Original paper

Status of PD-1 and PD-L1 expression in invasive urothelial carcinoma of the bladder with mismatch repair protein deficiency

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It has been reported that mismatch repair deficient (d-MMR) tumors show sensitivity to immune checkpoint inhibitors. We aimed to evaluate the correlation of d-MMR and PD-1/PD-L1 expression in invasive urothelial carcinoma of the bladder. Tissue microarray (TMA) tissues were stained PD-1/PD-L1 and MMR proteins. The expression ratio of these markers has been compared with histopathologic parameters. d-MMR tumors were more superficial muscle invasive ($p = 0.012$). When the d-MMR, and PD-1/PD-L1 expression ratios were examined, a significant correlation was obtained between the d-MMR and PD-L1 expression ratio of $> 5\%$ in both the tumor and immune cells ($p = 0.02$ and $p = 0.004$, respectively). The expression ratio was higher in the patients without MMR loss. PD-1 and PD-L1expression in those with MSH6 loss was one or none. When PD1/PDL1 expression was compared with histopathological parameters, a significant relationship was observed between tumor grade and depth of muscle invasion. PD-L1 expression was not observed in the superficial muscle invasive tumors. This study was shown the status of d-MMR and PD-1/PD-L1 in invasive urothelial cancers and their correlation with prognostic markers. PD-1/PD-L1 expression may contribute to the progression and poor prognosis of bladder cancer. However, further studies are required to research the clinical utility.

Key words: bladder, urothelial cancer, PD-1, PD-L1, MSI.

Introduction

Many genetic and epigenetic factors play a role in bladder carcinogenesis. DNA repair mechanism is required to prevent DNA mutations that can be fatal to cells [1]. Mismatch repair (MMR) genes encode a set of DNA repair proteins that cooperatio to recognize and repair DNA mismatches. These proteins are MSH2, MSH6, MLH1 and PMS2. Any defect in these proteins results in an inactive DNA repair process that increases ratios of pathogenic change in genes of the cell growth cycle, leading to defects in tumor suppressor genes and oncogenes, which in turn leads to an increased risk of cancer. DNA repair dysfunction may allow the formation of a high-risk urothelium for malignant transformation in the bladder. Dysfunction of MMR genes may present as absence or reduction of MMR gene expression or microsatellite instability (MSI) phenotype [2].

Lynch syndrome (LS) is an autosomal dominant inherited disorder caused by a germ line pathogenic variant in the DNA mismatch repair (MMR) genes (MLH1, MSH2, MSH6, and PMS2) or deletion of the 3' end of EPCAM in the upper part of MSH2 [3].

The cancers most commonly related with LS are colon cancer, endometrial cancer, ovarian cancer and malignancies affecting the stomach, small intestine, prostate, breast, brain, and hepato-biliary tract [4-8]. In a review article, it was emphasized that urothelial cancers are the third most common type of cancer in LS-related tumors [2].

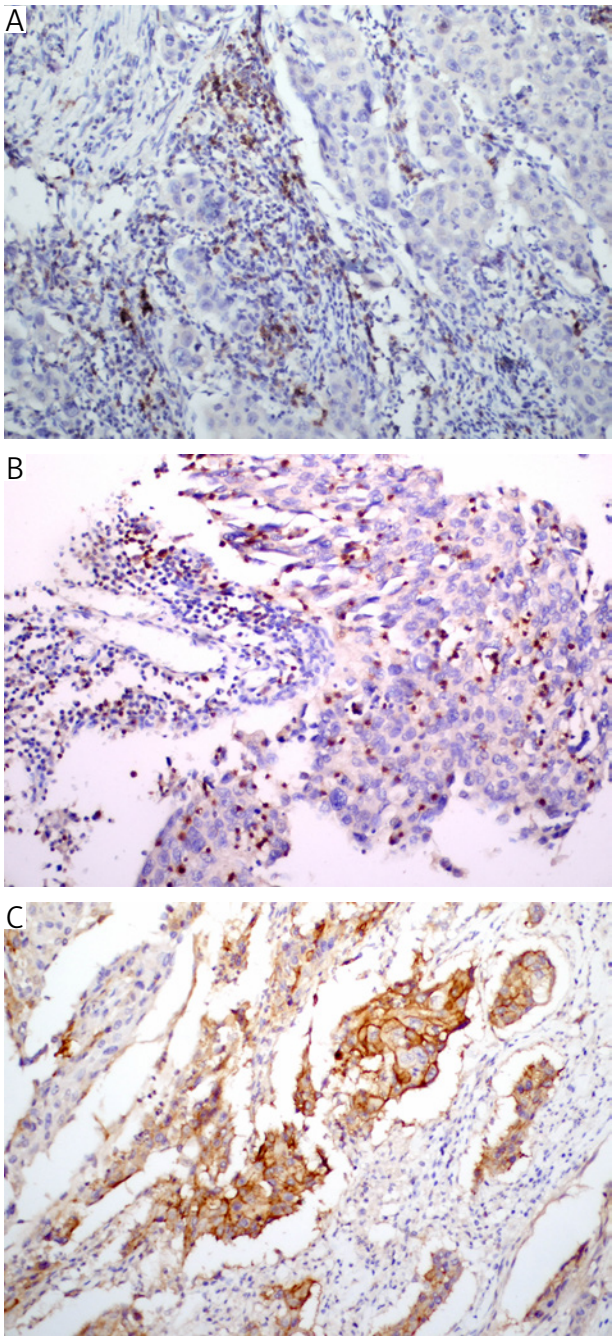

Fig. 1. A) While staining was not observed in tumor cells, staining was followed in immune cells., PD-1 × 200. **B**) Positive membranous expression was observed in PD-1-immune cells, but not expression in tumor cells, PD-L1 × 200. **C**) Positive membranous expression was observed in tumor cells, PD-L1 × 200

Genetic studies on urothelial carcinomas have also indicated that there is a correlation between microsatellite instability (MSI) and urothelial carcinomas. The incidence of MSI in urothelial carcinomas has been reported between 1.1% and 28% in publications [9].

The first test to examine the loss of MMR proteins is immunohistochemistry, which is currently recommended only for colorectal and endometrial carcinomas [10-16]. This technique has also been applied in urothelial carcinomas in recent studies [4].

Urothelial carcinomas with MSI benefit greatly from adjuvant cisplatin-based chemotherapy [17, 18]. Recently, the immune checkpoint inhibitors (ICIs) pembrolizumab and atezolizumab, which target PD-1 or PD-L1, have been approved for patients not eligible for first-line cisplatin-based chemotherapy [19, 20]. The importance of identifying urothelial carcinoma patients with MSI is becoming increasingly evident, as specific treatments are required for better prognosis.

The aim in this study was to assess the prognostic significance of MSI, and PD-1 and PD-L1 expression, which are used as predictive biomarkers in immunotherapy, in patients undergoing radical cystectomy, as well as the presence of PD-1 and PD-L1 expression in patients with MSI.

Material and methods

Study group

This was a retrospective cohort study that included 99 patients with tumors who underwent radical cystectomy.

Resection materials were re-evaluated under light microscopy by 2 pathologists, 1 of whom was experienced in the field of uropathology, and the diagnosis was confirmed.

Construction of the tissue microarray

For the tissue microarray (TMA), the samples were obtained from paraffin blocks with viable tumor areas that were as free of necrosis as possible. For each case, 2 tissue samples with a diameter of 5 mm, one from the tumor center and 1 from the tumor periphery, were taken to represent tumor heterogeneity.

Immunohistochemical analysis procedure

Four-micron thick unstained sections were sequentially cut from the TMA blocks and stained for PD-1 (ab137132, abcam, Cambridge, MA, USA), PD-L1 (ab205921, abcam, Cambridge, MA, USA), MLH-1 (1/100, Cell Marque, Clone G168-728), MSH-2 (1/200, Cell Marque, cloneG219-1129),

and MSH-6 (1/200 Thermo Fisher, clone 44). Immunohistochemical expression was performed using a Leica Bond-Max automatic expression device (Leica Microsystems, Wetzlar, Germany).

Analysis of immunohistochemical study results

Two pathologists evaluated all of the stained slides for PD-1, PD-L1, MLH1, MSH2, and MSH6. Membranous staining of PD-1 and PD-L1 was accepted as positive (Fig. 1A-C). For PD-1 and PD-L1, the expression ratios in both the immune and tumor cells was evaluated. The positive cut-off values were accepted as >5% and 10% [19, 21].

MMR protein expression was assessed in tumor nuclei with complete nuclear loss required for a case to be considered as mismatch repair deficiency (d-MMR). Nuclear expression was assessed as proficiency (p-MMR) (Fig. 2). Peritumoral and intra tumoral lymphocytes served as an internal control.

Statistical analyses

The mean ± standard deviation was used to represent descriptive statistics for age variables that fit the normal distribution. The number (n) and percentage (%) were used for the categorical variables (age group, grade, muscle invasion, MLH-1, MSH-2, MSH-6 repair gene losses). Cross-tables were created to compare the categorical variables according to the immune markers. The χ^2 test was used to identify different categories. Appropriate chi square values were given according to cell ratios with an expected value < 5 (expected count < 5 cell ratio). Pairwise comparisons with Bonferroni correction were performed to determine the different groups in the muscle invasion variables in which there was a difference as a result of the χ^2 test. MS-Excel 2010 and IBM SPSS Statistics for Windows 22.0 (IBM Corp., Armonk, NY, USA) were used for the statistical analyses and calculations. In the statistical decisions, $p < 0.05$ was accepted as statistically significant.

Results

Clinicopathological characteristics

The ages of the patients ranged between 38 and 95 years and the mean age was 66.9 ±10.7 years. The number of patients over the age of 65 was 56. All of the patients except for 1 were male. Of the 99 urothelial cancer samples included in the study, 14 (14.1%) were low grade, 80 (80.8%) were high grade, and 5 (5.1%) were other types of rare urothelial cancers (plasmacytoid variant etc.). While invasion of the lamina propria was observed in 35 (35.4%) of the samples, 7 (7.1%) of the 64 samples with muscle invasion were superficial muscle invasive and 57 (57.6%) were deep muscle invasive. While 60 (60.6%) of the examined samples had not invasion to perivesical adipose tissue, 39 (39.4%) had invasion to perivesical adipose tissue. Lymph node metastasis was not observed in any of the samples.

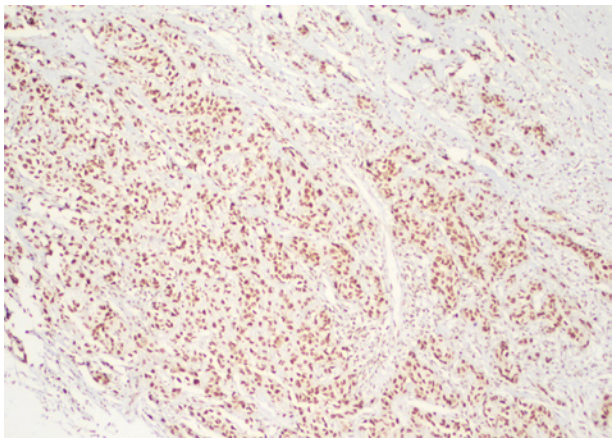

Fig. 2. As with MMR proteins, MLH1 and MSH6, nuclear expression of MSH2 was observed in tumor cells (MSH2 × 100)

Relationship between the MSI status and clinicopathological features

The distribution of MLH1, MSH2, and MSH6 was analyzed in the samples according to MMR status. Loss of MLH1, MSH2, and MSH6 repair genes was observed in 13 (13.1%), 21 (21.2%), and 15 (15.2%) of the samples, respectively. Loss of MLH1-MSH2 was observed in 3 cases and MLH1-MSH2-MSH6 in 1 case. Loss of MSH2 was the most common. The number of samples with missing any repair gene was calculated as 32 (32.3%). In 67 (67.7%) samples, loss of MLH1, MSH2, and MSH6 genes was not observed.

When the MSI status was examined according to the presence of muscle invasion of the tumor, muscle invasion was not observed in 12 of the d-MMR tumors, while there was superficial muscle invasion in 6 and deep muscle invasion in 14. It is not significant correlation was observed between the groups with and without muscle invasion ($p = 0.320$). When muscle invasion and MSI conditions were compared, a significant correlation was observed between muscle invasion and MSI status ($\chi^2 = 10.301$; $p = 0.005$). A reanalysis was performed to determine whether the relationship was due to superficial or deep muscle invasion. It was determined that 85.7% (6/7) of the cases with d-MMR had superficial muscle invasion, and 75.4% (43/57) of the cases with p-MMR had deep muscle invasion ($\chi^2 = 8.192$; $p = 0.012$). In the analysis performed to determine whether there is a relationship between MSI condi-

Table I. Associations between expression of MSI status with clinicopathological features

Subgroups	Loss of MLH-1			Loss of MSH-2			Loss of MSH-6			MSI		
	Present	Absent	p-value	Present	Absent	p-value	Present	Absent	p-value	d-MMR	p-MMR	p-value
Overall (n = 99)	13 (13.1)	88 (86.9)	< 0.001	21 (21.2)	78 (78.8)	< 0.001	15 (15.2)	84 (84.8)	< 0.001	32 (32.3)	67 (67.7)	< 0.001
Age (years) (n = 99)												
≤ 65 (n = 43)	6 (14.0)	37 (86.0)	0.832	13 (30.2)	30 (69.8)	0.054	6 (14.0)	37 (86.0)	0.771	17 (39.5)	26 (60.5)	0.179
> 65 (n = 56)	7 (12.5)	49 (87.5)		8 (14.3)	48 (85.7)		9 (16.1)	47 (83.9)		15 (26.8)	41 (73.2)	
Histologic grade (n = 94)*												
Low grade (n = 14)	1 (7.1)	13 (92.9)	0.494	2 (14.3)	12 (85.7)	0.488	1 (7.1)	13 (92.9)	0.329	4 (28.6)	10 (71.4)	0.704
High grade (n = 80)	11 (13.8)	69 (86.3)		18 (22.5)	62 (77.5)		14 (17.5)	66 (82.5)		27 (33.8)	53 (66.3)	
Muscle invasion												
Absent (n = 35)**	3 (8.6)	32 (91.4)	0.002	10 (28.6)	25 (71.4)	0.088	7 (20.0)	28 (80.0)	0.277	12 (34.3)	23 (65.7)	0.005
SMP (n = 7)	4 (57.1)[a]	3 (42.9)[b]		3 (42.9)	4 (57.1)		2 (28.6)	5 (71.4)		6 (85.7)[a]	1 (14.3)[b]	
DMP (n = 57)	6 (10.5)	51 (89.5)		8 (14.0)	49 (86.0)		6 (10.5)	51 (89.5)		14 (24.6)	43 (75.4)	
Muscle invasion												
Absent (n = 35)	3 (8.6)	32 (91.4)	0.321	10 (28.6)	25 (71.4)	0.185	7 (20.0)	28 (80.0)	0.277	7 (20.0)	28 (80.0)	0.320
Present(n = 64)	10 (15.6)	54 (84.4)		11 (17.2)	53 (82.8)		2 (28.6)	5 (71.4)		8 (12.5)	56 (87.5)	
İnvades perivesical tissue												
Absent (n = 60)	9 (15.0)	51 (85.0)	0.495	16 (26.7)	44 (73.3)	0.100	11 (18.3)	49 (81.7)	0.273	23 (38.3)	37 (61.7)	0.113
Present (n = 39)	4 (10.3)	35 (89.7)		5 (12.8)	34 (87.2)		4 (10.3)	35 (89.7)		9 (23.1)	30 (76.9)	

** Others (n = 5) excluded, a-b denotes different subgroups, SMP – tumor invades superficial muscularis propria (inner half); DMP – tumor invades deep muscularis propria (outer half)*

***Group showing lamina propria invasion but not muscle invasion, d-MMR – mismatch repair deficiency, p-MMR – mismatch repair proficiency*

Table II. Associations between expression of PD-L1 in tumor cells/immune cells with clinicopathological features and MSI status

Subgroups	Tumor cells						İmmune cells					
	5% cut-off value			10% cut-off value			5% cut-off value			10% cut-off value		
	Positive	Negative	p-value	Positive	Negative	p-value	Positive	Negative	p-value	Positive	Negative	p-value
Overall (n = 99)	38 (38.4)	61 (61.6)	0.021	22 (22.2)	77 (77.8)	<0.001	39 (39.4)	60 (60.6)	0.035	24 (24.2)	75 (75.8)	< 0.001
Age (years) (n = 99)												
≤ 65 (n = 43)	17 (39.5)	26 (60.5)	0.837	11 (25.6)	32 (74.4)	0.481	15 (34.9)	28 (65.1)	0.421	10 (23.3)	33 (76.7)	0.841
> 65 (n = 56)	21 (37.5)	35 (62.5)		11 (19.6)	45 (80.4)		24 (42.9)	32 (57.1)		14 (25.0)	42 (75.0)	
Histologic grade (n = 94)*												
Low (n = 14)	4 (28.6)	10 (71.4)	0.521	4 (28.6)	10 (71.4)	0.470	5 (35.7)	9 (64.3)	0.899	4 (28.6)	10 (71.4)	0.777
High (n = 80)	30 (37.5)	50 (62.5)		16 (20.0)	64 (80.0)		30 (37.5)	50 (62.5)		20 (25.0)	60 (75.0)	
Muscle invasion												
None (n = 35)**	16 (45.7)	19 (54.3)		7 (20.0)	28 (80.0)		12 (34.3)	23 (65.7)		8 (22.9)	27 (77.1)	
SMP (n = 7)	1 (14.3)	6 (85.7)	0.244	0 (0.0)	7 (100.0)	0.265	0 (0.0)[a]	7 (100.0)[b]	0.012	0 (0.0)	7 (100.0)	0.255
DMP (n = 57)	21 (36.8)	36 (63.2)		15 (26.3)	42 (73.7)		27 (47.4)	30 (52.6)		16 (28.1)	41 (71.9)	
Muscle invasion												
None (n = 35)	16 (45.7)	19 (54.3)	0.267	7 (20.0)	28 (80.0)	0.694	12 (34.3)	23 (65.7)	0.442	8 (22.9)	27 (77.1)	0.812
SMP (n = 64)	22 (34.4)	42 (65.6)		15 (23.4)	49 (76.6)		27 (42.2)	37 (57.8)		16 (25.0)	48 (75.0)	
İnvades perivesical tissue												
Absent (n = 60)	24 (40.0)	36 (60.0)	0.682	13 (21.7)	47 (78.3)	0.869	23 (38.3)	37 (61.7)	0.789	13 (21.7)	47 (78.3)	0.458
Present (n = 39)	14 (35.9)	25 (64.1)		9 (23.1)	30 (76.9)		16 (41.0)	23 (59.0)		11 (28.2)	28 (71.8)	
MSI (n = 99)												
p-MMR (n = 67)	31 (46.3)	36 (53.7)	0.020	17 (25.4)	50 (74.6)	0.275	33 (49.3)	34 (50.7)	0.004	20 (29.9)	47 (70.1)	0.060
d-MMR (n = 32)	7 (21.9)	25 (78.1)		5 (15.6)	27 (84.4)		6 (18.8)	26 (81.3)		4 (12.5)	28 (87.5)	
Loss of MLH-1												
Absent	36 (41.9)	50 (58.1)	0.067	20 (23.3)	66 (76.7)	0.525	37 (43.0)	49 (57.0)	0.057	23 (26.7)	63 (73.3)	0.135
Present	2 (15.4)	11 (84.6)		2 (15.4)	11 (84.6)		2 (15.4)	11 (84.6)		1 (7.7)	12 (92.3)	
Loss of MSH-2												
Absent	33 (42.3)	45 (57.7)	0.122	19 (24.4)	59 (75.6)	0.324	34 (43.6)	44 (56.4)	0.100	21 (26.9)	57 (73.1)	0.230
Present	5 (23.8)	16 (76.2)		3 (14.3)	18 (85.7)		5 (23.8)	16 (76.2)		3 (14.3)	18 (85.7)	
Loss of MSH-6												
Absent	37 (44.0)[a]	47 (56.0)[b]	0.006	22 (26.2)[a]	62 (73.8)[b]	0.025	38 (45.2)[a]	46 (54.8)[b]	0.005	23 (27.4)	61 (72.6)	0.085
Present	1 (6.7)[a]	14 (93.3)[b]		0 (0.0)[a]	15 (100.0)[b]		1 (6.7)[a]	14 (93.3)[b]		1 (6.7)	14 (93.3)	

** Others (n = 5) excluded, a-b denotes different subgroups, SMP – tumor invades superficial muscularis propria (inner half); DMP – tumor invades deep muscularis propria (outer half)*

***Group showing lamina propria invasion but not muscle invasion, d-MMR – mismatch repair deficiency, p-MMR – mismatch repair proficiency*

Table III. Associations between expression of PD-1 in immune cells with clinicopathological features and MSI status

Subgroups	5% cut-off value			10% cut-off value		
	Positive	Negative	p-value	Positive	Negative	p-value
Overall (n = 99)	61 (61.6)	38 (38.4)	0.021	38 (38.4)	61 (61.6)	0.021
Age (years) (n = 99)						
≤ 65 (n = 43)	26 (60.5)	17 (39.5)	0.837	17 (39.5)	26 (60.5)	0.837
> 65 (n = 56)	35 (62.5)	21 (37.5)		21 (37.5)	35 (62.5)	
Histologic grade (n = 94)*						
Low grade (n = 14)	2 (14.3) [a]	12 (85.7) [b]	<0.001	1 (7.1) [a]	13 (92.9) [b]	0.012
High grade (n = 80)	55 (68.8) [a]	25 (31.3) [b]		34 (42.5) [a]	46 (57.5) [b]	
Muscle invasion						
None (n = 35)**	22 (62.9)	13 (37.1)	0.172	13 (37.1)	22 (62.9)	0.354
SMP (n = 7)	2 (28.6)	5 (71.4)		1 (14.3)	6 (85.7)	
DMP (n = 57)	37 (64.9)	20 (35.1)		24 (42.1)	33 (57.9)	
Muscle invasion						
Absent (n = 35)	22 (62.9)	13 (37.1)	0.851	13 (37.1)	22 (62.9)	0.851
Present (n = 64)	39 (60.9)	25 (39.1)		25 (39.1)	39 (60.9)	
İnvades perivesical tissue						
Absent (n = 60)	38 (63.3)	22 (36.7)	0,663	23 (38,3)	37 (61,7)	0.990
Present (n = 39)	23 (59.0)	16 (41.0)		15 (38.5)	24 (61.5)	
MSI						
p-MMR (n = 67)	45 (67.2)	22 (32.8)	0.100	28 (41.8)	39 (58.2)	0.313
d-MMR (n = 32)	16 (50)	16 (50)		10 (31.3)	22 (68.8)	
Loss of MLH-1						
Absent (n = 86)	55 (64.0)	31 (36.0)	0.219	33 (38.4)	53 (61.6)	0.995
Present (n = 13)	6 (46.2)	7 (53.8)		5 (38.5)	8 (61.5)	
Loss of MSH-2						
Absent (n = 78)	49 (62.8)	29 (37.2)	0.635	32 (41.0)	46 (59.0)	0.298
Present (n = 21)	12 (57.1)	9 (42.9)		6 (28.6)	15 (71.4)	
Loss of MSH-6						
Absent (n = 84)	54 (64.3)	30 (35.7)	0.196	34 (40.5)	50 (59.5)	0.311
Present (n = 15)	7 (46.7)	8 (53.3)		4 (26.7)	11 (73.3)	

** Others (n = 5) excluded, a-b denotes different subgroups, SMP – tumor invades superficial muscularis propria (inner half); DMP – tumor invades deep muscularis propria (outer half)*

***Group showing lamina propria invasion but not muscle invasion, d-MMR – mismatch repair deficiency, p-MMR – mismatch repair proficiency*

tions and lamina propria and superficial muscle invasion, it was determined that d-MMR tumors showed more superficial muscle invasion than p-MMR tumors. There was a relationship between the groups with lamina propria and superficial muscle invasion according to the d-MMR ($\chi^2 = 4.375$; $p = 0.036$). However, when Bonferroni correction was made, it was concluded that there was no difference between these groups ($p = 0.036 * 3 = 0.108$). There was not correlation between the groups with lamina propria and deep muscle invasion according to the d-MMR ($\chi^2 = 1.011$; $p = 0.945$). When the depth of invasion was evaluated according to MMR proteins, only a significant correlation was found between MLH1 and invasion. Just as in the d-MMR group, the MLH1 group showed superficial muscle invasion compared to the p-MMR group. This was statistically significant ($p = 0.002$).

It is not significant correlation was observed between MSI status and tumor grade, invasion into perivesical adipose tissue, and age. No significant re-

lationship was found between tumor grade and loss of MLH1, MSH2 and MSH6 ($p > 0.05$) (Table I).

Relationship between the PD-1 and PD-L1 expression with clinicopathological features

The expression ratio of > 5% with PD-L1 was 38.4% (38) in the tumor cells and 39.4% (39) in the immune cells. The expression ratio of > 10% with PD-L1 was 22.2% (22) in the tumor cells and 24.2% (24) in the immune cells (Table II).

PD-1 was not detected in the tumor cells. In the immune cells, while the expression ratio of > 5% was 61.6% (61), the expression ratio of > 10% was 38.4% (38) (Table III).

When the PD-1 and PD-L1 expression ratios were examined to determine if they differed according to the tumor grade, no significant results were obtained for PD-L1 in either the tumor or immune cells. In the immune cells, a significant correlation was observed for both > 5% and > 10% with PD-1 ($p > 0.001$ and $p = 0.012$, respectively). The expression ratio was significantly higher in patients with a high grade tumor when compared to those with a low grade (Tables II and III).

When the PD-1 and PD-L1 expression ratio were examined to determine if they differed according to the muscle invasion, the immune cells for PD-1 and the tumor cells for PD-L1 is not significantly correlation detected. A strong association was observed with PD-L1 in immune cells, with only > 5% expression rate ($p = 0.012$). No expression was observed for PD-L1 in the superficial invasive tumors (Tables II and III).

It is not association was found between PD-1 and PD-L1 and age as well as with invasion to perivesical adipose tissue.

Associations between the expression of PD-1 and PD-L1 in the tumor cells/immune cells with MSI status

According to MMR protein expression status, when the PD-1, PD-L1 expression rates are analyzed, strong relationship were observed between the d-MMR group and > 5% expression ratio of PDL-1 in both tumor and immune cells ($p = 0.02$ and $p = 0.004$, respectively). The expression rates were higher in cases p-MMR compared to those with d-MMR.

In addition, the PD-1 and PD-L1 expression status of cases with loss of MLH1, MSH2, and MSH6 were also analyzed. According to the loss of MLH1 and MSH2, there was not significant relationship ($p > 0.05$) between the PD-L1 expression ratio in the tumor/immune cells (for > 5% and > 10%) (Table II).

When the PD-L1 expression was considered according to loss of MSH6, significant correlation was found between both ratio of > 5% and ratio of > 10% in the tumor cells ($p = 0.006$ and $p = 0.025$, respectively). While there was 1 case with > 5% expression in the tumor cells, there were no cases with > 10% expression. Only 1 case was detected in the group with > 5% expression in immune cells, which was statistically significant ($p = 0.005$). Although 1 case was detected in the group with > 10% expression in immune cells, it is not statistically significant correlation was found (Table II).

According to the loss of MLH1, MSH2 and MSH6, it is not significant correlation was observed between the PD-1 in the tumor/immune cells (for > 5% and > 10%) ($p > 0.05$) (Table III).

Isolated loss of MSH6 was detected in 7 cases. None of these cases indicated PD-L1 expression in the immune and tumor cells at ratio of > 5% and > 10%. Similarly, there was no expression of > 5% and > 10% for PD-1 in the tumor cells. On the other hand, the number of cases with expression of > 5% and > 10% for PD-1 in the immune cells became prominent, with 3 cases each. Since there were very few cases with isolated MSH6 loss, it was not possible to analyze them statistically.

Discussion

Urothelial cancers may be a component of LS [17]. Previous molecular genetic studies have shown a relationship between urothelial carcinomas and MSI. In immunohistochemical studies, while 1.1% to 28% of urothelial carcinomas were found to show MSI, this ratio was reported to be up to 45% in studies using PCR-based methods [2, 9, 22, 23]. Among these, MSH2 loss is the most common [1, 2, 17]. In the current study, it was found that the ratio of MSI was 32% and the loss of MSH2 among the MMR proteins was the most common, in accordance with the literature.

Some studies have shown a correlation between the histologic features of urothelial carcinomas and the presence of MSI [24-26]. In urothelial carcinomas with MSI, these histologic features were found to be high-grade papillary tumors in pTa or pT1 stages, usually without prominent nuclear pleomorphism. In the current study, when d-MMR tumors were compared according to the presence of deep and superficial muscle invasion, it was determined that d-MMR tumors showed more superficial muscle invasion than p-MMR tumors ($p = 0.012$). The relationship between d-MMR and p-MMR groups according to muscle invasion was statistically significant ($p = 0.005$). However, no significant result was found between low and high grade.

Approximately 75% of patients with urothelial carcinoma are non-invasive and have a favorable prognosis with transurethral resection, intravesical chemotherapy and immunotherapy. However, the remaining 25% muscle-invasive bladder cancer usually show a poor prognosis despite systemic treatment. In recent years, immune check point inhibitors (ICIs), particularly PD-1, PD-L1, and cytotoxic T lymphocyte-associated antigen 4, have brought a new development in the treatment of urological tumors, especially advanced urothelial cancer. These markers have also been associated with prognosis [27].

The positive cut-off value for PD-1/PD-L1 has not been standardized for ICI treatment. Wang *et al.* correlated well PD-L1 expression in tumor cells with the pathological grade, clinical stage, recurrence, and postoperative prognosis of bladder cancer in their study including 50 bladder cancers, in which PD-L1 positive expression ratio was >10 [21]. Chen *et al.* used positive cut-off values of 1%, 5%, 10%, and 50% in tumor cells and 1% in immune cells and applied TMA blocks in a study of 96 cases of invasive urothelial carcinoma and found that a 5% cut-off value for PD-L1 may be a good positive value in PD-1/PD-L1 inhibitor treatment [28]. These two cut-off values were used in the current study. However, as the study was retrospective, further prospective studies involving patients undergoing immunotherapy are needed to confirm these values.

In a comprehensive study including 318 radical cystectomies, Boorjian et al. found that > 5% PD-L1 expression in urothelial tumor cells was significantly related with increasing pathological stage [29]. In this study, there was strong association in the expression ratio of > 5% with PD-L1 in the immune cells ($p = 0.012$). PD-L1 expression was not observed in the superficial muscle invasive tumors.

Currently, treatment of anti-PD-1 have been approved in metastatic and advanced urothelial carcinoma. In a study by Kumar *et al.*; PD-1/PD-L1 expression was investigated immunohistochemically in 116 patients who underwent transurethral resection of the bladder and were diagnosed with urothelial carcinoma. In the study, a high correlation was found between PD-1 and tumor grade. High expression detected in high grade tumors [30]. In our study; we detected expression rates of > 5% and > 10% with PD-1 in immune cells, significantly higher in high-grade tumors than low-grade tumors ($p > 0.001$ and $p = 0.012$, respectively according to the percentage of expression).

Recent studies have shown that patients with multiple tumor types exhibiting MMR protein loss may respond to PD-1/PD-L1 blockade [31, 32]. In the literature, there are very few studies showing PD-1/PD-L1 expression in bladder cancers with loss of MMR. In a study including 201 cases of high-grade muscle invasive bladder urothelial cancer conducted by Hodgson *et al.*, a positive significant correlation was found between PD-L1 positivity and loss of MMR (PD-L1 positive in 3 of 4 cases) [17]. In the current study, negative significant association were obtained between d-MMR and the expression ratio of >5% of PDL-1 in both the tumor and immune cells ($p = 0.02$ and $p = 0.004$, respectively). The expression ratio was lower in tumors with loss of MMR. This result may be due only to the absence of high-grade tumors in our study. It may also be due to the fact that the cases in the study in the article included only high-grade invasive tumors and did not include low-grade tumors for comparison.

In addition, MLH1, MSH2 and MSH6 loss states and PD-1, PD-L1 expression ratios also differ. There are very few studies comparing them. In a study of 50 cases, Zavalishina *et al*. immunohistochemically evaluated the MSI phenotype and its relationship with PD-L1 in T1-T3 urothelial tumors. As a result of their study, while the decreased PMS2 and MLH1 expression was observed in PD-L1 positive cases, significant expression was detected with MSH6 [33]. Moreover, it can be said that there was no MSH6 loss in PD-L1 positive cases. In the present study, > 5% expression with PD-L1 in the immune/tumor cells and 10% expression with PD-L1 in the immune cells was found in only 1 case each with MSH6 loss. In tumor cells, no case with PD-L1 expression at the > 10% cut-off value was observed. This may indicate that MSI patients with loss of MSH6 may have a limitation to utilize the immunotherapy.

In a review article presented by Bellmunt *et al*., it was reported that the response ratio to immunotherapy in metastatic urothelial carcinomas was 20-30% and this ratio increased up to 39% at higher doses [34]. Since there are very few studies evaluating immunotherapy in urothelial cancer patients with MSI, studies showing response ratio to this treatment could not be found.

In studies involving MMR deficiency patients to whom immunotherapy was applied, MMR protein losses were mostly evaluated as high and low MSI, and MMR proteins were not evaluated separately. This may be attributed to the small number of patients. In one study, it was reported that patients with MSH6 loss would not benefit from immunotherapy. In this study conducted by Liu *et al*., 15 Chinese families with LS diagnosed clinically according to Amsterdam II criteria were identified. In these patients, immunohistochemical expression was observed to be negative with MSH6 but positive with MLH1, MSH2, and PMS2. While it was thought that these patients would benefit from PD-1 immune checkpoint blockade treatment, on the contrary, it was observed that tumors progressed rapidly after 4 sessions of anti-PD-1 treatment [35]. In the current study,

expression was observed in a maximum of 1 case in a patient with loss of MSH6. These results were found to be statistically significant ($p < 0.05$). This may explain why patients with MSH6 loss could not benefit from ICI treatment. However, comprehensive multicenter studies with multiple cases are required to support this.

The limitations of this study were that it included a small number of patients, it was single centered and retrospective, the MSI status was determined only by immunohistochemistry, and the results could not be correlated with PCR. Therefore, further research is required to interpret and confirm the results.

Conclusions

This study was conducted to indicate the MSI and PD-1/PD-L1 status in invasive urothelial carcinomas undergoing cystectomy and their relationship with prognostic markers. The PD-1/PD-L1 expression ratio was higher in muscle invasive and high-grade urothelial cancers. This suggests that PD-1 and PD-L1 expression may contribute to the progression and poor prognosis of bladder cancer. Loss of MMR proteins, especially MLH1 loss, was more common in the superficial muscle invasive tumors than in the deep muscle invasive tumors. The histomorphological findings of the MSI tumors indicated that they were more superficial tumors in this study, as reported in some other studies. PD-1/PD-L1 expression ratios were low in the d-MMR invasive urothelial cancers and no expression was observed in patients with MSH6 loss. Our study results suggested that the reason why a group of patients with MMR loss did not benefit from immunotherapy might be due to the loss of MSH6. However, comprehensive studies are required to research the clinical utility of d-MMR as a predictive biomarker of immune therapy response.

The authors declare no conflict of interest.

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