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Efficacy of Urinary Mast Cell Mediators in Patients with Primary High-Grade Non-Muscle Invasive Bladder Cancer Treated with BCG Immunotherapy

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Abstract

Background: Mast cells play a critical role in tumor-associated immune pathways. We aimed to prospectively investigate the urinary mast cell mediators in patients with non-muscle invasive bladder cancer (NMIBC) treated with Bacillus Calmette-Guérin (BCG) immunotherapy. Methods: Nineteen patients who have received immunotherapy due to NMIBC and 19 healthy participants were enrolled. Urine samples were collected to assay N-methylhistamine, histamine and tryptase levels immediately before the first BCG instillation, immediately after the third and sixth instillations, and four weeks after the sixth instillation in patients with NMIBC and at a single visit in healthy participants. Cystoscopic examinations were performed on the patient with NMIBC at three-month intervals for two years. The changes in urinary markers due to BCC response, BCG instillation, and the presence of NMIBC were assessed. Results: The average age was 56.1 ± 10.5 years in patients with NMIBC. Fourteen patients had high-grade Ta tumors, and 5 had high-grade T1 tumors. While 12 patients responded, 6 presented with recurrence and 1 with progression. There was no correlation between the levels of mast cell mediators and BCG response. The N-methylhistamine and histamine levels were increased significantly with the onset of immunotherapy and N-methylhistamine levels were decreased significantly when immunotherapy was terminated. Pre-BCG estimated marginal means of N-methylhistamine were significantly higher in patients with NMIBC than healthy participants. Conclusions: This is the first study to determine that urine histamine and N-methylhistamine levels showing the change with immunotherapy. However, these mediators were not found to predict the patients' response to immunotherapy.

- Study Design and Patient Selection

The present study protocol was reviewed and approved by the Institutional Review Board of the Istanbul University-Cerrahpasa School of Medicine (approval number: 21263603-806.01.03-400654). Informed consent was obtained by all subjects when they were enrolled. This study was conducted with the financial support of the Istanbul University-Cerrahpasa Board of Scientific Research Projects (Number: 30651). The inclusion and exclusion criteria of the study are given below.

Inclusion criteria:

- Patients diagnosed with high-grade NMIBC,

- Patients completing TUR and re-TUR treatment,

- Patients with no contraindication for BCG,

- Demographic data and medical records being available.

Exclusion criteria:

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- Patients with metastatic involvement of any region,

- Patients with any other malignancy.

Nineteen patients who were planned to be treated with BCG for high-grade NMIBC between February 2016 and November 2017 and 19 healthy participants for comparison were enrolled in the study. Healthy participants were selected randomly, and urinary ultrasonography and urinary analyses were performed to exclude bladder malignancies.

- Treatment Protocol

The patients with NMIBC received weekly intravesical BCG for six weeks, at least two weeks after the last TUR session. In each immunotherapy, 40 mg/ml of SII- Onco BCG (Serum Institute of India, Pune, India) was dissolved with 40 cc of isotonic NaCl and administered through a 12-Fr urethral catheter. This solution was retained in the bladder for 2 hours, after which urine collection was performed.

In the process of obtaining urine samples from each patient, the first visit was organized immediately before the first BCG instillation, the second visit immediately after the third instillation, the third visit immediately after the sixth instillation, and the last visit four weeks after the sixth instillation. In healthy participants, urine samples collection was performed at a single visit after the exclusion of bladder cancer and urinary tract infection using ultrasonography and urinalysis.

- Laboratory analysis

The samples were immediately centrifuged at 5,000 rpm and 4 °C for 10 min to remove cells and debris, and stored in aliquots at –70 °C until analysis. The urinary N-methylhistamine, histamine and tryptase levels*were* measured using *test kits* by the sandwich enzyme-linked immunosorbent assay (Genzyme Corporation, Cambridge, MA, USA). The specific biotinylated detection antibody and avidin horseradish peroxidase conjugate were added to the wells and incubated. After removing the non-washing parts, the blue color was created in the boxes by adding a substrate solution. By adding the stop solution, the reaction was stopped, and the blue color was observed to turn yellow. Optical density (OD) was determined spectrophotometrically at a wavelength of 450 $\pm$ 2 nm. Since the OD value was proportional to mast cell mediator levels in the samples, the N-methylhistamine, histamine, and tryptase levels concentrations were calculated using standard curve graphics.

- Follow-up

All patients with NMIBC were planned to undergo two-year maintenance BCG therapy in accordance with the Southwest Oncology Group Study (SWOG) protocol [10]. The patents were examined by cystoscopy and cytology at three-month intervals for two years [11]. They underwent CT urography imaging every six months [11]. Recurrence was defined as histopathologically confirmed detection of any tumor after BCG induction therapy. Progression was defined as an increase in the stage of the muscle-invasive disease. After two years of follow-up, the patients were classified as non-responders to immunotherapy in case of recurrence or progression and responders to immunotherapy if there was no recurrence or progression.

- Statistical Analysis

Statistical analysis was performed using the SPSS ver. 22.0 (IBM Corporation, NY, USA). The prognostic value of each mast cell mediators and clinical parameters were assessed by chi-square test. The differences in the urinary mast cell mediators levels between the patients with NMIBC and healthy participants were examined using the Mann–Whitney U test. The relationship between the urinary mast cell mediator levels and the patients' response to immunotherapy was also evaluated using the Mann–Whitney U test. The serial changes in the urinary mast cell mediators measured at four different visits were analyzed by the Wilcoxon signed rank and Friedman tests. A p-value of less than 0.05 was accepted as statistically significant.

Results

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The average age at the time of immunotherapy was 56.1 (37-79) years in patients diagnosed with NMIBC. There were 13 men and 6 women. Fourteen patients were diagnosed with Ta high-grade and 5 with T1 high-grade bladder cancer. During the follow-up, while 18 patients completed two-year maintenance BCG therapy, one underwent radical cystectomy. The cystoscopic evaluations undertaken at three-month intervals revealed that 7 of 19 patients did not respond to immunotherapy (non-responders) and 12 patients responded well (responders). Among the non-responders, recurrence was observed in six patients and progression in one patient. The average time to recurrence or progression was 9.4 (3-18) months. The mean age was 52.2 $\pm$ 10.5 years in responders and 61.4 $\pm$ 10.6 years in non-responders ($p = 0.098$). The remaining baseline clinicopathological findings are shown in **Table 1** .

The serial changes in estimated marginal means of urinary N-methylhistamine, histamine, and tryptase levels in responders and non-responders are shown in **Figure 1** . There was no statistically significant difference between the immunotherapy responders and non-responders in terms of the urinary N-methylhistamine, histamine and tryptase levels ($p > 0.05$). However, a statistically significant increase was observed in the estimated marginal means of urinary N-methylhistamine ($p = 0.027$) and histamine ($p = 0.004$) levels measured at the second visit compared to the first visit in patients treated with BCG (**Table 2**). Although there were no statistically significant differences between the second and third visits ($p = 0.053$ and $p = 0.26$), a statistically significant decrease was detected in the estimated marginal means of N-methylhistamine levels measured at the last visit compared to the previous visit in patients treated with BCG ($p = 0.013$). Concerning the urinary tryptase levels, no statistically significant differences were obtained in terms of immunotherapy response and changes of urinary tryptase levels at different visits in patients treated with BCG ($p > 0.05$).

The estimated marginal means of urinary N-methylhistamine, histamine, and tryptase levels measured at the first visit before immunotherapy in patients with NMIBC and measured at a single visit in healthy participants are given in **Table 2** . There were no statistically significant differences in the estimated marginal means of urinary histamine ($p = 0.307$) and tryptase ($p = 0.816$) levels between the patients diagnosed with NMIBC and healthy participants. However, the estimated marginal means of urinary N-methylhistamine levels were measured to be significantly higher in patients with NMIBC ($p = 0.005$).

The mast cell response to initial intravesical BCG immunotherapy between BCG responders and non-responders is also evaluated. The increase in the estimated marginal means of urinary N-methylhistamine was 27.24 nmol/ml in responders and 41.51 nmol/ml in non-responders ($p = 340$). The increase in the estimated marginal means of urinary histamine was 19.03 nmol/ml in responders and 27.64 nmol/ml in non-responders ($p = 801$). The increase in the estimated marginal means of urinary tryptase was 5.21 nmol/ml in responders and the increase in the estimated marginal means of urinary tryptase was 7.51 nmol/ml in non-responders ($p = 108$)

(Figure 1).

Discussion

In the present study, we observed that urinary N-methylhistamine and histamine levels were increased significantly with the onset of immunotherapy and N-methylhistamine levels were decreased significantly when immunotherapy was terminated. Although we did not find statistically significant differences between the responders and non-responders, the estimated marginal means of Pre-BCG N-methylhistamine were significantly higher in patients with NMIBC than healthy participants.

A few studies have shown valuable changes in the BCG-induced urinary immune microenvironment. In this field, IL-17+ mast cell, interleukins, TNF-α, IFN-γ, and soluble ICAM-1 levels have been examined [7, 12, 13]. But there is no available data in the literature that can clearly determine mast cell activation in patients with NMIBC treated with BCG. In our study, we determined that the urinary N-methylhistamine and histamine levels increased with BCG immunotherapy and N-methylhistamine decreased with the termination of this BCG immunotherapy. Tryptase is considered to be an unstable mast cell mediator, and therefore there was no statistically significant change in the tryptase levels due to BCG immunotherapy.

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Clinically applicable tools to predict disease recurrence and progression are much needed. Studies on predicting immunotherapy response started with measuring purified protein derivative (PPD)-associated BCG response. In a study, the median recurrence-free survival was 25 months in the PPD-negative group and was not available in the PPD positive group ($p < 0.05$) [14]. But there was only one study about the mast cell-related immunotherapy response. [7]. This study has confirmed the predictive value of IL-17+ mast cells in patients with NMIBC treated with BCG immunotherapy, and higher numbers of IL-17+ cells have found associated with improved event-free survival. However, there is no available data in the literature to determine urinary N-methylhistamine, histamine, and tryptase levels in patients with NMIBC. In our study, the lack of a statistically significant difference between the mast cell mediators and immunotherapy response can be explained with the small size of the patient group. The quantitative differences regarding the samples suggest that statistically significant differences could be found in further studies designed with larger patient groups.

There are a few studies on urinary markers in identifying NMIBC patients. Although some studies have reported promising results in determining bladder cancer with urinary immune markers, there were no mast cell-related markers in the literature [15, 16]. In our study, increased urinary N-methylhistamine levels were found in patients with NMIBC compared to healthy participants. These results can be discussed in several aspects. The evaluation of the samples obtained at the first visit before BCG instillation in patients with NMIBC excluded the BCG-related immunological response. However, the samples were taken after the re-TUR procedure, suggesting that a resection-induced mast cell activation may have been effective. Therefore, it can be considered that resection-associated mast cell activation alone can be effective against tumor cells.

A few studies suggest that a decrease in immune system function in elderly patients may weaken the BCG response. Kanematsu et al. were the first to report significantly reduced protection from tumor recurrence and reduced tuberculin skin test reactivity in patients aged >80 years treated with BCG [17]. A phase 2 study revealed that patients older than 80 years had the poorest recurrence-free survival, and thus being over 80 was an independent predictor of recurrence (hazard ratio: 1.56) [18]. Furthermore, age also found to be an independent predictor of progression by the Club Urologico Espanol de Tratamiento Oncologico (CUETO) group [19]. Although in our study, the mean age was reported higher in immunotherapy non-responders, these differences were not found statistically significant due to the low number of patients ($p = 0.098$).

Despite the promising results, our study has certain limitations. First, it was a single-center study with a relatively low number of cases. Second, it is considered that increasing the number of visits could provide more detailed information about the changes in the mast cell mediators. Third, although all patients with NMIBC were high grade, excluding other clinical and pathological conditions that could affect immunotherapy response can be considered as another limitation. Future studies with larger sample size and longer follow-up are needed to predict BCG response at the beginning of the treatment.

Conclusions

This is the first study to determine that urinary N-methylhistamine and histamine levels were increased significantly with receiving immunotherapy and N-methylhistamine levels were decreased significantly when immunotherapy was terminated. Although there are no statistically significant differences between the immunotherapy responders and non-responders, the estimated marginal means of Pre-BCG N-methylhistamine levels are significantly higher in patients with NMIBC than healthy participants. These results are promising for further studies to be conducted on mast cell activation.

Acknowledgments:

Conflict of Interest : The authors declare that they have no conflict of interest.

Funding Source : None.

Ethical approval: This study was approved by the institutional review board of the Medical Faculty of Istanbul University-Cerrahpasa.

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Informed consent: Informed consent was obtained from all individual participants included in the study.

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Table 1 – Patient characteristics: **(a)** baseline clinical outcomes; **(b)** tumor characteristics.	**Table 1** – Patient characteristics: **(a)** baseline clinical outcomes; **(b)** tumor characteristics.	**Table 1** – Patient characteristics: **(a)** baseline clinical outcomes; **(b)** tumor characteristics.	**Table 1** – Patient characteristics: **(a)** baseline clinical outcomes; **(b)** tumor characteristics.	**Table 1** – Patient characteristics: **(a)** baseline clinical outcomes; **(b)** tumor characteristics.	**Table 1** – Patient characteristics: **(a)** baseline clinical outcomes; **(b)** tumor characteristics.	**Table 1** – Patient characteristics: **(a)** baseline clinical outcomes; **(b)** tumor characteristics.	**Table 1** – Patient characteristics: **(a)** baseline clinical outcomes; **(b)** tumor characteristics.	**Table 1** – Patient characteristics: **(a)** baseline clinical outcomes; **(b)** tumor characte
(a)	**(a)**	**(a)** **Total (n = 19)**	**(a)**	**(a)** **Responders (n = 12)**	**(a)**	**(a)** **Non-responders (n = 7)**	**(a)**	**(a)** P value
Age *(years) (mean ± SD)*		56.1 ± 10.5		52.2 ± 10.5		61.42 ± 10.6		0.098
Gender, *n* Female Male		6 13		4 8		2 5		0.622
Complaint Hematuria LUTS Flank pain		10 5 4		6 2 4		4 3 0		0.171
Smoking, *n* Yes No		14 5		8 4		6 1		0.603
Occupational risk, *n* Yes No		4 15		2 10		2 9		0.475
Familial cancer history, *n* Yes No		7 12		4 8		3 4		0.526
(b)	**(b)**	**(b)**	**(b)**	**(b)**	**(b)**	**(b)**	**(b)**	**(b)**
Tumor stage T1 Ta Cis		5 14 0		11 1 0		4 3 0		0.058
Tumor size <3 cm [?]3 cm		10 9		7 5		3 4		0.650

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Number of tumor Single Multiple		12 7		7 3		5 4		0.603
Tumor morphology Papillary Non-papillary		16 3		13 1		3 2		0.359
Additional treatment, n Maintenance BCG Radical cystectomy		18 1		14 0		4 1		0.249
SD = standard deviation; LUTS = lower urinary tract symptoms; BCG = Bacillus Calmette-Guérin	SD = standard deviation; LUTS = lower urinary tract symptoms; BCG = Bacillus Calmette-Guérin	SD = standard deviation; LUTS = lower urinary tract symptoms; BCG = Bacillus Calmette-Guérin	SD = standard deviation; LUTS = lower urinary tract symptoms; BCG = Bacillus Calmette-Guérin	SD = standard deviation; LUTS = lower urinary tract symptoms; BCG = Bacillus Calmette-Guérin	SD = standard deviation; LUTS = lower urinary tract symptoms; BCG = Bacillus Calmette-Guérin	SD = standard deviation; LUTS = lower urinary tract symptoms; BCG = Bacillus Calmette-Guérin	SD = standard deviation; LUTS = lower urinary tract symptoms; BCG = Bacillus Calmette-Guérin	SD = standard deviation; LUTS = lower urinary tract symptoms; BCG = Bacillus Calmett Guérin

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Table 2. Comparison of estimated marginal means of urinary mast cell activation markers between the different visits in patients treated with a six-week course of Bacillus Calmette-Guérin therapy and between the patients diagnosed with NMIBC (Vi-	**Table 2.** Comparison of estimated marginal means of urinary mast cell activation markers between the different visits in patients treated with a six-week course of Bacillus Calmette-Guérin therapy and between the patients diagnosed with NMIBC (Vi-	**Table 2.** Comparison of estimated marginal means of urinary mast cell activation markers between the different visits in patients treated with a six-week course of Bacillus Calmette-Guérin therapy and between the patients diagnosed with NMIBC (Vi-	**Table 2.** Comparison of estimated marginal means of urinary mast cell activation markers between the different visits in patients treated with a six-week course of Bacillus Calmette-Guérin therapy and between the patients diagnosed with NMIBC (Vi-	**Table 2.** Comparison of estimated marginal means of urinary mast cell activation markers between the different visits in patients treated with a six-week course of Bacillus Calmette-Guérin therapy and between the patients diagnosed with NMIBC (Vi-	**Table 2.** Comparison of estimated marginal means of urinary mast cell activation markers between the different visits in patients treated with a six-week course of Bacillus Calmette-Guérin therapy and between the patients diagnosed with NMIBC (Vi-	**Table 2.** Comparison of estimated marginal means of urinary mast cell activation markers between the different visits in patients treated with a six-week course of Bacillus Calmette-Guérin therapy and between the patients diagnosed with NMIBC (Vi-	**Table 2.** Comparison of estimated marginal means of urinary mast cell activation markers between the different visits in patients treated with a six-week course of Bacillus Calmette-Guérin therapy and between the patients diagnosed with NMIBC (Vi-	**Table 2.** Comparison of estimated marginal means of urinary mast cell activation markers between the different visits in patients treated with a six-week course of Bacillus Calmette-Guérin therapy and between the patients diagnosed with NMIBC (Vi-	**Table 2.** Comparison of estimated marginal means of urinary mast cell activation markers between the different visits in patients treated with a six-week course of Bacillus Calmette-Guérin therapy and between the patients diagnosed with NMIBC (Vi-	**Table 2.** Comparison of estimated marginal means of urinary mast cell activation markers between the different visits in patients treated with a six-week course of Bacillus Calmette-Guérin therapy and between the patients diagnosed with NMIBC (Vi-	**Table 2.** Comparison of estimated marginal means of urinary mast cell activation markers between the different visits in patients treated with a six-week course of Bacillus Calmette-Guérin therapy and between the patients diagnosed with NMIBC (Vi-	**Table 2.** Comparison of estimated marginal means of urinary mast cell activation markers between the different visits in patients treated with a six-week course of Bacillus Calmette-Guérin therapy and between the patients diagnosed with NMIBC (Vi-	**Table 2.** Comparison of estimated marginal means of urinary mast cell activation markers between the different visits in patients treated with a six-week course of Bacillus Calmette-Guérin therapy and between the patients diagnosed with NMIBC (Vi-	**Table 2.** Comparison of estimated marginal means of urinary mast cell activation markers between the different visits in patients treated with a six-week course of Bacillus Calmette-Guérin therapy and between the patients diagnosed with NMIBC (Vi-	**Table 2.** Comparison of estimated marginal means of urinary mast cell activation markers between the different visits in patients treated with a six-week course of Bacillus Calmette-Guérin therapy and between the patients diagnosed with NMIBC (Vi-	**Table 2.** Comparison of estimated marginal means of urinary mast cell activation markers between the different visits in patients treated with a six-week course of Bacillus Calmette-Guérin therapy and between the patients diagnosed with NMIBC (Vi-	**Tab 2.** Compari- son of es- ti- mate marg mean of urinary mast cell activation mark ers between the dif- fer- ent vis- its in pa- tient treat with a six- week c Baci lus Cal Gué therapy and between the pa- ti- ents diagnosed with NMI (Vi-

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Figure Legend

Figure 1. Serial changes in the estimated marginal means of urinary N-methylhistamine, histamine and tryptase levels in patients treated with a six-week course of bacillus Calmette-Guérin therapy.

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