Does the Urinary Mast Cell Mediators Predict the Immune Response to BCG in Patients with Primary High-Grade Non-Muscle Invasive Bladder Cancer?

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Abstract

Background: Mast cells play a critical role in tumor-associated immune pathways. We aimed to determine whether the urinary mast cell mediators predict the immune response 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 $\pm$ 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 significantly decreased when immunotherapy was terminated. Pre-BCG estimated marginal means of N-methylhistamine were significantly higher in patients with NMIBC than healthy participants. Conclusions: Our study is the first study to identify the changes in mast cell mediators with the onset of immunotherapy and with the presence of bladder cancer. However, these mediators were not found to predict the patients' response to immunotherapy.

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Keywords: BCG; bladder cancer; immunotherapy; mast cell; mediator

Running Title: Mast Cell Mediators in Bladder Cancer

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Abstract

Background: Mast cells play a critical role in tumor-associated immune pathways. We aimed to determine whether the urinary mast cell mediators predict the immune response 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 $\pm$ 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 significantly decreased when immunotherapy was terminated. Pre-BCG

estimated marginal means of N-methylhistamine were significantly higher in patients with NMIBC than healthy participants.

Conclusions: Our study is the first study to identify the changes in mast cell mediators with the onset of immunotherapy and with the presence of bladder cancer. However, these mediators were not found to predict the patients' response to immunotherapy.

Keywords: BCG; bladder cancer; immunotherapy; mast cell; mediator.

Introduction

Bladder cancer is the 10th most common malignancy worldwide [1]. Approximately 70-80 % of patients present with non-muscle invasive bladder cancer (NMIBC) limited to the mucosa and lamina propria, and the remaining 20-30 % of patients present with muscle-invasive or metastatic disease [2]. Intravesical Bacillus Calmette-Guérin (BCG) is the most effective therapy for NMIBC [3]. Optimal improvements in recurrence and progression rates can be observed with intravesical BCG immunotherapy applied after transurethral resection (TUR). However, the rate of non-responders is seen up to 80 % in patients treated with BCG immunotherapy during long follow-up periods [4].

The immune microenvironment of bladder tissue plays a critical role in anti-tumor mechanisms. It is showed that different inflammatory cells infiltrate oncogenic tissue, including mast cells, neutrophils, macrophages, and lymphocytes, as a marker of the cancer-induced immune response [5]. Different mechanisms have been evaluated about interactions between bladder cancer, BCG immunotherapy, and immune response. After intravesical BCG immunotherapy, the antigen 85 complex adheres to the bladder wall through fibronectin and initiates an acute immune response in the bladder wall, but many questions remain unclear [6].

Clinical trials based on BCG induced acute immune response have examined T cells, cytokines, and macrophages. In these trials, bladder mast cell activity is thought to play a critical role in tumor pathways and angiogenesis [7]. The bladder mast cell contains many granules, each of which can secrete many immunoactive molecules. Of these mediators, elevated expression of IL 17, N-methylhistamine, histamine, and tryptase may accept as a marker of mast cell activation [7-9]. Although one study has confirmed the predictive value of IL-17+ mast cells in patients with NMIBC treated with BCG immunotherapy [7], there is no study in the literature investigating urinary N-methylhistamine, histamine, and tryptase levels. It is considered that the determination of the mast cell activation can contribute to the increase of our clinical approach to BCG immunotherapy. Furthermore, the determination of mast cell activation in patients with NMIBC who receive immunotherapy will benefit the development of mast cell-based new treatment approaches.

We aimed to investigate the changes in urinary mast cell mediators level in patients with NMIBC due to the instillation of BCG and the presence of NMIBC. In addition, we assessed the differences between responders and non-responders to immunotherapy in terms of urinary mast cell mediator levels.

Material and Methods

- 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 procedures,

- Patients with no contraindication for BCG,

- Demographic data and medical records being available.

Exclusion criteria:

- Patients with Ta low-grade bladder cancer,

- Patients with metastatic involvement of any region,

- Patients with any other malignancy.

- Patients on immunosuppressive drugs like chemotherapy, steroids, or HIV medications.

- Patients with a previous history of tuberculosis infection or BCG instillation.

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 excluding 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. The reaction was stopped by adding the stop solution, and the blue color was observed to turn yellow. Optical density (OD) was determined spectrophotometrically at a wavelength of 450 ± 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 according to 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 predictive 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, Friedman, and Post-Hoc tests. A p-value of less than 0.05 was accepted as statistically significant.

Results

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. There was no patient with carcinoma in situ. 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 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 changes ($p > 0.05$). 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 last visit compared to the third visit ($p = 0.013$). 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 significantly higher in patients with NMIBC than healthy participants ($p = 0.005$).

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 after initial BCG was 27.24 nmol/ml in responders and 41.51 nmol/ml in non-responders ($p = 0.340$). 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 ($p = 0.801$). The increase in the estimated marginal means of urinary tryptase was 5.21 nmol/ml in responders and 7.51 nmol/ml in non-responders ($p = 0.108$) **(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 significantly decreased 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 determine mast cell activation in patients

with NMIBC treated with BCG. Our study 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 an unstable mast cell mediator, and therefore there was no statistically significant change in the tryptase levels due to BCG immunotherapy.

Clinically useful 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 been 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 by the small size of the patient group. The samples' quantitative differences 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 was 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 was also 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 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 another limitation. Future studies with a larger sample size and longer follow-up are needed to predict BCG response at the beginning of the treatment.

Conclusions

According to our knowledge, 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 significantly decreased 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.

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Table 1 – Patient characteristics: **(a)** baseline clinical outcomes; **(b)** tumor characteristics.

(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		51.42 ± 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 5		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		5 14		1 11		4 3		0.508
Tumor size <3 cm [?]3 cm		10 9		7 5		3 4		0.650
Number of tumors Single Multiple		12 7		9 3		3 4		0.603
Tumor morphology Papillary Non-papillary		16 3		11 1		5 2		0.359

Additional treatment, *n* Maintenance BCG Radical cystectomy		18 1		12 0		6 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 Calmet Guérin

Table 2. Comparison of estimated marginal means of urinary mast ce

N-methylhistamine *(nmol/ml) (mean ± SD)*
Histamine *(ng/ml) (mean ± SD)*
Tryptase *(ng/ml) (mean ± SD)*
SD = standard deviation; IL= interleukin; TNF-α = tumor necrosis fa