a very heterogeneous distribution within the tumor. Hence, immunohistochemical examination with modified tissue microarray method may cause false negativity in terms of PD-L1 expression. Therefore, it is more appropriate to use whole tissue sections in the evaluation of PD-L1 expression. However; complete tissue sections can be obtained in prospective studies.

The first limitation of our study is the small number of cases and retrospective design. Some cases could not be included in the study because we couldn't reach the tissue specimens of the patients diagnosed before 2006 and some of the samples were very small and insufficient to evaluate PD-L1 expression. In the beginning, we had aimed to evaluate the relationship between PD-L1 expression and prognostic factors but due to the small number of PD-L1 positive cases, statistical analysis could not be performed.

To conclude, although PD-L1 positivity rate is higher in adult tumors and is often associated with advanced stage disease with poor prognosis, it is very low in childhood tumors. This situation may be due to differences in the pathogenesis of childhood and adult tumors. However, prospective studies with larger populations are needed to clarify this issue.

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Author contribution

The authors confirm contribution to the paper as follows: study conception and design: SŞ, AP, AO, FGP, CK; data collection: SŞ; analysis and interpretation of results: SŞ, AP, CK; draft manuscript preparation: SŞ, CK. All authors reviewed the results and approved the final version of the manuscript.

Ethical approval

The study protocol was approved by the Institutional Ethics Committee of Gazi University Faculty of Medicine with the decision number 11/2017-546 of December 01, 2017.

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Conflict of interest

All the authors declare no conflict of interest.

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