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ORIGINAL ARTICLE

Assessment of *ex-vivo* efficacy of immunotherapeutic agents in intermediate-risk and high-risk non-muscle invasive bladder cancer

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Summary

Purpose: *To study the ex-vivo efficacy of immunotherapeutic agents as an oncogram in primary bladder cancer cell cultures obtained from patients with intermediate-risk and high-risk non-muscle invasive bladder cancer (NMIBC) and its possible relationships with clinicopathologic features were evaluated.*

Methods: *Primary bladder cancer cell cultures were produced from each tissue sample. Each culture was divided into 5 drug administration groups. In Group-1, mononuclear cells (MNCs) were isolated from blood samples and non-treated primary cells only were used. Besides MNCs, mitomycin-C (MMC) for Group-2, Bacillus Calmette Guérin (BCG) for Group-3, nivolumab for Group-4 and ipilimumab for Group-5 were applied. Viability tests were performed using WST-1. Expressions of programmed death-1, its ligands (PD-1, PD-L1 and PD-L2, respectively) and cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) were investigated immunohistochemically (IHC).*

Results: *Tumoral PD-1 positivity was found to be significantly associated with worse pathologic outcomes. All agents caused lower viability rates. Good response rates of PD-L1 positive cases were significantly higher compared to PD-L1 negative cases after the MMC (66% vs 0%, p=0.045) and nivolumab (66% vs 0%, p=0.045) treatments. Also, good response rate after MMC was found higher only in high-risk cases with peritumoral PD-1 positive than negatives (87.5% vs 20%, p=0.032).*

Conclusions: *Drug responses, namely oncogram results of MMC, BCG, nivolumab and ipilimumab were found significantly higher than in controls. Although, there were some differences based on IHC between the patients, nivolumab response rate was better especially in PD-L1 positive patients.*

Key words: *Bacillus Calmette Guerin (BCG), ipilimumab, nivolumab, non-muscle invasive bladder cancer (NMIBC), Oncogram*

Introduction

Bladder cancer (BC) is one of the most morbid and fatal cancers among urological malignancies [1]. At the time of diagnosis, 70-75% of patients are diagnosed with non-muscle invasive bladder cancer (NMIBC) [2]. Those are treated with transurethral resection of the bladder tumor (TUR-BT) with or without additional intravesical treatments. They are followed-up by cystoscopy according to European Organization for Research and Treatment of Cancer (EORTC) risk groups [2]. On the other

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hand, 25-30% of patients have muscle-invasive bladder cancer (MIBC). Those are treated with radical cystectomy (RC) with or without systemic neoadjuvant and adjuvant treatments according to European Urology (EAU) Guidelines [1,3,4].

According to EORTC risk groups, NMIBC patients are divided into three risk groups as low-risk, intermediate-risk and high-risk groups [2]. Especially in intermediate-risk and high-risk NMIBCs, intravesical Bacillus Calmette Guérin (BCG) immunotherapy is recommended to reduce the risk of tumor recurrence after the primary TUR-BT or repeat TUR (Re-TUR) [2,5]. Recurrence and progression rates are seen in 40-50% for patients with high-risk NMIBC despite BCG immunotherapy [2]. Therefore, additional treatment modalities are needed in patients with BCG-refractory NMIBC. Similarly, because of need for additional treatments for patients with post-platinum and cisplatin ineligible metastatic MIBC, immune checkpoint inhibitors have been gone into the use for the treatment of these patients [6-10]. Also for patients with BCG-refractory NMIBC, immune checkpoint inhibitors may fill the treatment needing area [3,11].

Previous studies have shown that primary cell culture obtained from patients with bladder cancer can be grown for an oncogram and by this way it can be tested for chemotherapy and immunotherapy sensitivities with drug administration *in vitro* [12,13].

We aimed to measure the *ex-vivo* efficacy of immunotherapeutic agents (BCG, nivolumab and ipilimumab) and a chemotherapeutic agent (mitomycin-C (MMC) and MNCs in BC primary cell cultures obtained from patients with intermediate-risk and high-risk NMIBC and to investigate their possible relationships with clinicopathologic features.

Methods

After receiving ethical approval by Ethics Committee (Approval number: 3320-GOA, 2017/24-23), we prospectively investigated patients who underwent cystoscopy and TUR-BT due to BC that was diagnosed by radiological imaging between 2018-2019. After obtaining informed consent, at least 5x5x5 mm fresh tumor tissue was obtained from each patient during the TUR-BT procedure and peripheral blood sample was also obtained from each patient prior to surgery.

Laboratory steps: Collection of bladder cancer tissues and peripheral blood samples

The obtained each tumor tissue was transferred to laboratory in sterile transfer medium (1% penicillin/streptomycin (P/S) and Roswell Park Memorial Institute medium (RPMI)) in cold conditions. Peripheral blood samples were also transferred with EDTA blood collection tube at cold conditions. Mononuclear cells (MNCs) that were separated with using ficoll paque solution from peripheral blood and all tissue were centrifuged and slowly frozen in freezing medium (complete RPMI containing 20% fetal bovine serum (FBS) and 5% dimethyl sulfoxide (DMSO)) to -80°C.

Laboratory steps: Primary cell culture of the bladder cancer and applications of the drugs

Before the experiments of primary cell culture, human T24 bladder cancer cell line was cultured at 37°C humidified 5% CO_2 incubator. T24 cells were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 1% L-glutamine, 1% penicillin/streptomycin (P/S) and 10% FBS. Then, the drugs concentrations were determined by applying different doses (10-80mM for MMC, 1.2-13.0µg/ml for BCG, 0.5-50µg/ml for nivolumab and 0.5-50µg/ml for ipilimumab) on T24 cell line. Proliferation rates were calculated according to control cells absorbances after the WST-1 test. After that, LC50 doses of MMC, BCG, nivolumab and ipilimumab were determined as 60µM, 8µg/ml, 5µg/ml and 10µg/ml at 24 h, respectively.

After the determination of doses of drugs for the applications on cultures, all samples were defrosted. Then, all cell suspensions were plated into 96-well plates with 10^5 cells per well. One ml Leibovitz's L-15 medium containing 20% FBS was added into each well and the wells were incubated in 5% CO_2 at 37°C in an incubator. After the incubation of the cells for 24 h, the cells reached approximately 80% confluence. Drugs doses were confirmed with an experiment for primary cell culture for one patient.

The 80% confluent tumor cells in the well plates for each culture were divided into 5 different groups. The control group (Group 1) had only medium (Leibovitz's L-15 medium with 20% FBS) applied. Also, 60mM MMC (mitomycin C®) (Group 2), 8µg/ml BCG (BCG, SII®) (Group 3), 5µg/ml nivolumab (Obdivo®) (Group 4) and 10µg/ml ipilimumab (Yervoy®) (Group 5) applied, respectively. The obtained MNCs (1000 cells/well) were included into all conditions (applicated well plates).

Laboratory steps: Viability tests with trypan blue and water-soluble tetrazolium salt (WST-1)

The treated cultures were harvested from the 96-well plates at the end of 24 h and the harvested cells were centrifuged. Then, 10µl supernatant and 10µl trypan blue were added to a new Eppendorf tube. The cells were counted in the EVE™ cell counting device. In addition, to check the viability results, WST-1 assay was also used for each culture. Good response rate was defined as >23% viability rate decreases after each drug application according to the median viability dropping rates of the drug groups.

Laboratory steps: Immunohistochemical examinations

Immunhistochemical examinations were performed by an expert uropathologist (KY) in paraffin blocks of the patients with BC after routine pathological examination

in the pathology laboratory. In the examinations, 1/100 diluted PD-1, PD-L1, PD-L2 and CTLA-4 antibodies were applied for 1 h without washing. After washing with PBS, steps of streptovidin-biotin secondary antibody and DAB coloring, ground staining was performed with hematoxylin. Then, samples were evaluated under light microscope. The strongest intensity within a sample was selected as intensity score (IS) of the sample. Scores of 0 and +1 were classified as negative and scores of 2+ and 3+ as positive.

Pathological and clinical data of the patients

Patients with NMIBC were treated and followed-up according to the EORTC risk table. Patients were not treated with these drugs at the same time. Diagnosis of NMIBC was made by an uropathologist using immunohistochemical staining. Pathologic and clinical data, routine intravesical therapy results (for NMIBC) and follow-up data of the patients were compared to *ex-vivo* viability results of the drug application to the cell cultures for each case.

Statistics

Data were analyzed using the SPSS 22.0 statistical package (SPSS, Chicago, Ill). Wilcoxon test was used for comparison of drug groups according to their own controls. Also, the Mann-Whitney U test, Kruskal-Wallis test and x^2 test were used for analysis of patient data. Statistical significance was set at $p<0.05$.

Results

Twenty NMIBC cases investigated had a mean age of 69.6 years (±11.2, range:52-89). Clinicopathologic data of the patients are given in Table 1. As a result, 7 patients were evaluated in intermediate-risk group and 13 patients in high-risk group. Pathologic and clinical data, EORTC risk group data, those receiving additional treatment findings and laboratory results (primary cell culture viability rates after MMC, BCG, nivolumab and ipilimumab administration) for each patient are given in Table 2, separately. According to the findings, viability rates were higher after BCG administration in four high-risk patients who had recurrence (MS3 71.4%, MS18 75.2%, MS22 74.1%) and MS25 76.3%) compared to some patients who had not any recurrence (MS4 53.7% and MS13 35.6%) after the intravesical BCG treatment. However, viability rates of these four patients with recurrence were lower than two patients with no recurrence (MS14 99.4%) and MS21 (82.9%). For MS14, the lowest rate of viability was found with MMC (86.7%) than other immunotherapy drugs. On the other hand, in BCG treated intermediate-risk patients, viability rate of BCG was lower in a patient with no recurrence than in a patient with recurrence (MS7 54.5%) vs MS8 (67.1%) (Table 2).

Table 1. Patients' demographics and clinicopathological data

	n (%)
Age (years)	69.6±11.2 (52-89)
Gender	
Male	18 (90)
Female	2 (10)
Tumor diameter (cm)	58.5±34.3 (25-150)
Number of tumors	3.8±3.8 (1-10)
Pathological stage	
Ta	7 (35)
T1	13 (65)
Tumor grade	
Low-grade	8 (40)
High-grade	12 (60)
EORTC risk table for NMIBC	
Intermediate-risk	7 (35)
High-risk	13 (65)
Type of urothelial carcinoma	
Pure UC	17 (85)
UC with squamous differentiation	2 (10)
UC with sarcomatoid differantiation	1 (5)
UC with glandular differantiation	1 (5)
Histological pattern of UC	
Papillary	18 (90)
Solid	3 (15)
Inverted	1 (5)
Micropapillary	2 (10)
Concomittant CIS presence	5 (25)
Presence of muscle layer in the specimens	11 (55)
Vascular invasion	2 (10)
Postoperative early single dose instilation (with MMC)	5 (25)
Re-TUR	7 (35)
Tumor presence in Re-TUR specimens	5 (71.4)
Intravesical treatment	
Untreated	8 (40)
BCG	11 (55)
MMC	1 (5)
Recurrence in the follow-up	
in untreated patients	6 (75)
in patients under BCG treatment	5 (45.5)
in patients under MMC treatment	0 (0)
Follow-up time (years)	16.9±7.3 (3-24)

EORTC: European Organization for Research and Treatment of Cancer, NMIBC: Non-muscle invasive bladder cancer, UC: Urothelial carcinoma, MMC: Mitomycin C, Re-TUR: Repeat transurethral resection, CIS: Carcinoma in situ, BCG: Bacillus Calmette Guerin

Viability rates of BC cell cultures after drug applications and comparison results with own controls are given in Table 3. Viability rates were significantly decreased after the applications compared to own controls (p<0.001). Viability rates after the applications according to immunohistochemical (IHC) findings of immune checkpoints in all cases are shown in Table 4. All comparisons showed no statistical difference in viability rates of the same drug between negative and positive IHC results. On the other hand, cell viability rates of tumoral CTLA-4 positive cases were detected to be lower than negative patients after all immunotherapeutic applications (BCG, nivolumab and ipilimumab). However, this situation was not valid for the MMC (Table 4). Also, when we look at the significant response rates (>23% of viability rates decrease) after the drug applications, some findings were glittered (Table 5). Only in PD-L1 positive cases, good response rates were found to be significantly higher compared to PD-L1 negative cases after the MMC (66 vs 0%, p=0.045) and nivolumab (66 vs 0%, p=0.045) administrations (Table 5).

Table 2. Pathological results, clinical stage, received additional treatment findings, recurrence status, primary cell culture viability rates after MMC, BCG, Nivolumab and Ipilimumab applying of each patient.

PN	*P Stage*	*Histology*	*Grade*	*CIS*	*EORTC Risk group*	*ISI*	*IV therapy*	*Rec*	*Primary Culture viability rates after drug administration (%)*				
									Control	*MMC*	*BCG*	*Nivo*	*Ipili*
MS1	T1	UC-Src	High	+	High	-	-	+	99	100	64.9	71.6	72.8
MS3	T1	UC	High	-	High	-	BCG	+	100	71.8	71.4	78.2	88.3
MS4	T1	UC	High	+	High	-	BCG	-	100	61.4	53.7	55.8	58.7
MS6	T1	UC	High	-	High	-	-	+	99	74.9	57.7	81.2	76.4
MS7	Ta	UC	Low	-	Intermed	-	BCG	-	97	57.2	54.5	48.6	50.5
MS8	Ta	UC	Low	-	Intermed	-	BCG	+	95	71.5	67.1	70.6	78.3
MS12	T1	UC	High	+	High	-	-	+	95	71.9	83.6	80.3	72.4
MS13	T1	UC	High	-	High	-	BCG	-	95	42	35.6	40.4	33.4
MS14	T1	UC	High	-	High	-	BCG	-	99	86.7	99.4	100	90.3
MS15	Ta	UC	Low	-	Intermed	-	-	-	99	76.5	89.7	75.1	75.2
MS16	T1	UC-Squ	High	-	High	-	MMC	-	96	48.5	48.7	47.3	48.5
MS18	T1	UC-Squ	High	+	High	-	BCG	+	100	68.8	75.2	61.7	100
MS20	Ta	UC	Low	-	Intermed	MMC	-	+	95	71.3	80.4	79.4	88.2
MS21	T1	UC	Low	-	High	-	BCG	-	99	81.5	82.9	75.3	80.6
MS22	T1	UC	High	+	High	MMC	BCG	+	97	80.3	74.1	79	91.2
MS24	T1	UC	High	+	High	MMC	-	+	98	70.2	64.8	55.9	54
MS25	T1	UC	High	-	High	-	BCG	+	99	76.3	96.7	83.5	84.2
MS27	Ta	UC	Low	-	Intermed	MMC	-	+	98	71.4	59.4	55.1	67
MS28	Ta	UC	Low	-	Intermed	-	BCG	-	98	82.4	77.8	77.1	75.7
MS30	Ta	UC	Low	-	Intermed	MMC	-	-	98	77.3	79.7	66.6	49

PN: Patient number, P stage: Pathologic stage, UC: Urothelial carcinoma, Squ: squamous differentiation, Src: sarcomatoid differentiation, CIS: Carcinoma in situ, ISI: Immediate single instilation of chemotherapy, IV: Intravesical, Rec: Recurrence, MMC: Mitomycin C, BCG: Bacillus Calmette Guerin, Niv: Nivolumab, Ipili: Ipilimumab

Table 3. Viability rates and comparative results of groups after the drug administration

	n	*Viability rates (%)*	*Median rates of the viability decrease (%)*	*p**
Control	20	97.8±1.8 (95-100)	-	-
MMC	20	72.1±12.9 (41.9-100)	23.6	<0.001
BCG	20	70.8±16.4 (35.6-99.4)	25.9	<0.001
Nivolumab	20	69.1±14.9 (40.4-100)	23.8	<0.001
Ipilimumab	20	71.7±17.6 (33.4-100)	22.7	<0.001

MMC: Mitomycin C, BCG: Bacillus Calmette Guerin.
*Analysis of viability rates in other groups compared to the control group using the Wilcoxon test.

Looking at the viability rates after the applications according to the T stage and tumor grades, it was observed that the viability rates were similar between Ta and T1 stage groups and also low-grade and high-grade groups.

In addition, only tumoral PD-1 positivity of all IHC results were found to be significantly higher in high-risk cases (61.5%), T1 stage cases (61.5%) and high-grade cases (66.7%) compared to intermediate-risk cases (14.3%), Ta stage cases (14.3%)

Table 4. Mean viability rates of bladder cancer cell cultures after drug applications according to immunohistochemical (IHC) findings of immune checkpoints in all cases

Immune checkpoints	*IHC staining*	*n=20*	*Viability rates of drug applications*				
			Control	*MMC*	*BCG*	*Nivolumab*	*Ipilimumab*
Tumoral CTLA-4	Negative	18	97.8±1.8	71.3±11.2	72.1±16.7	70.1±14.9	72.9±17.8
	Positive	2	98±1.4	78.6±30.3	59.7±7.3	60.1±16.3	61.6±15.8
Peritumoral CTLA-4	Negative	9	97.8±1.4	75.4±13.6	72.8±14.6	68±12.6	68.9±15.2
	Positive	11	97.8±2.1	69.3±12.2	69.2±18.2	70.1±17.1	74.1±19.8
Tumoral PD-1	Negative	11	97.2±1.7	68±13.2	68.9±18.6	64.4±15.2	66.9±17.6
	Positive	9	98.6±1.7	77±11.2	73.3±13.9	74.9±13	77.7±16.6
Peritumoral PD-1	Negative	7	98.1±1.5	79.6±10.9	78.4±16	74.4±15.7	76.7±13
	Positive	13	97.6±1.9	68±12.3	66.8±15.7	66.3±14.2	69±19.6
PD-L1	Negative	2	97.5±0.7	81.3±1.5	75.9±2.6	78.1±1.3	83.5±10.9
	Positive	18	97.8±1.9	71±13.2	70.3±17.2	68.1±15.4	70.4±17.9
Tumoral PD-L2	Negative	19	97.9±1.7	72.1±13.2	70.2±16.5	68.5±15	71.7±18.1
	Positive	1	95	71.9	83.6	80.3	72.4
Peritumoral PD-L2	Negative	16	98.2±1.4	74.7±11.6	73.6±14.7	71±14.2	74.5±16.5
	Positive	4	96.3±2.5	61.7±14	60±20.3	61.8±17.5	60.7±20

CTLA-4: Cytotoxic T-lymphocyte Associated Antigen-4, PD-1: Programmed Death-1, PD-L1: Programmed Death-Ligand 1, PD-L2: Programmed Death-Ligand 2, IHC: Immunohistochemical, MMC: Mitomycin C, BCG: Bacillus Calmette Guerin. Mann-Whitney U test was performed for the comparison of viability rates of the same drug between negative and positive IHC results that showed no statistical significance between IHC groups.

Table 5. Good response rates of bladder cancer cell cultures after drug applications according to immunohistochemical (IHC) findings of immune checkpoints in all cases. n (%)

Immune checkpoints	*IHC staining*	*n=20*	*Good response rates of the drug applications*			
			MMC	*BCG*	*Nivolumab*	*Ipilimumab*
Tumoral CTLA-4	Negative	18	11 (61.1)	9 (50)	10 (55.6)	7 (38.9)
	Positive	2	1 (50)	2 (100)	2 (100)	2 (100)
Peritumoral CTLA-4	Negative	9	4 (44.4)	4 (44.4)	6 (66.7)	5 (55.6)
	Positive	11	8 (72.7)	7 (63.6)	6 (54.5)	4 (36.4)
Tumoral PD-1	Negative	11	7 (63.6)	6 (54.5)	8 (72.7)	6 (54.5)
	Positive	9	5 (55.6)	5 (55.6)	4 (44.4)	3 (33.3)
Peritumoral PD-1	Negative	7	3 (42.9)	3 (42.9)	4 (57.1)	3 (42.9)
	Positive	13	9 (69.2)	8 (61.5)	8 (61.5)	6 (46.2)
PD-L1	Negative	2	0 (0) *	0 (0)	0 (0) *	0 (0)
	Positive	18	12 (66) *	11 (61.1)	12 (66) *	9 (50)
Tumoral PD-L2	Negative	19	11 (57.9)	11 (57.9)	12 (63.2)	9 (47.4)
	Positive	1	1 (100)	0 (0)	0 (0)	0 (0)
Peritumoral PD-L2	Negative	16	8 (50)	8 (50)	9 (56.3)	7 (43.8)
	Positive	4	4 (100)	3 (75)	3 (75)	2 (50)

Chi-square test was performed for the comparison of good response rates of the same drug between negative and positive IHC results.
*Significant statistical analysis with p=0.045 between PD-L1 positive and negative cases of each MMC and nivolumab group.
CTLA-4: Cytotoxic T-lymphocyte Associated Antigen-4, PD-1: Programmed Death-1, PD-L1: Programmed Death-Ligand 1, PD-L2: Programmed Death-Ligand 2, IHC: Immunohistochemical, MMC: Mitomycin C, BCG: Bacillus Calmette Guerin

and low-grade cases (12.5%), separately (p values were 0.043, 0.043 and 0.017, respectively).

During follow-up, recurrence was observed in 6 of 8 untreated patients and no statistically significant difference was found for IHC results between patients with and without recurrence. However, peritumoral PD-1 was positive for 2 of 2 (100%) patients without any recurrence, while peritumoral PD-1 positivity was observed only in 2 of 6 (33.3%) patients with recurrence (p=0.102). When we look at the drug response rates of the cases with and without any recurrence, no good response of MMC was observed in 2 of 2 patients with recurrence, while good response for MMC was observed in 5 of 6 patients with recurrence (p=0.035). On the other hand, recurrence was found in 5 of 11 patients treated with BCG in follow-up and there was no statistical difference for IHC results between patients with and without recurrence. When we look at the drug response rates, there was a good response in 2 of 5 recurred patients after the applications of BCG and nivolumab, while good response was not observed in all of them after ipilimumab application (p>0.05).

In addition, no good response rates after immunotherapeutic agents were found to be statistically significant across all studied markers. However, good response rate after MMC was only found to be higher in high-risk cases with peritumoral PD-1 positive than negatives (87.5 vs 20%, p=0.032).

Discussion

All immunotherapeutic and chemotherapeutic agents caused lower viability rates than their own controls in primary BC cells. As expected, some non-recurred cases (MS4 and MS13) during the follow-up were presented with lower viability rates than the majority of recurred (MS3, MS18, MS22 and MS25) cases in cell cultures after the BCG application. However, paradoxically some non-recurred cases (MS14 and MS21) had higher viability rates compared to some recurred cases (MS3, MS18 and MS22) after the BCG administration. On the other hand, MMC was more cytotoxic than other immunotherapeutic agents in some case cultures (MS14). These findings have shown that patients with similar clinicopathologic features can have different outcomes within themselves.

In the management of NMIBC, some intravesical treatment modalities are recommended by the guidelines because of the nature of high recurrence and progression rates of the disease. However, the treatment of NMIBC remains a challenge for the patients and clinicians. Currently, an ISI of chemotherapy with MMC or epirubicin is routinely used in daily practice of low-risk and intermediate-risk patients with low recurrence rate and score after TUR-BT for decreasing recurrence [2,14-16]. In addition, intravesical chemotherapy of MMC and immunotherapy of BCG are recommended for patients with intermediate-risk and high-risk NMIBC, respectively [2,5]. However, especially in patients with BCG-refractory NMIBC, management of the disease has not been clear yet. In this context, intravesical gemcitabine, MMC with microwave-induced hyperthermia, recombinant adenovirus IFN-α with Syn3 (rAd-IFNα/Syn3 (Adstiladrin), docetaxel combinations, ALT-803 (interleukin-15 super-agonist) and immune checkpoint inhibitors constitute possible treatment choices [2,3,11,17-20]. When we look at the current NMIBC management picture, we aimed to assess BCG and MMC from the standard treatments and nivolumab and ipilimumab from the immune checkpoint inhibitors in the current study.

For BC cell culture, BC cell lines which were previously defined or primary cell culture obtained from the patients' cancer tissue / urinary exfoliated BC cells are used for *ex-vivo* researches [13,21-25]. In the current study, we investigated drug response rates using the oncogram method that was previously defined by us [13].

In previous studies, it was reported that the expression of the immune checkpoint receptors or ligands (CTLA-4, PD-1 and PD-L1) has been observed in BC tumor cells and tumor infiltrating immune cells in a majority of localized BCs [3]. However, with using different methods or antibody clones for IHC evaluation reveals various results in statistical analysis [3]. PD-L1 positivity defined with the range of 7-69% in the literature and CTLA-4 positivity have been found to be associated with advanced disease and poor survival. PD-1 positivity (8-53%) has been associated with T stage [3,11]. PD-L2 is mainly expressed in antigen presenting cells [11]. Therefore, we assessed IHCs of CTLA-4, PD-1, PD-L1 and PD-L2 in terms of tumoral and peritumoral areas in TUR-BT pathologies. Immune checkpoint inhibitors that block CTLA-4 (ipilimumab) or PD-1 (pembrolizumab, nivolumab and durvalumab) or PD-L1 (atezolizumab and avelumab) pathways promote antitumor immunity, reactivating T-lymphocyte proliferation and activity [26,27]. PD-L2 is another targetable ligand of PD-1, but its prognostic significance remains controversial [27]. These five immunotherapeutic agents are currently in

use in second-line therapy for patients who progressed during or after cisplatin-based chemotherapy and had PD-L1 positive in terms of eligibility for immunotherapy. In addition, using of immune checkpoint inhibitors is also on the agenda in first-line therapy for metastatic urothelial carcinoma, in neoadjuvant therapy prior to radical cystectomy for MIBC and in BCG refractory NMIBC [28]. Among the immune checkpoint inhibitors, particularly pembrolizumab has been approved by FDA for the treatment of patients with BCG-unresponsive, high-risk NMIBC with CIS who are ineligible for cystectomy [29]. In KEYNOTE-676 study, a randomized-controlled Phase III study of BCG with or without pembrolizumab in patients with persistent or recurrent high-risk NMIBC after BCG induction was planned [30]. In the combination arm, intravenous 200mg pembrolizumab every 3 weeks and intravesical 50mg BCG induction therapy once weekly for 6 weeks have been administered. It was reported by the authors that the combination therapy may indicate a more effective option for high risk patients [30]. On the other hand, the efficiency of nivolumab and ıpilimumab in different conditions according to the IHC results for patients with treatment naive NMIBC is not clear yet. At this point, it can be thought that personalized treatment can be included in the management of NMIBC with the using of additional IHC or cell culture (oncogram) results. In parallel with the stated point, we plan our hypothesis to answer the question of which drug for which patient by the using of IHC and *ex-vivo* cell culture (oncogram) testing.

When we look at the IHC findings of the current study, tumoral PD-1 positivity was found to be significantly associated with worse pathological results (T1, high grade or high-risk patients). Good response rates of PD-L1 positive cases were found to be significantly higher compared to PD-L1 negative cases after the MMC and nivolumab administrations (66 vs 0% in both drug groups; p=0.045). Besides, cell viability rates of tumoral CTLA-4 positive cases were detected to be lower than negative patients (non-significantly) after all immunotherapeutic (BCG, Nivolumab and Ipilimumab) applications. Also, good response rate after MMC was only found to be higher especially in the sub-group of high-risk NMIBC with peritumoral PD-1 positive than negatives (87.5 vs 20%, p=0.032). When we look at the follow-up data, good response rate for MMC in cell cultures was detected in 5 of 6 untreated patients who had a recurrence in follow-up (p=0.035). This finding shows that these patients can gain a benefit from the MMC treatment after TUR-BT. On the other hand, good response rate was not observed for all drug administrations in a majority of recurred cases after the BCG treatment. In parallel, BCG failure in cell cultures can be similarly associated with failure of other immunotherapeutic agents in BCG unresponsive cases. Thus, it can be required to select for these patients according to the IHC and drug response rates in decision making before the treatments of nivolumab and ipilimumab after the BCG failure.

The major limitation of this study is that pembrolizumab and atezolizumab were not investigated because of drug accessing problems. We also did not study the nivolumab and ipilimumab combination due to the limited access to drugs. However, this is the first study to evaluate immune checkpoint inhibitors in NMIBC *in vitro* and an oncogram study that includes *ex-vivo* drug responses and additional IHC results and to assess the applicability of the method for personalized treatment of NMIBC in the routine daily practice. Additionally, our large and comprehensive oncogram study is also going on with more drugs and next generation sequence analysis.

In conclusion, although the drug responses, oncogram results obtained from the *ex-vivo* applications of MMC, BCG, nivolumab and ipilimumab, were found to significantly differ compared to the own controls, they differ on the basis of patients. It was found that nivolumab response rate was better, especially in PD-L1 positive patients. Accordingly, it can be said that the drug responses observed after *ex-vivo* applications can be evaluated with the clinical characteristics, histopathologic and immunohistochemical examination results of the patients, and can provide important information in the selection of the most appropriate immunotherapeutic drug (in the decision of personalized treatment) after evaluating drug alternatives before intravesical treatment in patients with intermediate-risk and high-risk NMIBC.

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Conflict of interests

The authors declare no conflict of interests.

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