JBUON 2021; 26(5): 2122-2130
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

Relationship between survival outcomes and microsatellite instability, tumor infiltrating lymphocytes and programmed cell death ligand-1 expression in patients with bladder cancer and radical cystectomy

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Summary

Purpose: *Platin-based chemotherapies are first-line treatment methods after surgery in bladder cancer. Recently, novel immunotherapies emerged after platin-based regimens. The purpose of this study was to evaluate the prognostic significance of microsatellite instability (MSI), tumor infiltrating lymphocytes (TILs) and programmed cell death ligand-1 (PD-L1) expression which are used as predictive biomarkers in immunotherapy.*

Methods: *Clinical and pathological features of bladder cancer patients who underwent radical cystectomy were retrospectively analyzed from their records in this single-center study. PD-L1, PD-L1 on TIL, PMS2, MSH2, MSH6 and MLH1 immunohistochemistry staining were carried out to archieve resected tumor specimens of the eligible patients. MSI was evaluated according to existing of PMS2, MSH2, MSH6 and MLH1.*

Results: *MSI was high in 24.6% of 61 patients. PD-L1 expression on tumor cells and PD-L1 expression on TIL were positive in 14.8% and 16.4% of the patients, respectively. Intratumoral TIL rate was >10% in 12 patients (19.7%). There was no statistically significant relationship between PD-L1, PD-L1 on TIL, MSI and TIL rate and patients' characteristics including sex, stage, pathologic grade and lymph node status. There was a positive trend between MSI-high patients and overall survival (OS) (p=0.089). Univariate analysis did not reveal any significant difference at 3-years OS with PD-L1 tumor expression and PD-L1 expression on TIL and TIL rate >10% (p=0.822, p=0.638, p=0.318, respectively).*

Conclusion: *This study revealed that there is a positive trend between OS and MSI but no prognostic significance of PD-L1 and TIL which are proven predictive biomarkers of immunotherapy in patients with bladder cancer.*

Key words: *bladder cancer, urothelial carcinoma, PD-1, PD-L1, MSI, TIL, immunotherapy*

Introduction

Urinary bladder cancer was the sixth most common cancer worldwide among men in 2012 and responsible for nearly 200.000 deaths estimated in 2018 [1,2]. Currently no suitable screening method exists for early detection or prevention [3]. A 22-25% reduction in risk of death is obtained with cisplatin-based combinations in adjuvant treatment, but overall survival (OS) in metastatic disease is still 13-15 months [4]. Hopefully, the molecular basis of invasive bladder cancer is capable to help improve targets for therapies. In particular, when programmed cell death-1 receptor and its ligand

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Received: 27/10/2020; Accepted: 03/12/2020

(PD-1 and PD-L1, respectively) are expressed intensely on tumors or surrounding T-lymphocytes, tumor cells can escape from the immune system and its response [5]. Therefore, blocking this PD-1/PD-L1 pathway by novel checkpoint inhibitors is the main approach of bladder cancer immunotherapy. Atezolizumab (anti-PD-L1), avelumab (anti-PD-L1) and pembrolizumab (anti-PD-1) are mostly investigated and started to be routinely used immunotherapeutics in urothelial carcinoma. Their response rates are 15%, 16% and 28% (atezolizumab, avelumab and pembrolizumab, respectively) in the metastatic setting after platinum-based chemotherapy [6-8]. Studies are proceeding to consolidate their use in the adjuvant setting. Immunohistochemically detected presence of PD-1/PD-L1 axis has predictive value for immunotherapy. In previous studies response rates were higher with strong immune or tumor cell PD-L1 expression by immunohistochemistry (IHC) (15% vs 26% for atezolizumab, 16% vs 40% for avelumab, 28% vs 33% for pembrolizumab) [6-8]. Previous studies have shown different results of PD-L1 expression on prognosis. Different methods to determine PD-L1 positivity might interfere with the results which vary from no correlation to poor prognostic effect on survival [9-16].

Infiltration of tumor with lymphocytes plays major role in anti-tumor activity of the immune system. The existence of tumor-infiltrating lymphocytes (TILs) is associated with favorable prognosis in bladder cancer as well as in other cancers. CD8+ T-lymphocytes are dominant members among TILs in anti-tumor immunity [17-21]. Also, local Bacillus Calmette Guerin (BCG) treatment for urothelial carcinoma probably has its benefits through immune response due to T-cell migration to the tumor site [22].

Bladder cancer is classified as T-staging regarding tissue invasion and grade according to its histopathological features. Although this classification predicts prognosis, tumors having the same T stage and grade may have various outcomes in different patients. This behavior is associated to genetic alterations. Genetic instability and replication errors commonly occur as a result of defects in mismatch repair (MMR) genes. When defective mismatch repair fails to stabilize microsatellites (simple sequence repeats) and their length (number of repetitive sequences) it causes microsatellite instability (MSI). In a previous study MSI was found to be correlated with tumor stage and grade as well as with poor prognosis regardless of grade [23,24].

PD-1/PD-L1 pathway, TILs and MSI have an arguably predictive and prognostic value for bladder cancer. This study aimed to determine the prognostic effects of immunohistochemically detected tumor PD-L1, PD-L1 expression on TILs, MSI and TIL count on survival outcomes in patients with radical cystectomy of bladder urothelial carcinoma treated with the same treatment and followed-up in a single center.

Methods

Patient selection

Patients with high-grade invasive urothelial carcinoma subjected to radical cystectomy between 2008-2017 were included in the study. Archived hematoxylin and eosin (H&E) stained slides of selected patients were evaluated for diagnosis and re-classified according to WHO classification [25].

Histopathological evaluation

All slides of the cases were investigated and blocks which contained both non-neoplastic epithelia and invasive tumor were studied. Tumor grade, stage and subtype differentiation were evaluated from original sections of selected blocks. All histopathological evaluations were performed by two different pathologists.

Intratumoral tumor TILs were calculated as the average number of absolute lymphocyte count in five consecutive high power field (HPF) containing tumor and lymphocytes [26].

The density of stromal TILs was scored as weak (0-10 %), moderate (11-50 %) and strong (≥51 %) according to the percentage of stromal area infiltrated by mononuclear cells in the adjacent stroma of invasive tumor segments [26].

Immunohistochemical evaluation

Four μm sections from selected blocks for immunohistochemical evaluation were transferred on poly-l-lysine coated slides. PD-L1 (SP-142 clone, Ventana Roche), MSH-2 (G219-1129 Mouse monoclonal antibody, Cell Marque), MSH-6 (44 Mouse monoclonal antibody, Ventana Roche), MLH-1 (M1 clone, Mouse monoclonal antibody, Ventana Roche), PMS-2 (EPR3947 Rabbit monoclonal antibody, Cell Marque) determinants were carried out via Benchmark XT immunohistochemical (HIC) staining device. Splenic and placental tissues were used as positive controls for PD-L1. Non-neoplastic epithelia, stromal cells or inflammatory cells detected in the investigated slide were used as positive controls for MSI determinants.

Evaluation of PD-L1 expression

After standard deparaffinization, rehydration and antigen retrieval (CCV1 antigen retrieval solution, 64 min in 100°C), IHC staining was carried out with PD-L1 primary monoclonal mouse antibody (1:50 dilution, 60 min in 37°C). Following 10 min with OptiView DAB IHC detection kit (Ventana) and 30 min with OptiView Amplification kit (Ventana), rinsing with distilled water and hematoxylin counterstaining were performed. Xylene changes were performed and they were coverslipped for investigation.

PD-L1 expression was evaluated separately on tumor intratumoral TILs (iTILs) and stromal TILs (sTILs).

Evaluation of PD-L1 expression on tumor: Staining density was scored as weak, moderate and strong. Staining pattern were determined as complete membranous, incomplete membranous and cytoplasmic. Staining extent was calculated in percentage as proportion of staining-positive tumor to whole tumor. Tumors were accepted as PD-L1 positive when ≥1% of tumor cells expressed PD-L1 in IHC staining [27] (Figure 1a).

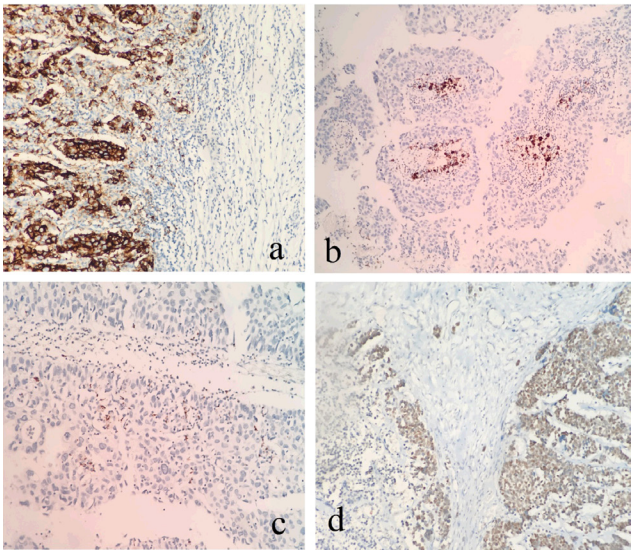

Figure 1. Immunohistochemical staining images. **(a)** Strong membranous and cytoplasmic PD-L1 expressed urothelial carcinoma (x200); **(b)** Strong PD-L1 positivity in urothelial carcinoma sTIL (x200); **(c)** Urothelial carcinoma iTILs positive for strong PD-L1 staining (x200); **(d)** Positive staining with PMS-2 (urothelial carcinoma, x200).

Evaluation of PD-L1 expression in TILs: PD-L1 staining density was scored separately on iTILs and sTILs. The percentage of PD-L1 positive lymphocyte count in the tumor area was calculated for iTILs whereas the ratio between PD-L1 positive lymphocyte count in tumor-adjacent stroma and invasive tumor area was calculated for sTIL [28] (Figure 1b and 1c).

Evaluation of microsatellite instability

Following deparaffinization, slides prepared from selected formalin-fixed, paraffin-embedded blocks incubated for antigen retrieval with CC1 solution during 60 min for each PMS-2, MLH1, MSH2 and MSH6. PMS-2, MLH1, MSH2 and MSH6 primary antibodies were incubated in 40°C for 16 min, 24 min, 16 min and 16 min, respectively. Rinsing with distilled water and hematoxylin counterstaining were performed. Deparaffinization with xylene and rehydration were performed and slides were coverslipped for investigation.

Positive staining referred to ≥1% specific nuclear staining on tumor cells while no staining meant negative result [29]. Positive staining meant mismatch repair (MMR) proteins were present in the tumor tissue and therefore stated as MSI-low (MMR-proficient). One or more negative staining of four MMR proteins meant MSI-high (MMR-deficient). (Figure 1d).

Statistics

The characteristics of each group were compared with chi-square and Mann-Whitney U tests (whichever

Table 1. Patient characteristics and x^2 results

		Tumor PD-L1			*TIL PD-L1*			*MSI*			*iTIL*		
	n (%)	*Positive n (%)*	*Negative n (%)*	*p*	*Positive n (%)*	*Negative n (%)*	*p*	*MSH n (%)*	*MSLow n (%)*	*p*	*<10% n (%)*	*>10% n (%)*	*p*
Total	61 (100)	9 (14.8)	52 (85.2)		10 (16.4)	51 (83.6)		15 (24.6)	46 (75.4)		49 (80.3)	12 (19.7)	
Sex				0.563			0.185			0.09			0.252
Male	56 (91.8)	8 (88.9)	48 (92.3)		8 (80)	48 (94.1)		12 (80)	44 (95.7)		46 (93.9)	10 (83.3)	
Female	5 (8.2)	1 (11.1)	4 (7.7)		2 (20)	3 (5.9)		3 (20)	2 (4.3)		3 (6.1)	2 (16.7)	
Age				0.367			0.262			0.099			0.119
≤64	34 (55.7)	6 (66.7)	28 (53.8)		7 (70)	27 (52.9)		11 (73.3)	23 (50)		25 (51.0)	9 (75)	
>64	27 (44.3)	3 (33.3)	24 (46.2)		3 (30)	24 (47.1)		4 (26.7)	23 (50)		24 (49.0)	3 (25)	
pT				0.258			0.432			0.467			0.252
T1 T2	11 (18) 12 (19.7)	2 (22.2)	21 (40.4)		3 (30)	20 (39.2)		5 (33.3)	18 (39.1)		20 (40.8)	3 (25)	
T3 T4	21 (34.4) 17 (27.9)	7 (77.8)	31 (59.6)		7 (70)	31 (60.8)		10 (66.7)	28 (60.9)		29 (59.2)	9 (75)	
Lymph node				0.250			0.860			0.605			0.524
N0	45 (73.8)	8 (88.9)	37 (71.2)		8 (80)	37 (72.5)		11 (73.3)	34 (73.9)		37 (75.5)	8 (66.7)	
N1	16 (26.2)	1 (11.1)	15 (28.8)		2 (20)	14 (27.5)		4 (26.7)	12 (26.1)		12 (24.5)	4 (33.3)	
p Grade				0.437			0.395			0.358			0.321
Low Grade	5 (8.2)	0 (0)	5 (9.6)		0 (0)	5 (9.8)		2 (13.3)	3 (6.5)		5 (10.2)	0 (0)	
High Grade	56 (91.8)	9 (16.1)	47 (83.9)		10 (100)	46 (90.2)		13 (86.7)	43 (93.5)		44 (89.8)	12 (100)	

was more appropriate). OS was defined as the period from patients' diagnosis date to death due to any reason or last follow-up. For survival analysis, Kaplan-Meier curves and Log-rank test in univariate analysis and Cox regression in multivariate analysis were used. Parameters which were significant on OS in univariate analysis, were evaluated in multivariate analysis. Results with $p<0.05$ were considered significant. All analyses were performed using the SPSS software package (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.).

Results

Fifty-six males (91.8%) and 5 females (8.2%) of total 61 patients were eligible for the study. Median age was 61.72 (35-81). In pathological examination; 18% of patients (n=11) had T1 disease while 19.7% (n=12) had T2, 34.4% (n=21) had T3 and 29.9% of them (n=17) had T4 disease. 56 (91.8%) patients had high grade tumors while other 5 (8.2%) patients had low grade tumors. Forty-five patients (73.8%) had N0 disease and 16 patients (26,2%) N1 disease. PMS2, MSH2, MSH6 and MLH1 expression was detected with IHC in 54 (88.5%), 53 (86.9%), 47 (77%) and 55 (90.2%) patients, respectively. Therefore, MSI was high in 15 patients (24.6%). PD-L1 expression in tumor cells and PD-L1 expression on TIL were positive in 14,8% (n=9) and 16.4% (n=10) of patients, respectively. Intratumoral TIL rate was <10% in 49 patients (80.3%) while >10% in 12 patients (19.7%). In Chi-square test, there was no statistically significant relationship between PD-L1, PD-L1 on TIL, MSI and TIL rate and patients' characteristics including sex, stage, pathologic grade, lymph node status. Table 1 shows patient characteristics and Chi-square test results.

Three-year OS rate was calculated as 43%. There was a positive trend with MSI-high patients and 3-years OS in univariate analysis. (MSI-high: 65.7% vs MSI-low: 38.5%; p=0.089) (Figure 2a). Univariate analysis did not reveal any significant difference at 3-year OS with PD-L1 expression

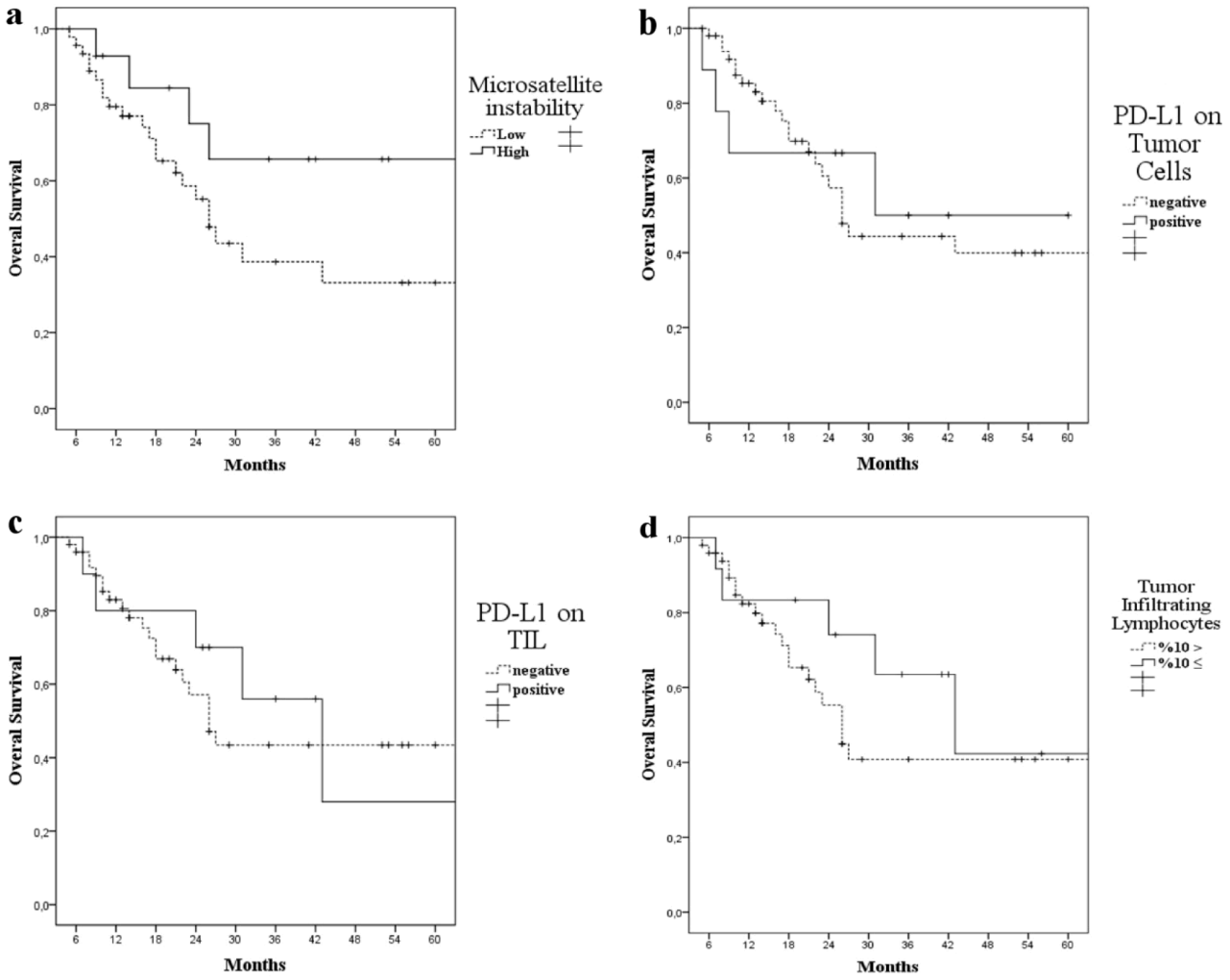

Figure 2. Kaplan-Meier curves of univariate analyses of MSI **(a)**, PD-L1 on tumor cells **(b)**, PD-L1 on TILs **(c)** and TIL rate **(d)** on 3-year OS (p=0.089, 0.082, 0.638 and 0.318, respectively).

Table 1. Univariate and multivariate analyses results

	Univariate		*Multivariate*		
	3-year OS (%)	*p value*	*HR*	*95% CI*	*p value*
Total		43.0			
Sex					
Male	44.6	0.869			
Female	60.0				
Age			2.49	1.136-5.465	0.023
<64	60.9	0.011			
>64	20.5				
Stage			3.667	1.349-9.970	0.011
pT1-2	77.1	0.004			
pT3-4	29.3				
Lymph nodes					
N0	48.5	0.051			
N1	25.6				
Tumor PD-L1					
Positive	50.0	0.822			
Negative	44.4				
TIL PD-L1					
Positive	55.0	0.638			
Negative	43.4				
MSI					
MSH	65.7	0.089			
MSLow	38.5				
iTIL					
<10%	40.8	0.318			
>10%	63.5				

on tumor cells (positive: 50% vs negative: 44.4%; p=0.822), PD-L1 expression on TIL (positive:55% vs negative:43.4%; p=0.638) and TIL rate (<10%: 40.8% vs >10%: 63.5%; p=0.318) (Figure 2b, 2c and 2d). Three-year OS was statistically different with age (<64: 60.9% vs >64: 20.5%), stage (pT1-2: 77.1% vs pT3-4: 29.3%) and lymph node status (N0: 48.5% vs N1: 25.6%).

Age and stage showed statistically significant difference in multivariate analysis. (p=0.023, p=0.011, respectively). Table 2 shows univariate and multivariate results.

Discussion

MSI and inactivation of mismatch repair proteins have established roles for tumorigenesis and genetic cancer syndromes such as Lynch syndrome. Additionally, mismatch repair deficient or MSI-high phenotype appears to be a pan-cancer biomarker which predicts response to immunotherapy. MSI-high tumors express abundant proteins which provide antigens to immune system to develop response. Therefore, the US Food and Drug Administration (FDA) granted approval to pembrolizumab for MSI-high or MMR-deficient solid tumors regardless of anatomic site in 2017. In a study where Le et al investigated PD-L1 blockade (pembrolizumab) on MMR-deficient tumors with 12 different tumor-sites; 53% of the patients had durable objective response and their further functional analysis revealed that there was a neoantigen-specific T-cell response to mutant neopeptides [30].

MMR-deficiency is identified using IHC with loss of one or more of the four mismatch repair proteins (MSH2, MSH6, MLH1, PMS2). MMR-deficiency is considered to be MSI-high [31] Although there is 92% consistency between four antibodies (PMS2, MSH2, MSH6, MLH1) IHC and PCR methods to determine MSI status in CRC, these two methods have not been tested between bladder cancer [32].

Vaish et al investigated MSI-high phenotype using PCR and its prognostic effects in bladder cancer. They found that MSI-high was associated with high stage and high grade. They also found that patients with MSI-high tumors had more recurrences than MSI-low patients regardless of the tumor

grade [23]. Jin et al evaluated only IHC detected MSH2 and found that low staining of MSH2 protein related to recurrence [33]. Saetta et al also investigated IHC detected MLH1 and MSH2 proteins and MSI status via molecular methods. In their study, reduced MLH1 expression predicted shorter disease-free survival. However, the presence of MSI did not show prognostic significance [34]. On the other hand, reduced expression of MSH2 and MLH1 proteins determined with IHC correlated to fewer recurrence rates. In the same study where MSI was investigated via PCR, MSI-high status had no relation with outcome. Interestingly, there was a discordance between MSH2/MLH1 loss and MSI status which acknowledged that none of the patients who had reduced MMR proteins expression showed MSI [35]. Mylona et al also found that loss of MSH6 and MSH2 associated with favorable OS [36]. All previous studies investigated IHC detected MMR-deficiency/MSI status and their correlation with outcome had different patient characteristics such as stage and only focused on few MMR proteins. To our knowledge, this is the first study to evaluate MSI status with four IHC detected MMR proteins in regard of OS. Our results showed that MSI-high/MMR-deficient patients had slightly better but not statistically significant OS than those with MSI-low patients (p=0.089). These results require elucidation with multi-center prospective clinical trials which would comprise diverse nature of bladder cancer and use different techniques to establish a standard.

Checkpoint inhibitors as immunotherapeutics are currently used in metastatic bladder cancer. Atezolizumab, durvalumab, avelumab, nivolumab and pembrolizumab have been approved and recommended for second-line treatment after platin-based chemotherapy in metastatic bladder cancer. Furthermore, immunotherapy gains ground with more ongoing clinical investigations such as atezolizumab monotherapy vs atezolizumab plus chemotherapy vs chemotherapy plus placebo in first-line (clinicaltrials.gov; NCT02807636), avelumab in post-platin maintenance treatment (NCT02603432), pembrolizumab in post-platin maintenance treatment (NCT02500121), durvalumab in adjuvant setting (NCT03732677) and nivolumab in adjuvant setting (NCT02632409). There are trials and approvals for 5 checkpoint inhibitors and 4 different PD-L1 assigning methods with various IHC antibodies and cut-off values [37]. This situation arises controversies against the predictive value of PD-L1. Moreover, the prognostic significance and its effects on survival of PD-L1 should be fully understood in order to determine its predictive role.

Bellmunt et al showed that 20% of patients with metastatic bladder cancer have PD-L1 positive on tumor cells and 37.1% of them have PD-L1 on TIL. Also in the Bellmunt et al study, PD-L1 positivity on TILs was found to be associated with longer OS but PD-L1 had no significant prognostic effect when present on tumor cells. This prognostic effect is attributed to PD-L1 positive TILs that are more antigen specific and ready to response. They included both invasive and non-invasive disease in their study [9]. Nakanishi et al reported that patients with high PD-L1 expression on tumor cells have worst outcome when compared to those with PD-L1 negative tumors in their study where invasive and non-invasive disease was evaluated collectively [10]. Moreover, PD-L1 expression on tumor cells was correlated with poor prognosis in organ-confined bladder cancer in two different studies [11,12]. Pichler et al investigated the prognostic effects of PD-L1 expression on tumor cells and on TIL in patients with high-risk for recurrence after radical cystectomy and found that patients with PD-L1 expression on immune cells had higher recurrence rates and shorter recurrence-free survival than those without expression. There was no difference between tumor cell PD-L1 positive and negative patients regarding recurrence rates and recurrence-free survival [13]. Faraj et al study also evaluated PD-L1 expression and disease outcome (OS) and found no correlation [14]. In another study, where Huang et al investigated PD-L1 mRNA expression via microarray analyses, PD-L1 was found to be associated with shorter OS [15]. However, Breyer et al found that high PDL-1 mRNA levels in pT1 non-muscle invasive bladder cancer patients were associated with favorable survival rates (recurrence-free survival, progression-free survival and carcinoma-specific survival) [16]. In the current study, PD-L1 expression on urothelial carcinoma cells or on TIL detected via IHC showed no correlation with OS. These results are consistent with the Pichler et al and Faraj et al studies but conflicting other studies mentioned above. These controversies may arise from various disease stages and PD-L1 assessment methods such as IHC methods and pathologist dependency by nature. These results regarding the prognostic effect of PD-L1 expression in bladder cancer shows that this controversy requires clarification with further investigations.

Invasion of tumor and its stroma with immune cells (specifically with T-lymphocytes) has key role for anti-tumor immunity. Previous studies showed that the presence of immune infiltration correlates to better survival [17-21]. However, cancer cells create a tumor microenvironment which is

established with tumor-induced interactions such as PD-1/PD-L1 axis to disrupt immune cell function and escape immune surveillance. Accordingly, there are also studies that report no correlation between TIL invasion and better prognosis [38]. In bladder cancer Sharma et al demonstrated in their study that infiltration of CD8+TILs was associated with better disease free survival in muscle-invasive disease [17]. Faraj et al found that CD8+TIL density which was defined as ≥60 CD8+TIL per HPF was associated with favorable OS [14]. Zhang et al showed that TIL presence (>1% positive expression of CD8+TILs) was a favorable prognostic factor on OS in non-organ-confined disease, but was an unfavorable factor in organ-confined disease [18]. Our results showed that there is no correlation between OS and TIL rate which was defined as presence of >10% TIL per HPF.

In the current study, we evaluated histopathologically detected lymphocytic immune cells, not specifically CD8+ cells, and this should be taken into consideration as a limitation of the study. In breast cancer, high level of TIL presence has prognostic significance and correlated with longer survival rates. This prognostic effect identified both non-specific TILs (via H&E) and CD8+TIL (IHC) [39,40]. But in bladder cancer, the prognostic significance of TILs (both non-specific and CD8+) has not been identified entirely and detecting methods are still depended to pathologists. Because various methods and cut-off levels may contribute to different study results, immune infiltration of tumor and tumor microenvironment requires further comprehensive investigations.

The study limitations are the small number of patients, short follow-up time, single-center and retrospective design, detecting only non-specific TILs and detecting MSI status only with IHC and not in correlation with PCR. Therefore, the results should be interpreted with caution and further investigations are necessary for verification.

In conclusion, our study provides evidence of the prognostic effects of PD-L1, TIL and MSI-high status in bladder cancer. Although there is no relationship between OS and expression of PD-L1 on tumor cells, PD-L1 on TILs and presence of TILs, our findings suggest that there is a trend with MSI-high patients and favorable outcome. Since this is the first study investigating the MSI status in bladder cancer with four-antibody IHC testing and there is lack of data on validating PCR and IHC methods, further studies should focus on testing methods as well as on clinical significance. The importance of patient randomization with clinical trials concerning MSI status is emphasized in this study so that to achieve reliable evaluation of the data.

Authors' contribution

D. Tural: Protocol/project development, data analysis, manuscript writing/editing.
E. Akar: Data collection or management, data analysis, manuscript writing/editing.
HF. Baytekin: Data collection or management.
D. Canoglu: Data collection or management.
M. Yilmaz: Manuscript writing/editing.
V. Tugcu: Protocol/project development, manuscript writing/editing.

Ethics approval

Local ethics committee's (Bakirkoy Sadi Konuk Training and Research Hospital Ethics Committee) approval was obtained prior to the study (No.2017/285).

Acknowledgements

Results of this study have been presented as an abstract at the American Society of Clinical Oncology (ASCO) 2019 Genitourinary Cancers Symposium on 14-19 February in 2019 in San Francisco, USA.

Conflict of interests

The authors declare no conflict of interests.

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