Original Article

Programmed death ligand 1 expression in bladder rhabdomyosarcoma and its association with clinicopathological features

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Received July 12, 2017; Accepted September 25, 2017; Epub October 1, 2017; Published October 15, 2017

Abstract: The aim of this study was to detect PD-L1 expression in bladder rhabdomyosarcoma and its association with clinicopathological features and patient prognosis. PD-L1 expression was detected in paraffin-embedded sections obtained from 34 patients with bladder rhabdomyosarcoma via immunohistochemistry. Immunohistochemistry results were statistically analyzed to determine their association with patient clinicopathological features and survival outcomes. PD-L1-positive staining was observed in 47.1% (16/34) of patients. Metastatic tumor cells in the lymph nodes of two patients were positive for PD-L1 expression. PD-L1 expression was significantly different with regard to muscularis invasion, but the expression did not affect patient survival outcomes. We confirmed PD-L1 expression in bladder rhabdomyosarcoma, suggesting that PD-1/PD-L1 inhibitors are potential therapeutic agents for patients with bladder rhabdomyosarcoma.

Keywords: Urinary bladder, rhabdomyosarcoma, programmed death ligand 1, expression, overall survival

Introduction

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma present in children and is rare in adults. RMS accounts for 5% of all pediatric cancers and is 1.4 times more common in males than in females [1, 2]. As malignant tumor cells progress from immature mesenchymal stem cells to undergo myogenesis, RMS can develop in multiple locations throughout the body [3]. Approximately 20%-25% of patients are localized in the genitourinary tract, mostly in the bladder and prostate [4]. The main symptoms of bladder RMS are hematuria and dysuria. Histological classification continues to be one of the strongest predictors of patient outcomes in RMS [5]. In 2013, the World Health Organization amended the classification of RMS subtypes as alveolar RMS, embryonal RMS (ERMS), pleomorphic RMS, and sclerosing/spindle cell RMS. ERMS, accounting for approximately 70% of childhood RMS, is the most prevalent subtype and is often diagnosed in the first decade of life [6]. Outcomes of children with bladder RMS have improved in the past few decades because of research that focuses on how to maximally preserve bladder function and implement multimodal therapy, including systemic chemotherapy, surgery, and radiotherapy [7]. The cure rates for non-metastatic RMS have gradually increased from 25% in the 1970s to 70% in the 1990s, and to over 80% in the 2000s [8].

The programmed death 1 (PD-1)/programmed death ligand 1 (PD-L1) pathway plays a significant role in tumor immune escape [9]. PD-1, an immunoinhibitory molecule, is expressed on various kinds of cells, including T cells, B cells, and dendritic cell, whereas its ligand, PD-L1, is mainly expressed on antigen-presenting cells [10]. PD-L1 is also found in multiple types of tumor cells, including melanoma, RCC, non-small cell lung cancer, and urothelial and pancreatic cancer; PD-L1 expression could predict the survival outcome of these patients [11-16]. When PD-L1 binds to PD-1, the activation of tumor-infiltrating T cells is downregulated,
Significance of PD-L1 in bladder rhabdomyosarcoma

![Image](90x427 to 522x720)

Figure 1. Immunohistochemical analysis of PD-L1 protein expression in bladder rhabdomyosarcoma. Immunohistochemical staining cases with positive (weak, moderate, strong) and negative expressions are shown.

Table 1. Characteristics and relationship between PD-L1 expression and clinicopathologic variables in bladder rhabdomyosarcoma

<table>
<thead>
<tr>
<th>Parameters</th>
<th>N (%) or mean (95% CI)</th>
<th>PD-L1 expression</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td>Positive (%)</td>
<td>Negative (%)</td>
</tr>
<tr>
<td>Male</td>
<td>29 (85.3%)</td>
<td>14 (48.3%)</td>
<td>15 (51.7%)</td>
</tr>
<tr>
<td>Female</td>
<td>5 (14.7%)</td>
<td>2 (40.0%)</td>
<td>3 (60.0%)</td>
</tr>
<tr>
<td>Age (Y)</td>
<td>4.1 (2.6-5.7)</td>
<td>3.1 (2.5-3.7)</td>
<td>5.1 (2.1-8.1)</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>2.8 (2.1-3.4)</td>
<td>2.6 (1.8-3.4)</td>
<td>2.9 (1.8-4.0)</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>2 (5.9%)</td>
<td>1 (50.0%)</td>
<td>1 (50.0%)</td>
</tr>
<tr>
<td>Negative</td>
<td>32 (94.1%)</td>
<td>15 (46.9%)</td>
<td>17 (53.1%)</td>
</tr>
<tr>
<td>Muscularis invasion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>16 (47.1%)</td>
<td>11 (68.8%)</td>
<td>5 (31.3%)</td>
</tr>
<tr>
<td>Negative</td>
<td>18 (52.9%)</td>
<td>5 (27.8%)</td>
<td>13 (72.2%)</td>
</tr>
<tr>
<td>Tumor number</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unifocal</td>
<td>28 (82.4%)</td>
<td>15 (53.6%)</td>
<td>13 (46.4%)</td>
</tr>
<tr>
<td>Multifocal</td>
<td>6 (17.6%)</td>
<td>1 (16.7%)</td>
<td>5 (83.3%)</td>
</tr>
<tr>
<td>Resection margin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>10 (29.4%)</td>
<td>7 (70.0%)</td>
<td>3 (30.0%)</td>
</tr>
<tr>
<td>Negative</td>
<td>24 (70.6%)</td>
<td>9 (37.5%)</td>
<td>15 (62.5%)</td>
</tr>
</tbody>
</table>

A recent study demonstrated that PD-L1 is also expressed in some subtypes of soft tissue sarcoma, including RMS, and acts as an independent prognostic factor [18]. However, the role of PD-L1 in bladder RMS remains unknown. Furthermore, several anti-PD-1/PD-L1 antibodies have been approved to treat some specific solid tumors. In this study, we detected PD-L1 expression in bladder RMS tissue from 34 patients and evaluated the association of PD-L1 expression with clinicopathological features and patient prognosis.

Materials and methods

Patients and tissue samples

Thirty-four patients were included in this retrospective study. All patient tissue samples were pathologically diagnosed with RMS between 2003 and 2016 at Zhujiang Hospital. Every patient inducing immunosuppression that enables tumor cells to evade the immune system and exacerbate disease [17].
underwent radical cystectomy, and their tissue samples were collected after surgery, fixed in formalin, and embedded in paraffin to evaluate PD-L1 expression. In addition, seven normal bladder tissues obtained from patients were histopathologically confirmed as normal controls. Follow-up data were available for all 34 patients, with durations ranging from 9.1 to 161.2 months (median, 41.6 months). This study was approved by the Institutional Review Board of Zhujiang Hospital, Guangzhou, China.

Immunohistochemistry

Three micrometer sections of formalin-fixed and paraffin-embedded samples were prepared for each patient. Immunohistochemical staining was performed using a rabbit monoclonal antibody for PD-L1 (1:200, #13684, CST, Danvers, MA, USA). After deparaffinization and rehydration in a graded ethanol series, heat-induced antigen retrieval was performed using EDTA buffer (CW0129S, CwBiotech, Beijing, China) and placing the samples in a microwave for 15 min. After 15-minute endogenous peroxidase activity was blocked using 3% hydrogen peroxide and the sections were blocked using normal goat serum (SP-9001, ZSGB-BIO, Beijing, China) for 15 min. The sections were subsequently incubated overnight at 4°C with diluted primary antibodies. For the negative control, PBS was used instead of the primary antibody to evaluate reaction specificity. Biotinylated anti-rabbit immunoglobulin and streptavidin conjugated to horseradish peroxidase (SP-9001, ZSGB-BIO, Beijing, China) both were then added and incubated for 15 min. The DAB system was used to detected signals. Finally, the sections were counterstained with hematoxylin and then mounted after dehydration.

Immunohistochemical evaluation

Immunohistochemical evaluation of PD-L1 expression was separately performed by two independent pathologists who were blinded to the clinical information data and clinical patient outcomes. As recommended by Remmele et al [19], combined the staining intensity and proportion of positive cells was used to assess PD-L1 expression. The staining intensity was graded as negative (0), weak (1), moderate (2), or strong (3). The proportion of positive cells was graded as follows: 0, no positive cells; 1, <10%; 2, 10%-50%; and 3, >50%. After multiplying the two scores, a score of 0 or 1 was considered as negative PD-L1 expression and scores of 2-9 were considered as positive PD-L1 expression.

Statistical analysis

Statistical analysis was performed using the SPSS 18.0 software (SPSS Inc., Chicago, IL). Differences in PD-L1 expression among clinicopathological features were determined using the chi-square test, except for age and tumor size, which were performed using an independent samples t-test. Survival time was defined as the time interval between the first diagnosis of RMS and death or the last follow-up. The Kaplan-Meier method with the log-rank test was used to plot survival curves, whereas the Cox univariate analysis was used to assess the prognostic difference in each group. A P value of <0.20 in the univariate analysis was selected for the multivariate analyses. We used two-sided tests of significance for all analyses. P<0.05 was considered statistically significant.

Results

Immunohistochemistry

Positive staining was observed in 47.1% (16/34) of patients, whereas no positive staining was detected in the normal controls (Figure 1).
Significance of PD-L1 in bladder rhabdomyosarcoma

Table 2. Univariate and multivariate analyses for overall patient survival

<table>
<thead>
<tr>
<th>Variables</th>
<th>Univariate RR (95% CI)</th>
<th>p-value</th>
<th>Multivariate RR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male vs. female)</td>
<td>25.577 (0.4782123)</td>
<td>0.601</td>
<td>25.577 (0.4782123)</td>
<td>0.601</td>
</tr>
<tr>
<td>Tumor size (≥5 cm vs. &lt;5 cm)</td>
<td>4.531 (0.638-32.204)</td>
<td>0.131</td>
<td>2.863 (0.356-23.023)</td>
<td>0.323</td>
</tr>
<tr>
<td>Lymph node metastasis (positive vs. negative)</td>
<td>15.240 (2.116-109.748)</td>
<td>0.007</td>
<td>12.010 (1.505-95.836)</td>
<td>0.019</td>
</tr>
<tr>
<td>Muscularis invasion (positive vs. negative)</td>
<td>1.509 (0.212-10.726)</td>
<td>0.681</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor number (unifocal vs. multifocal)</td>
<td>0.036 (0.1642.146)</td>
<td>0.543</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resection margin (positive vs. negative)</td>
<td>1.128 (0.117-10.860)</td>
<td>0.917</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PD-L1 expression (positive vs. negative)</td>
<td>0.369 (0.038-3.551)</td>
<td>0.388</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The metastatic tumor cells in the lymph nodes of two patients also exhibited positive PD-L1 expression. The combined score was 0 and 1 in 12 and 5 patients who were considered negative, respectively. PD-L1 expression level was significantly higher in RMS cells than in normal bladder tissue cells (P=0.031).

Association between PD-L1 expression and clinicopathological features

Baseline clinicopathological features of the 34 patients are shown in Table 1. There were 29 males and 5 females. The mean age at the time of diagnosis was 4.1 years (95% CI, 2.6-5.7). The mean tumor size was 2.8 cm (95% CI, 2.1-3.4 cm). Only two patients had lymph node metastasis. The tumor cells of 16 patients demonstrated muscularis invasion, whereas those of the other 18 patients were located in the subepithelium. Tumor cells remained in the section margin after surgery in 10 patients. Furthermore, we found that gender, age, tumor size, lymph node metastasis, tumor numbers, and resection margin did not significantly associate with PD-L1 expression. However, PD-L1 expression did significantly associate with muscularis invasion status (P=0.037).

Association between survival outcomes and PD-L1 expression

During the median follow-up duration of 41.6 months, four patients (11.8%) died. Survival curves indicated that overall survival (OS) of patients were not significantly different between PD-L1 positive and negative groups (P=0.369; Figure 2). The results of the univariate and multivariate survival analyses are summarized in Table 2. Only lymph node metastasis showed a significant association with OS and predicted a poor prognosis.

Discussion

To the best of our knowledge, this is the first study to evaluate PD-L1 expression in bladder RMS and assess its effect on patient clinicopathological features and survival outcomes. We found that PD-L1 expression tended to associate with tumor invasion but not with OS. RMS is a really rare cancer [20]. The primary site of RMS has been confirmed to be a prognostic factor. Favorable sites include the orbit/eye lid, head and neck, and genitourinary area, with the exceptions of the bladder, prostate, and extremities, which are considered unfavorable sites [21]. Primary RMS therapies include chemotherapy, radiotherapy, and surgery [22]. Several monoclonal antibodies have been evaluated in treatment efficacy against RMS, such as bevacizumab (antibody to the vascular endothelial growth factor), cixutumumab (antibody to the insulin-like growth factor-1 receptor), and temsirolimus (inhibitor of the mammalian target of rapamycin). These biological agents are regarded as adjuncts to chemotherapy [23, 24]. PD-1 was first reported by Ishida in 1992 [25], whereas PD-L1 was discovered in 1999 by Dong [26]. PD-L1 is expressed in several types of cancers, with variable positive ratios according to histological types. Moreover, PD-L1 expression indicates poor prognosis and adverse clinicopathological features [16]. With high PD-L1 expression, tumor cells could activate PD-1 on T cells, which downregulates T-cell activity and promotes tumor immune escape. With the exception of tumor and APC cells, PD-L1 is scarcely expressed in normal cells. Thus, it is possible to kill tumor cells by blocking the PD-1/PD-L1 pathway [27].

Several successful PD-1/PD-L1 inhibitors have been discovered, with some approved by the US FDA. Anti-PD-1 antibodies pembrolizumab
Significance of PD-L1 in bladder rhabdomyosarcoma

and nivolumab are approved to treat advance melanoma and NSCLC [28, 29]. In May 2016, the anti-PD-L1 antibody atezolizumab was approved to treat urothelial carcinoma [30]. Furthermore, a significantly higher overall response rate was observed in PD-L1-positive patients than in PD-L1-negative patients. The response rate of atezolizumab for urothelial carcinoma was 26%-46% in patients with positive PD-L1 expression, whereas it was 9.5%-15% in patients with negative PD-L1 expression [31, 32]. Therefore, determining PD-L1 expression for each tumor type is necessary.

We demonstrated that 47.1% of patients with bladder RMS had positive PD-L1 expression, indicating that PD-1/PD-L1 inhibitors may be promising means to treat bladder RMS. There are several ongoing clinical trials that are investigating the efficacy of PD-1/PD-L1 inhibitors in patients with sarcoma: atezolizumab (NCT02609984), nivolumab (NCT02304458), and pembrolizumab (NCT02301039, NCT026367-25, and NCT02888665). We look forward to the results of these trials and plan to conduct future studies to assess the effect of PD-1/PD-L1 inhibitors in patients with bladder RMS.

The limitations of this study were small sample size, relatively short duration of follow-up because of the low morbidity of bladder RMS, and different therapies received among our patient cohort. Such factors may have affected our statistical results. Therefore, larger patient sample sizes and longer duration of follow-up are necessary in future studies. In conclusion, this is first study to detect PD-L1 expression in bladder RMS and define its association with patient clinicopathological features and patient prognosis. Although we found no association between patient survival and PD-L1 expression in RMS cells, future studies with larger sample sizes and longer duration of follow-up are warranted.

Acknowledgements

This study was funded by grants from the Science and Technology Planning Project of the Guangdong Province (2016A020215109), the Guangdong Natural Science Foundation (No. 2015A030313291) and the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 17K11138).

Disclosure of conflict of interest

None.

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Significance of PD-L1 in bladder rhabdomyosarcoma


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