Int J Clin Exp Pathol 2017;10(10):10565-10570
www.ijcep.com /ISSN:1936-2625/IJCEP0061302

Original Article

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

Peng Zhou[1], Qiongren Wang[1], Chongshan Wang[1], Xiezhao Li[1], Wei Du[1], Chunxiao Liu[1], Masami Watanabe[2], Peng Huang[1,2], Abai Xu[1]

[1]Department of Urology, Zhujiang Hospital, Southern Medical University, Guangzhou, China; [2]Department of Urology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

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,

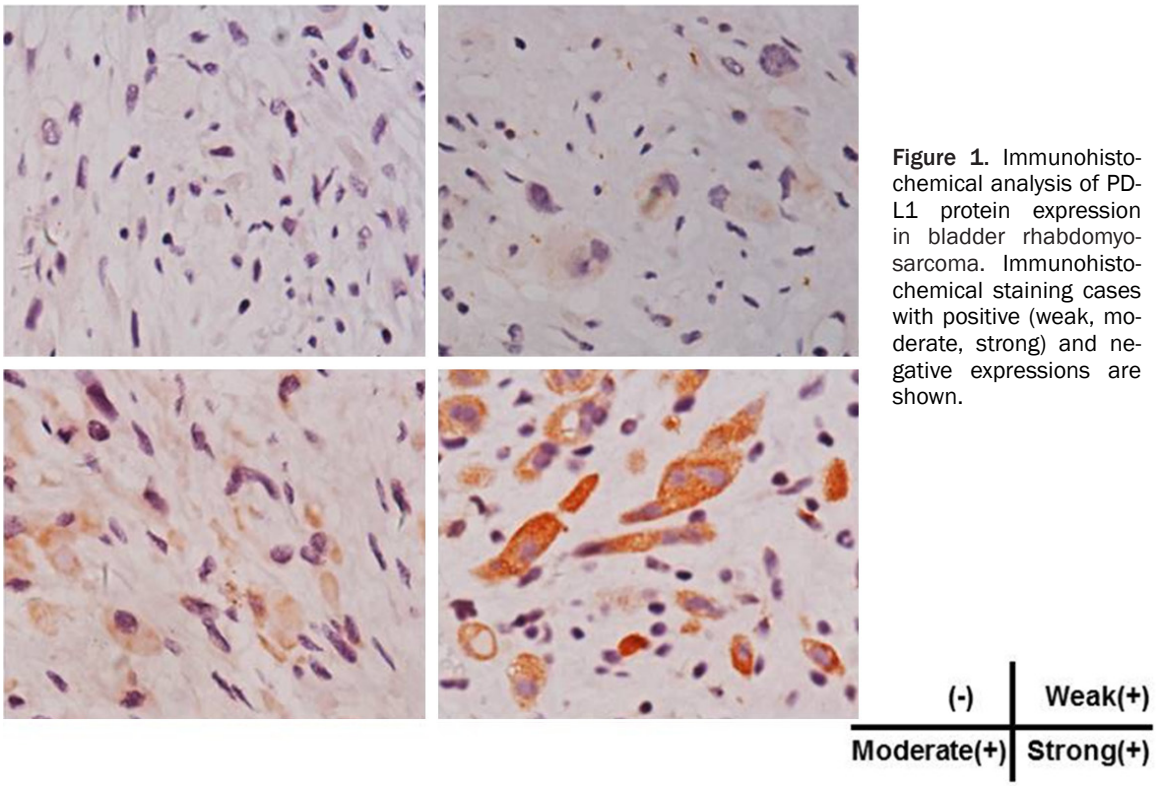

Figure 1. Immunohistochemical analysis of PD-L1 protein expression in bladder rhabdomyosarcoma. Immunohistochemical staining cases with positive (weak, moderate, strong) and negative expressions are shown.

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

Parameters	N (%) or mean (95% CI)	PD-L1 expression		*P*-value
		Positive (%)	Negative (%)	
Gender				0.732
Male	29 (85.3%)	14 (48.3%)	15 (51.7%)	
Female	5 (14.7%)	2 (40.0%)	3 (60.0%)	
Age (Y)	4.1 (2.6-5.7)	3.1 (2.5-3.7)	5.1 (2.1-8.1)	0.197
Tumor size (cm)	2.8 (2.1-3.4)	2.6 (1.8-3.4)	2.9 (1.8-4.0)	0.635
Lymph node metastasis				0.932
Positive	2 (5.9%)	1 (50.0%)	1 (50.0%)	
Negative	32 (94.1%)	15 (46.9%)	17 (53.1%)	
Muscularis invasion				0.017
Positive	16 (47.1%)	11 (68.8%)	5 (31.3%)	
Negative	18 (52.9%)	5 (27.8%)	13 (72.2%)	
Tumor number				
Unifocal	28 (82.4%)	15 (53.6%)	13 (46.4%)	
Multifocal	6 (17.6%)	1 (16.7%)	5 (83.3%)	
Resection margin				
Positive	10 (29.4%)	7 (70.0%)	3 (30.0%)	
Negative	24 (70.6%)	9 (37.5%)	15 (62.5%)	

inducing immunosuppression that enables tumor cells to evade the immune system and exacerbate disease [17].

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

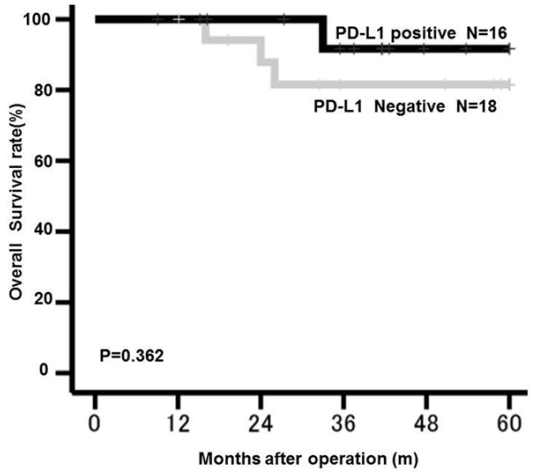

Figure 2. Survival analysis of bladder rhabdomyosarcoma patients (n=34) using the Kaplan-Meier method. Kaplan-Meier survival curves were constructed to assess overall survival (OS). The statistical significance was calculated by the log-rank test.

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**).

Table 2. Univariate and multivariate analyses for overall patient survival

Variables	Univariate		Multivariate	
	RR (95% CI)	*P*-value	RR (95% CI)	*P*-value
Gender (male vs. female)	25.577 (0-4782123)	0.601		
Tumor size (≥5 cm vs. <5 cm)	4.531 (0.638-32.204)	0.131	2.863 (0.356-23.023)	0.323
Lymph node metastasis (positive vs. negative)	15.240 (2.116-109.748)	0.007	12.010 (1.505-95.836)	0.019
Muscularis invasion (positive vs. negative)	1.509 (0.212-10.726)	0.681		
Tumor number (unifocal vs. multifocal)	0.036 (0-1642.146)	0.543		
Resection margin (positive vs. negative)	1.128 (0.117-10.860)	0.917		
PD-L1 expression (positive vs. negative)	0.369 (0.038-3.551)	0.388		

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

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 trails 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.

Address correspondence to: Peng Huang and Abai Xu, Department of Urology, Zhujiang Hospital, Southern Medical University, Guangzhou, China. Tel: +86-20-6164-3255; Fax: +86-20-62782725; E-mail: huangpeng509@gmail.com (PH); lc96xab@163.com (ABX)

References

[1] Yang L, Takimoto T and Fujimoto J. Prognostic model for predicting overall survival in children and adolescents with rhabdomyosarcoma. BMC Cancer 2014; 14: 654.

[2] Egas-Bejar D and Huh WW. Rhabdomyosarcoma in adolescent and young adult patients: current perspectives. Adolesc Health Med Ther 2014; 5: 115-25.

[3] Soleimani VD and Rudnicki MA. New insights into the origin and the genetic basis of rhabdomyosarcomas. Cancer Cell 2011; 19: 157-159.

[4] Rigamonti W, Iafrate M, Milani C, Capizzi A, Bisogno G, Carli M and Glazel GP. Orthotopic continent urinary diversion after radical cystectomy in pediatric patients with genitourinary rhabdomyosarcoma. J Urol 2006; 175: 1092-1096.

[5] Ferrer FA, Isakoff M and Koyle MA. Bladder/prostate rhabdomyosarcoma: past, present and future. J Urol 2006; 176: 1283-1291.

[6] Coindre JM. New WHO classification of tumours of soft tissue and bone. Ann Pathol 2012; 32 Suppl: S115-S116.

[7] Lott S, Lopez-Beltran A, Montironi R, MacLennan GT and Cheng L. Soft tissue tumors of the urinary bladder part II: malignant neoplasms. Hum Pathol 2007; 38: 963-977.

[8] Komasara L, Golebiewski A, Anzelewicz S and Czauderna P. A review on surgical techniques and organ sparing procedures in bladder/prostate rhabdomyosarcoma. Eur J Pediatr Surg 2014; 24: 467-473.

[9] Francisco LM, Sage PT and Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. Immunol Rev 2010; 236: 219-242.

[10] Keir ME, Butte MJ, Freeman GJ and Sharpe AH. PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol 2008; 26: 677-704.

[11] Hino R, Kabashima K, Kato Y, Yagi H, Nakamura M, Honjo T, Okazaki T and Tokura Y. Tumor cell expression of programmed cell death-1 ligand 1 is a prognostic factor for malignant melanoma. Cancer 2010; 116: 1757-1766.

[12] Thompson RH, Dong HD, Lohse CM, Leibovich BC, Blute ML, Cheville JC and Kwon ED. PD-1 is expressed by tumor-infiltrating immune cells and is associated with poor outcome for patients with renal cell carcinoma. Clin Cancer Res 2007; 13: 1757-1761.
[13] Velcheti V, Schalper KA, Carvajal DE, Anagnostou VK, Syrigos KN, Sznol M, Herbst RS, Gettinger SN, Chen LP and Rimm DL. Programmed death ligand-1 expression in non-small cell lung cancer. Lab Invest 2014; 94: 107-116.
[14] Nakanishi J, Wada Y, Matsumoto K, Azuma M, Kikuchi K and Ueda S. Overexpression of B7-H1 (PD-L1) significantly associates with tumor grade and postoperative prognosis in human urothelial cancers. Cancer Immunol Immunother 2007; 56: 1173-1182.
[15] Geng L, Huang DS, Liu JW, Qian YG, Deng JF, Li DL, Hu ZH, Zhang J, Jiang GP and Zheng SS. B7-H1 up-regulated expression in human pancreatic carcinoma tissue associates with tumor progression. J Cancer Res Clin Oncol 2008; 134: 1021-1027.
[16] Wang X, Teng FF, Kong L and Yu JM. PD-L1 expression in human cancers and its association with clinical outcomes. Oncotargets Ther 2016; 9: 5023-5039.
[17] Ichikawa M and Chen LP. Role of B7-H1 and B7-H4 molecules in down-regulating effector phase of T-cell immunity: novel cancer escaping mechanisms. Front Biosci 2005; 10: 2856-2860.
[18] Kim C, Kim EK, Jung H, Chon HJ, Han JW, Shin KH, Hu H, Kim KS, Choi YD, Kim S, Lee YH, Suh JS, Ahn JB, Chung HC, Noh SH, Rha SY, Kim SH and Kim HS. Prognostic implications of PD-L1 expression in patients with soft tissue sarcoma. Bmc Cancer 2016; 16: 434.
[19] Remmele W and Stegner HE. [Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue]. Pathologe 1987; 8: 138-140.
[20] Malempati S and Hawkins DS. Rhabdomyosarcoma: review of the children's oncology group (COG) soft-tissue sarcoma committee experience and rationale for current COG studies. Pediatr Blood Cancer 2012; 59: 5-10.
[21] Lawrence W Jr, Anderson JR, Gehan EA and Maurer H. Pretreatment TNM staging of childhood rhabdomyosarcoma: a report of the intergroup rhabdomyosarcoma study group. Children's cancer study group. Pediatric oncology group. Cancer 1997; 80: 1165-1170.
[22] Kieran K and Shnorhavorian M. Current standards of care in bladder and prostate rhabdomyosarcoma. Urol Oncol 2016; 34: 93-102.
[23] Wagner LM, Fouladi M, Ahmed A, Krailo MD, Weigel B, DuBois SG, Doyle LA, Chen H and Blaney SM. Phase II study of cixutumumab in combination with temsirolimus in pediatric patients and young adults with recurrent or refractory sarcoma: a report from the Children's oncology group. Pediatr Blood Cancer 2015; 62: 440-444.
[24] Benesch M, Windelberg M, Sauseng W, Witt V, Fleischhack G, Lackner H, Gadner H, Bode U and Urban C. Compassionate use of bevacizumab (Avastin((R))) in children and young adults with refractory or recurrent solid tumors. Ann Oncol 2008; 19: 807-813.
[25] Ishida Y, Agata Y, Shibahara K and Honjo T. Induced expression of Pd-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell-death. Embo J 1992; 11: 3887-3895.
[26] Dong HD, Zhu GF, Tamada K and Chen LP. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. Nat Med 1999; 5: 1365-1369.
[27] Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, Roche PC, Lu J, Zhu G, Tamada K, Lennon VA, Celis E and Chen L. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. Nat Med 2002; 8: 793-800.
[28] Franklin C, Livingstone E, Roesch A, Schilling B and Schadendorf D. Immunotherapy in melanoma: recent advances and future directions. Eur J Surg Oncol 2017; 43: 604-611.
[29] D'Argento E, Rossi S, Schinzari G, Strippoli A, Basso M, Cassano A and Barone C. From 2000 to 2016: which second-line treatment in advanced non-small cell lung cancer? Curr Treat Options Oncol 2016; 17: 59.
[30] Ratner M. Genentech's PD-L1 agent approved for bladder cancer. Nat Biotechnol 2016; 34: 789-790.
[31] Petrylak DP, Powles T, Bellmunt J, Braiteh FS, Loriot Y, Zambrano CC, Burris HA, Kim JW, Teng SL, Bruey JM, Hegde P, Abidoye OO and Vogelzang NJ. A phase Ia study of MPDL3280A (anti-PDL1): updated response and survival data in urothelial bladder cancer (UBC). J Clin Oncol 2015; 33.
[32] Rosenberg J, Petrylak D, Abidoye O, Van der Heijden MS, Hofman-Censits J, Necchi A, O'Donnell PH, Balmanoukian A, Loriot Y, Retz M, Perez-Gracia JL, Dawson NA, Balar A, Galsky MD, Fleming MT, Powles T, Cui N, Mariathasan S, Fine GD and Dreicer R. Atezolizumab in patients (pts) with locally-advanced or metastatic urothelial carcinoma (mUC): results from a pivotal multicenter phase II study (IMvigor 210). Eur J Cancer 2015; 51: S720.