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Short Communication

The PD-L1/PD-1 axis expression on tumor-infiltrating immune cells and tumor cells in pediatric rhabdomyosarcoma

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ABSTRACT

Background: Activation of immune checkpoints, e.g. PD-1/PD-L1 axis, in cancer microenvironment, enables evasion of host anti-cancer immune response and drives tumor progression. To date, there have been only a few studies analyzing PD-1/PD-L1 expression in pediatric malignancies.

Aim: In the current study, we aimed to assess PD-L1 and PD-1 expression in pediatric rhabdomyosarcoma (RMS) and to investigate their clinicopathological associations.

Materials and methods: The study enrolled 31 children with RMS. Tissue microarrays with representative tumor tissue samples were stained with anti-PD-1 NAT105 clone (Ventana, Roche) and two different antibodies against PD-L1: SP142 (Ventana, Roche) and 22C3 (DAKO). Adequate positive controls were applied. Their expression was assessed in tumor-associated immune cells (TAICs) and in the tumor cells separately.

Results: We did not detect any positive PD-L1 staining in analyzed tumors using SP142 antibody; however, in 11 cases (35.48%) its expression was revealed by means of 22C3 clone. The staining was restricted to TAICs in all cases, which no reaction in tumor cells. The 5-year relapse free survival (RFS) rate was significantly higher in PD-L1 positive cases (61.5% vs 25.0%, $p = 0.024$), but it most likely results from more frequent PD-L1 expression in low-stage RMS. PD-1 expression on TAICs was detected in 7 cases and did not influence the prognosis.

Conclusions: We found that PD-L1 expression on TAICs, as detected with the use of 22C3 clone but not SP142 antibody, tends to be associated with low-stage RMS in children. PD-1 expression on TAICs in RMS is neither associated with distinct clinical course nor with clinicopathological features.

1. Introduction

The significant role of the tumor immune microenvironment in cancer biology and its influence on the clinical course has been recognized in the recent years. Especially, the presence of tumor-infiltrating lymphocytes and macrophages (tumor-associated immune cells, TAICs), along with the expression of negative regulators of an immune response by inflammatory cells and tumor cells is now under thorough investigation. Activation of immune checkpoints, e.g. PD-1/PD-L1 axis, in cancer microenvironment, enables evasion of host anti-cancer immune responses and drives tumor progression. When PD-1 expressed by T lymphocytes binds PD-L1 on tumor cells it leads to inhibition of T-cell dependent cancer cell killing [1]. Introduction of PD-1/PD-L1 axis inhibitors has improved outcomes in numerous malignancies, especially in patients with advanced melanoma [2].

Immunohistochemical evaluation of the PD-1/PD-L1 expression in the tumor microenvironment was believed to predict response to PD-1/PD-L1 inhibitors, however, it turned out that its predictive value is relatively weak. The mutational burden of tumors and microsatellite instability status are better predictors of the efficacy of the immune checkpoint inhibitors, probably because tumors with multiple mutations are more immunogenic and thus, more responsive to immunotherapy [3]. Nevertheless, since the immunohistochemical assessment of PD-1/PD-L1 expression is a feasible and widely available method, it is still used in clinical practice to qualify patients for targeted immunotherapy. Moreover, many studies have shown that PD-1/PD-L1 expression has prognostic value in various cancers, including lung, breast and colorectal carcinomas [4]. Interestingly, some PD-1/PD-L1-

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positive cancers have better, whereas others present worse prognosis.

In most cases, those studies were conducted in adults and our knowledge about the role of immune checkpoints in pediatric malignancies remains limited. Pediatric tumors frequently display a relatively small amount of genetic abnormalities. Especially, soft tissue sarcomas are often driven by single translocations, as such tumors are likely to be less immunogenic. Nevertheless, there are promising, ongoing clinical trials incorporating immunotherapy in advanced pediatric cancers [3]. To date, there have been only a few studies analyzing PD-1/PD-L1 expression in pediatric malignancies, including lymphomas, neuroblastoma, Wilms tumor, glioblastoma, osteosarcoma, and rhabdomyosarcoma (RMS) [5–8]. A prognostic significance of PD-L1 expression was demonstrated in children with neuroblastoma [5].

Rhabdomyosarcoma is the most common soft tissue sarcoma in pediatric population. Four histological variants of RMS are currently recognized, two of which are frequent in children, i.e. embryonal (RME) and alveolar RMS (RMA). The latter is often driven by translocations t(2;13)(q35;q14) and t(1;13)(p36;q14), which generate PAX3/FKHR and PAX7/FKHR fusion genes, respectively [9]. On the other hand, RME is not associated with recurrent translocations, but rather show changes in various chromosome pairs. All cases of pediatric RMS are considered as high-grade sarcomas, and due to aggressive biology, the treatment is multimodal, including surgery, radiation therapy and multidrug chemotherapy. The prognosis in advanced, i.e. inoperable and/or metastatic cases, is still poor. New prognostic factors and targeted therapies, like immunotherapy, are highly anticipated to improve the outcomes of patients with advanced RMS.

In the current study, we aimed to assess PD-L1 and PD-1 expression in pediatric RMS and to investigate their clinicopathological associations.

2. Materials and methods

The study enrolled 31 children with RMS (13 girls and 18 boys), aged from 1 day (a congenital tumor) to 18 years, with a median age of 7.4 years at diagnosis. The group included 19 children with RME and 12 children with RMA. All patients included in the study were treated in pediatric oncology centers of the Polish Pediatric Solid Tumors Study Group in the years 1992-2013. The detailed demographic and clinicopathological information about the cohort is given in Table 1. The fusion status was unavailable, since it is not routinely performed in RMS diagnostic process according to CWS protocols used in Poland.

Archival microscopic slides of each case were reevaluated by the second independent pathologist being an expert in the field of diagnostics of childhood sarcomas. The tissue microarrays (TMAs) containing representative single punch biopsies of 5 mm diameter from each case were built utilizing a commercial Tissue-Tek® Quick-Ray™ Tissue Microarray System (Sakura Finetek USA, Inc company). The TMAs were composed only of pretreatment tissues, and no post-therapy tissues for comparison were available. TMAs were then stained with the following antibodies against PD-L1: SP142 (1:100 dilution, Ventana, Roche) and 22C3 (1:50 dilution, cat. no M3653, DAKO), and against PD-1: NAT105 (1:50 dilution, Ventana, Roche). Appropriate controls comprising the tonsil and placenta (positive) and omission of the primary antibodies (negative) were included in each staining. Only continuous membranous staining in > 1% of cells was considered positive. Immunohistochemistry results were evaluated by two pathologists. The whole cohort was screened for the presence of TAICs. The detection of any lymphocytes or macrophages by hematoxylin and eosin (H&E) staining was considered positive as proposed by Majzner et al. [5]. Immune cells forming peritumoral lymphoid follicles were not included as TAICs. PD-L1 and PD-1 staining in neoplastic cells and in the TAICs were evaluated separately.

In the same cohort, we have previously evaluated expression of several biomarkers, including cell cycle-related proteins (cyclin D1, p53, Ki-67), markers of hypoxia (hypoxia-inducible factor 1, HIF-1; vascular endothelial growth factor, VEGF; carbonic anhydrase IX, CA-IX; glucose transporter 1, GLUT-1), regulators of apoptosis (survivin), and extracellular matrix elements (fibronectin). The detailed methodology of the assessment of these markers is presented in our other papers [10,11].

Table 1
Correlations between clinicopathological variables and immunohistochemical PD-L1 status in tumor associated immune cells. * p values were calculated by Fisher's exact test.

Variables		PD-L1		p*
		Negative (N, %)	Positive (N, %)	
Sex	Female	8 (61.54)	5 (38.46)	1.0
	Male	12 (66.67)	6 (33.33)	
Histology	Embryonal	11 (57.89)	8 (42.11)	0.451
	Alveolar	9 (75.00)	3 (25.00)	
Location	Superficial	4 (44.44)	5 (55.56)	0.217
	Deep	16 (72.73)	6 (27.27)	
Stage	I-II	2 (28.57)	5 (71.43)	0.066
	III-IV	18 (75.00)	6 (25.00)	
Tumor size	T1	3 (60.00)	2 (40.00)	1.0
	T2	17 (65.38)	9 (34.62)	
Lymph nodes	N0	12 (60.00)	8 (40.00)	0.697
	N1	8 (72.73)	3 (27.27)	
Distant metastases	M0	10 (62.50)	6 (37.50)	1.0
	M1	10 (66.67)	5 (33.33)	
GLUT-1	Low	13 (59.09)	9 (40.91)	0.429
	High	7 (77.78)	2 (22.22)	
HIF-1	Low	7 (52.85)	6 (46.15)	0.449
	High	13 (72.22)	5 (27.78)	
CA-IX	Low	5 (45.54)	6 (54.55)	0.132
	High	15 (75.00)	5 (25.00)	
VEGF	Low	2 (28.57)	5 (71.43)	0.066
	High	18 (75.00)	6 (25.00)	
Ki-67	< 30%	6 (60.00)	4 (40.00)	1.0
	> 30%	14 (66.67)	7 (33.33)	
P53	Low	12 (66.67)	6 (33.33)	1.0
	High	8 (61.54)	5 (38.46)	
Cyclin D1	Low	12 (66.67)	6 (33.33)	1.0
	High	8 (61.54)	5 (38.46)	
Fibronectin	Low	5 (55.56)	4 (44.44)	0.683
	High	15 (68.18)	7 (31.82)	
Survivin	Low	7 (63.64)	4 (36.36)	1.0
	High	13 (65.00)	7 (35.00)	

3. Statistics

Kaplan-Meier curves were constructed to calculate survival rates in PD-L1(-) *vs* PD-L1(+) and PD-1(-) *vs* PD-1(+) patients, and then compared with F-Cox test, which is dedicated to small groups. Categorical variables were compared using two-tailed Fisher's exact test. P-value < 0.05 was considered statistically significant.

4. Results

We did not detect any positive PD-L1 staining in analyzed tumor sample, when using SP142 antibody. However, PD-L1 expression was revealed in 11 cases (35.48%) with used of the 22C3 clone. Of note, an appropriate positive reaction in control tissues was observed with SP142 clone. The positive PD-L1 staining in RMS samples was restricted to TAICs in all cases, with no reaction in tumor cells. Representative examples of positive staining in TAICs are presented in Fig. 1. TAICs were noted in all samples and the percentage of PD-L1 positive TAICs ranged from 1% to 10%.

PD-1-positive TAICs were displayed in 7 tumors (22.5%), ranging from 1% to 3% of immune infiltrates. Tumors cells were invariably PD-1 negative. In 2 cases peritumoral lymphoid follicles with PD-1(+) and PD-L1(+) cells were identified. Representative examples of positive staining in TAICs are presented in Fig. 2.

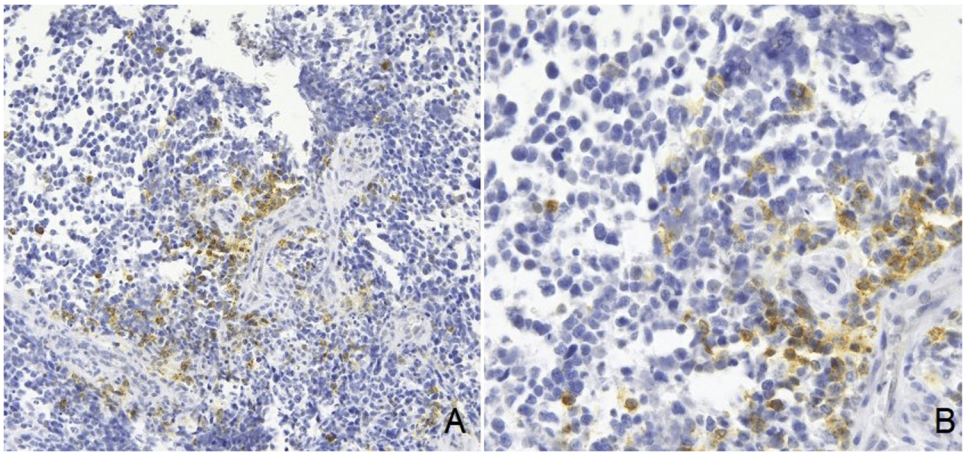

Fig. 1. Examples of positive staining in the tumor associated immune cells with the use of 22c3 anti-PD-L1 antibody in pediatric rhabdomyosarcoma (A–B).

No statistically significant associations between PD-L1 expression and various clinicopathological variables was found, however, there was a trend toward a reverse correlation between PD-L1 positivity in TAICs and the tumor stage, as well as with VEGF expression (Table 1). Similarly, presence of PD-1 positive cells correlated neither with clinical nor with pathological features. Three tumors (9.7%) co-expressed PD-1 and PD-L1, but there was no statistically significant relationship between these two markers (p = 0.67).

Seventeen of 31 patients (54.8%) in our cohort died from malignant disease, including 4 PD-L1(+) cases (4/11, 36.4%), and 13 PD-L1(-) (13/20, 65.0%). The median overall survival (OS) and relapse-free survival (RFS) in the whole cohort were 33 months and 15 months, respectively. The median OS in PD-L1(+) cases was 37 months, whereas in PD-L1(-) patients it was 30.5 months. The 5-year OS rate was higher in PD-L1 positive cases when compared to PD-L1 negative ones (59% vs 30%), however, this finding did not reach statistical significance (p = 0.078, F-Cox; Fig. 3). The 5-year RFS rate was significantly higher in PD-L1(+) cases (61.5% vs 25.0%, p = 0.024, F-Cox; Fig. 4). Nevertheless, these differences in survival are most likely associated with higher frequency of PD-L1-expressing TAICs in low-stage tumors. All 4 PD-L1(+) patients who died or had relapse manifested advanced disease (stage 3 or 4). On the opposite, high-stage PD-L1(+) RMS cases were not associated with better survival. The presence of PD-1(+) immune cells did not influence neither RFS (42.8% vs 35.6%, p = 0.273. F-Cox) nor OS (38.1% vs 39.2%, p = 0.528. F-Cox). The Kaplan-Meier curves are shown in Figs. 5 and 6.

In multivariate Cox proportional hazard regression analysis incorporating PD-1, PD-L1, tumor histology, stage, sex, and age of the patient, only histological type was included in multivariable model predicting OS or RFS.

5. Discussion

In the current study we investigated the potential prognostic value and clinicopathological correlations of PD-L1 and PD-1 expression in TAICs in pediatric RMS. To date, only a few studies have analyzed the expression and role of PD-1/PD-L1 axis in pediatric malignancies. The very first study on this issue by Chowdhury et al. demonstrated the relatively high frequency of PD-L1 positivity in tumor cells of various pediatric cancers, including alveolar and embryonal RMS (86% and 50% positive cases, respectively) [8]. They showed that PD-L1+ tumors have worse survival unless they are infiltrated with abundant CD8+ lymphocytes T. Nevertheless, the results of this study should be interpreted with caution since the utilized antibody was not validated for diagnostic purposes [8]. Another study, comprising 451 pediatric tumors, including 53 RMS cases, provided different conclusions. Only one case of RMS had PD-L1 positive tumor cells, whereas 30% of tumors were infiltrated with PD-L1 positive TAICs [5]. Pinto et al. reported that PD-L1 mRNA was detected in all cases of RMS, but it was absent in 15 of 23 cases by IHC [7]. Italian authors demonstrated PD-L1 expression on TAICs in 15/25 (60%) cases using IHC and a very small percentage of PD-L1-positive tumor cells in primary cell lines by flow cytometry analysis [12]. On the opposite to our study, they reported comparable results obtained with SP142 and 22C3 clone, however the latter showed weaker staining. Interestingly, enhanced PD-L1 expression was observed in post chemotherapy specimens when compared to

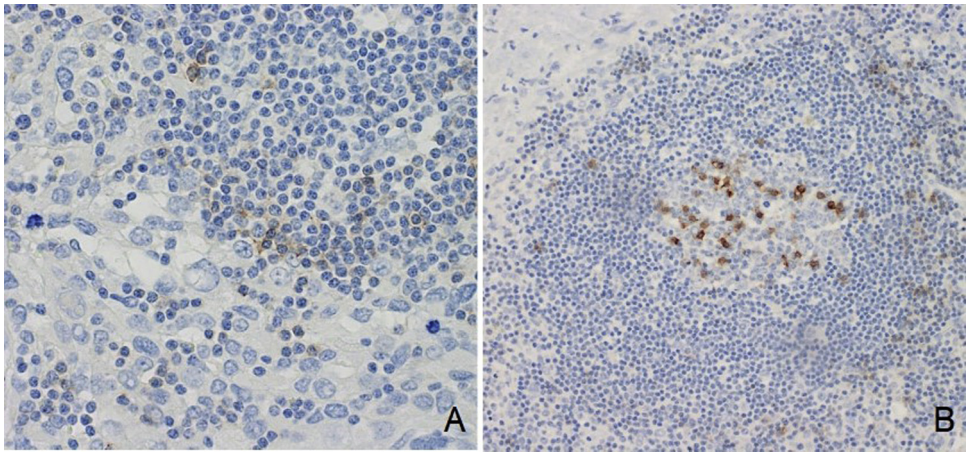

Fig. 2. Examples of positive PD-1 staining in scattered lymphocytes (A) and peritumoral lymphoid follicles (B).

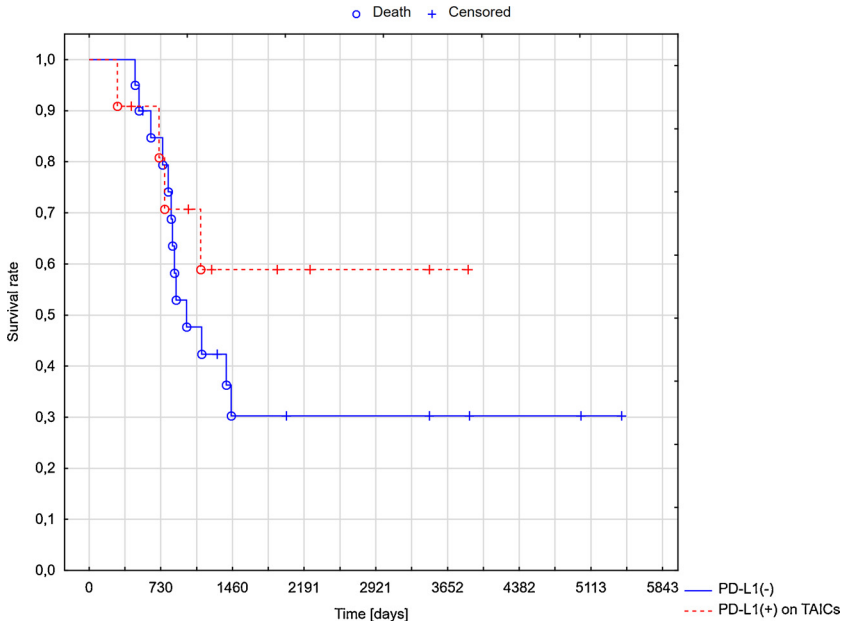

Fig. 3. Overall survival in pediatric rhabdomyosarcoma according to PD-L1 status.

pretreatment ones. The prognostic significance of these findings was not investigated. Recently, Kather et al. reported prognostic value of CD163(+) immune cell infiltrates in RME, but failed to demonstrate PD-L1 expression, whereas PD-1 was expressed by exceedingly rare intratumoral mononuclear cells in the minority of tumors tested [13].

In our study, the PD-L1 expression on TAICs was associated with better survival, but it rather resulted from the tendency of more frequent PD-L1(+) expression in lower-stage cases. Similar results were obtained by van Erp et al., who showed that PD-L1 expression on both tumor cells and CD8+ lymphocytes correlated with low IRS grade and lack of metastases in RMA [14]. This phenotype was also associated with better OS and RFS. Interestingly, these associations were not observed in RME, suggesting that clinical consequences of PD-L1 expression in soft tissue sarcomas are subtype dependent [14]. On the face of it, the fact that PD-L1 positivity could be associated with better prognosis seems paradoxical. However, the PD-L1 expression on lymphocytes is a marker of their activation, which may translate to the enhanced anti-tumor reaction. On the other hand, the PD-L1 expression on tumor cells may reflect increased anti-tumor response, and thus be associated with a more favorable prognosis. Similar observations were made in various malignancies, including breast, lung, head & neck, and vulvar cancer [15–18].

The tumor microenvironment undergoes constant changes due to various exogenous and endogenous stimuli. For example in tissue hypoxia tumor cells start to produce multiple substances, including HIF-1, VEGF, GLUT1, and CA-IX. Some of them induce angiogenesis, whereas others are involved in Warburg effect. Hypoxia modifies function of cytotoxic lymphocytes T and regulatory T cells. HIF-1 binds directly to a hypoxia response element in the PD-L1 promoter and is thought to be one of the crucial elements regulating PD-L1 expression in cancer cells [19]. Moreover, hypoxic microenvironment attracts immunosuppressive regulatory T cells, whereas in normoxic conditions,

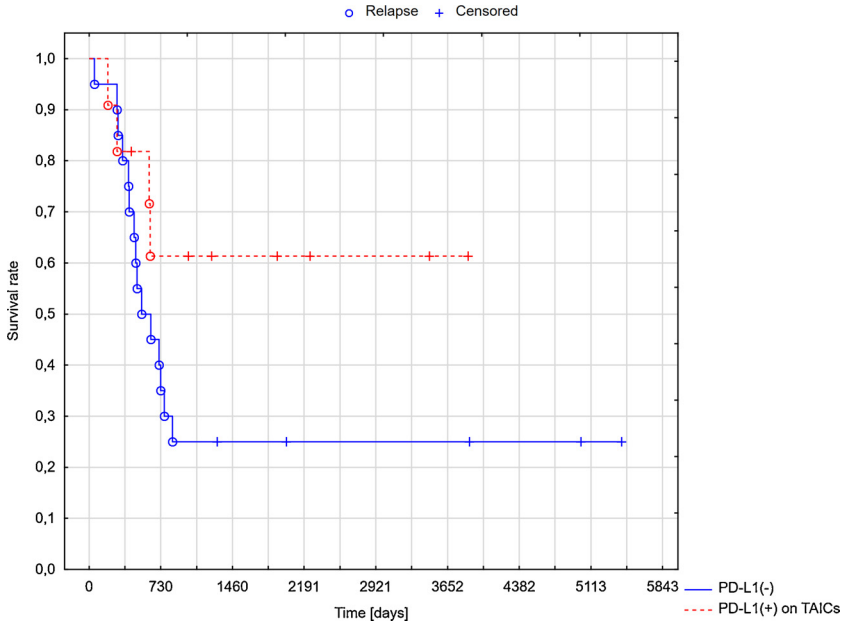

Fig. 4. Relapse-free survival in pediatric RMS according to PD-L1 status.

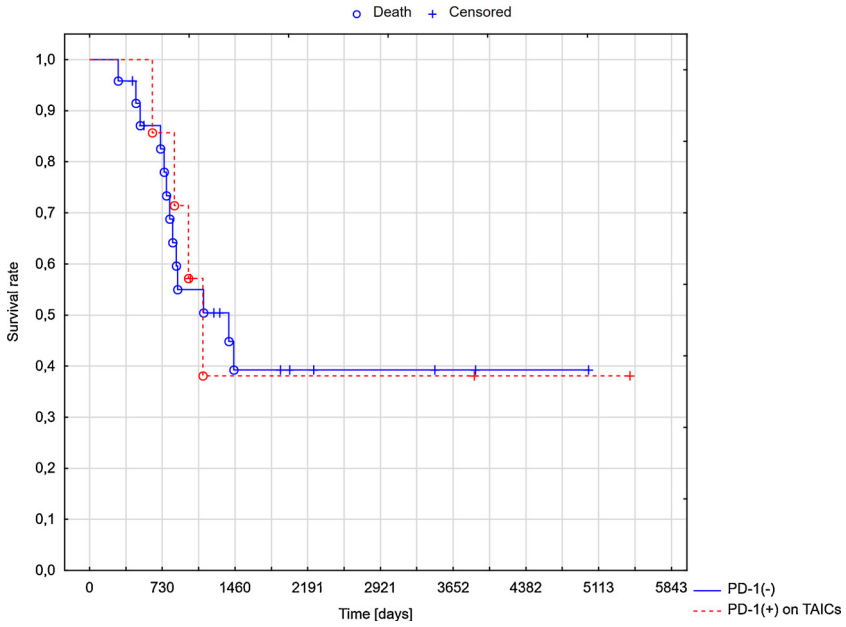

Fig. 5. Overall survival in pediatric rhabdomyosarcoma according to PD-1 status.

the cell recruitment signals shift, and immunocompetent PD-L1 positive lymphocytes are preferred [19]. It is consistent with our findings since we noted a trend toward an inverse correlation between the expression of PD-L1 in TAICs and tumor expression of VEGF. It suggests, that anti-tumor response in RMS might be attenuated in hypoxia.

A crucial issue in PD-L1 expression assessment is to define cut-off in terms of percentage of positive cells. We demonstrate, that in RMS expression of PD-L1 in > 1% carries prognostic value. In our study 22C3 clone was superior to SP142, since we did not find any expression of PD-L1 utilizing the latter one. Recently, Krawczyk et al. compared the efficacy of these two assays in non-small cell lung carcinoma, demonstrating stronger IHC reactions with the use of 22C3 [20]. However, in another study evaluation of PD-L1 expression with 22C3 assay resulted in an underestimation of PD-L1 expression compared to SP142 clone [21]. These discrepancies might be responsible for inconsistencies between studies and may lead to confusing results in real clinicopathological practice. A very recent study validating the IASLC PD-L1 testing guidelines suggests that evaluation of tissues older than 3 years increases risk of an underestimation of the PD-L1 status [22]. In our cohort, some samples were older than 15 years, which might have interfered with antigenicity of the tissues and lead to staining discrepancies between clones. PD-L1 reactivity depends on fixation time and tissue processing, and some archival non-buffered formalin-fixed samples in our cohort might have been inadequately fixed [23]. Our own experiences with PD-L1 staining in bladder urothelial carcinomas using SP142 antibody (data not published), suggests that intensity of its staining especially depends on quality of tissue processing and its age.

Due to the small size of the group, further studies are needed to exactly establish the criteria of PD-L1 positivity and the significance of the type of IHC PD-L1 assay in pediatric RMS.

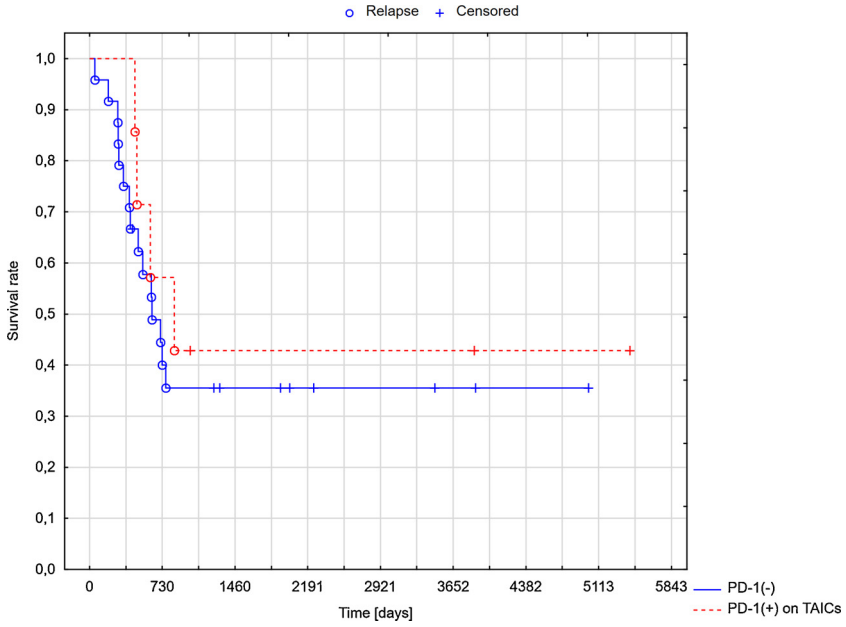

Fig. 6. Relapse-free survival in pediatric RMS according to PD-1 status.

6. Conclusions

Despite great progress in pediatric oncology, effective treatment of patients with advanced RMS remains a challenge. Here, we found that PD-L1 expression, detected on TAICs with the use of 22c3 clone but not SP142 antibody, tend to occur in low-stage cases of RMS. This finding might provide new piece of evidence of the potential usefulness of PD-1/PD-L1 axis inhibitors in RMS.

Declaration of Competing Interest

None.

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