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ЭКСПРЕССИЯ ГЕНОВ ДОМЕНОПОДОБНОГО РЕЦЕПТОРА 4 (NLR4) ДОМЕНА ОЛИГОМЕРИЗАЦИИ НУКЛЕОТИДОВ И УРОВЕНЬ ИНТЕРЛЕЙКИНА 1β (IL-1β) В ОБРАЗЦАХ МОЧИ ДО И ПОСЛЕ ВНУТРИПУЗЫРНОЙ ТЕРАПИИ БЦЖ ДЛЯ ЛЕЧЕНИЯ РАКА МОЧЕВОГО ПУЗЫРЯ

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Резюме. Рак мочевого пузыря (РМП) является седьмым по частоте злокачественным новообразованием у мужчин и одиннадцатым в мире. У 75% больных РМП выявляется рак, не инвазирующий мышечный слой (РНИМС). Иммунотерапия БЦЖ (бациллой Кальметта-Герена) остается стандартным методом интравезикальной терапии при РНИМС. Точный механизм профилактики рецидивов посредством БЦЖ остается неизвестным.

Целью данного исследования была оценка экспрессии гена NLR4 и уровней IL-1β в качестве возможных прогностических показателей рецидивов РНИМС и неудач БЦЖ-терапии и выявление различий в их уровнях между раком, инвазирующим мышечный слой (ИМС) и РНИМС, что может помочь в первичной дифференциальной диагностике.

Данное исследование проводили в группе из 30 больных РНИМС и 17 пациентов с ИМС. Образцы мочи брали до операции в стерильные сосуды. У пациентов с РНИМС брали еще 4 образца (как указано ниже). Определение экспрессии гена NLR4 проводили в дооперационном материале при ИМС и в 4 образцах при РНИМС, т.е. в дооперационном образце, в материале, взятом через 4 часа после 3-й инстилляции БЦЖ и в образцах, собранных при дальнейшем наблюдении (3 и 6 мес. после операции).

Отмечено статистически значимое повышение уровней экспрессии гена NLRP4 при РНИМС (CT=0,87±1,48), по сравнению ИМС (CT=2,82±2,07). Насколько нам известно, не найдено публикаций относительно сравнительной экспрессии генов при РНИМС и ИМС. Экспрессия генов в предоперационных пробах мочи была более высокой в случаях рецидивов, чем при их отсутствии. Далее, уровень экспрессии повышался до 21-кратного (по сравнению с предоперационным) в образцах, взятых после введения 3-й дозы БЦЖ Этот показатель значительно снижался до 1-кратного повышения над уровнем до операции через 3 мес. наблюдения и лишь в 0,9 раз к 6 мес. Экспрессия гена до опера-

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ции в случаях отсутствия метастазов была намного ниже, нежели в случаях рецидивирования РМП. Отмечено 11-кратное повышение экспрессии гена после 3-й инстилляции БЦЖ и последующее снижение до 5,6-кратного в образцах, взятых через 3 мес. по сравнению с дооперационными образцами.

Уровни IL-1β определяли при РНИМС и ИМС в образцах мочи до операции и при терапии БЦЖ в случаях РНИМС до и через 4 часа после 3-й дозы БЦЖ и на протяжении 3-месячного наблюдения этих случаев для анализа их возможного применения в первичном дифференциальном диагнозе между РНИМС и ИМС, а также в качестве прогностического фактора возможного рецидивирования при РНИМС. В целом уровень IL-1β был выше в дооперационных образцах (0,62±0,12 пг/мл) по сравнению с уровнями перед 3-й дозой индукционной БЦЖ-терапии (0,53±0,13 пг/мл). Его уровень был существенно выше через 4 часа после назначения 3-й дозы БЦЖ (1,96±0,62 пг/мл), нежели предыдущие значения. Эти показатели снижались до предоперационных уровней к 3 мес. наблюдения (0,57±0,099 пг/мл). Уровни IL-1β в образцах, собранных через 4 часа после 3-й дозы БЦЖ, были повышенными в случаях последующего метастазирования, нежели в случаях отсутствия метастазов. Эти значения снижались в обоих случаях и становились выше в нерецидивирующих случаях (0,64±0,05 пг/мл) по сравнению со больными, у которых уже были диагностированы метастазы к 3 мес наблюдения (0,45±0,05 пг/мл).

В заключение, при отслеживании экспрессии гена NLRP4 и уровней IL-1β в ходе лечения БЦЖ в 30 случаях метастазирующего и неметастазирующего РНИМС отмечено достоверное статистическое различие обоих показателей в образцах, взятых после 3-й дозы БЦЖ, с повышением у пациентов с последующим развитием метастазов через 3 и 6 мес. Если эти предварительные результаты будут подтверждены в последующих больших когортных исследованиях, они станут перспективными для прогнозирования таких случаев с возможностью раннего планирования индивидуализированного лечения, избегая БЦЖ-терапии у пациентов, более подверженных рецидивам, от вероятных побочных эффектов лечения БЦЖ. Определение экспрессии NLRP4 и уровней IL-1β поможет в прогнозировании неудач БЦЖ-терапии, что сграет существенную роль для своевременного радикального хирургического вмещательства. При сравнении экспрессии NLRP4 и уровней IL-1β при РНИМС и ИМС были отмечены повышение значения в неинвазивных случаях. Этот результат может служить в качестве возможного диагностического подхода, что является существенной проблемой. Поэтому здесь необходимо установить граничные диагностические значения экспрессии генов и уровня цитокинов.

Ключевые слова: рак мочевого пузыря, БЦЖ, IL-1β, внутрипузырная терапия БЦЖ, Nod-подобные рецепторы, опухолевый иммунитет

NUCLEOTIDE OLIGOMERIZATION DOMAIN-LIKE RECEPTOR 4 (NLR4) GENE EXPRESSION AND INTERLEUKIN 1β (IL-1β) LEVEL IN URINE SAMPLES BEFORE AND AFTER INTRAVESICAL BCG THERAPY FOR TREATMENT OF BLADDER CANCER

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Abstract. Bladder cancer is the 7th most commonly diagnosed cancer in males worldwide and the 11th when both genders are considered. Seventy five per cent of bladder cancer cases are non-muscle invasive bladder cancer (NMIBC). Bacillus Calmette–Guérin (BCG) immunotherapy remains the standard intravesical agent for NMIBC. The exact mechanism by which BCG prevents recurrence is unknown.

The aim of this study was to evaluate NLR4 gene expression and IL-1β as possible prognostic indicators for NMIBC recurrence and BCG treatment failure, and to detect the difference in their levels among muscle

invasive bladder cancer (MIBC) and NMIBC that may aid in primary differentiation between cases.

This study was conducted in 30 patients who had NMIBC and 17 patients who had MIBC. Urine samples were obtained in sterile cups before operation. From NMIBC cases, four more samples were obtained as mentioned below. Evaluation of NLR4 gene expression was performed in pre-surgical sample for MIBC and in 4 samples for NMIBC: pre-surgical sample, sample collected 4 hours after the 3rd dose of BCG instillation, and samples collected during follow up (3 and 6 months post-surgically).

There was statistical significant increase in NLRP4 expression levels in NMIBC (CT=0.87±1.48) compared to MIBC (CT=2.82±2.07). As far as we searched, no published results were found regarding comparative gene expression levels between NMIBC and MIBC cases. Gene expression in recurrent cases was higher in pre-surgical urine samples than in non-recurrent cases. The expression level further increased up to 21 fold than the pre-surgical level in the sample taken after injection of the 3rd dose of BCG. This level decreased distinctly to become 1-fold increase over pre-surgical level at the 3rd month follow up then to only 0.9-fold at the 6th month. In non- recurrent cases, gene expression level started pre-surgically in much lower levels than those encountered in recurrent cases. There were 11-fold increase in expression level after 3rd dose of BCG instillation and then decreased to be 5.6 folds higher in the sample taken at 3rd month follow up than in pre-surgical samples. Gene expression further decreased to become 4.1 fold higher in samples taken at 6 month follow up than the pre-surgical levels.

IL-1β levels were estimated for NMIBC and MIBC cases in urine samples pre-surgically and during BCG therapy in case of NMIBC before and 4 hours after the 3rd dose and during 3rd month follow-up of those cases for searching its possible use of for primary differentiation between NMIBC and MIBC, and also as a prognostic factor for possible recurrence in case of NMIBC cases. The level of IL-1β was generally higher in pre-surgical samples (0.62±0.12 pg/ml) when compared to its level before the 3rd dose of BCG induction therapy (0.53±0.13 pg/ml). Its level was distinctly higher four hours after administration of the 3rd dose BCG (1.96±0.62 pg/ml) than both previous levels. Levels decreased bellow pre-surgical level at 3rd month follow up (0.57±0.099 pg/ml). The levels of IL-1β estimated in samples collected four hours after the 3rd dose BCG was higher in cases that showed recurrence later on than non-recurrent cases. The levels decreased in both cases and became higher in non-recurrent cases (0.64±0.05 pg/ml) than in cases already developed recurrence at the 3rd month diagnosed during follow-up (0.45±0.05 pg/ml).

To conclude, on following NLRP4 gene expression and IL-1β levels during BCG administration among recurrent and non-recurrent cases of thirty NMIBC cases, there was a significant statistical difference in both levels for the samples collected after the third dose BCG, being higher in patients who showed subsequent recurrence at the 3rd and 6th month of follow-up. If these preliminary reported findings will be confirmed in upcoming larger cohort's studies, it could be promising in prognosis of such cases, with the possibility of early manipulation of individualized treatment schedule, keeping patients most probably prone to encounter recurrence safe from possible side effects of BCG therapy. The assessment of NLRP4 expression and IL-1β levels could help predict failure of BCG therapy, playing an appreciable role in early deciding radical surgery. When comparing NLRP4 expression and IL-1β levels between MIBC and NMIBC cases, increased values were noted among non-invasive ones. This finding may serve as a possible diagnostic tool, which represents a challenging issue. Hence, cut-off values for gene expression and cytokine level are to be specified.

Keywords: bladder cancer, BCG, IL-1β, intravesical BCG therapy, Nod-like receptors, tumor immunity

Introduction

Inflammasomes are part of the innate immunity essential in maturation of inflammatory cytokines such as interleukin 1β (IL-1β) in response to infection or autogenous danger signals [17]. Nucleotide oligomerization domain-like receptors (NLRs) are major components of pattern-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns or damage-associated molecular patterns from normal host tissues or tumor cells to initiate innate immune response [14]. NLR proteins can interact with endogenous ligands, inducing autoimmune diseases or antitumor response [6, 10].

Innate immune cells activated by a tumor share in developing antitumor immunity by recruitment of effector cells, or promoting tumor development by providing a pro-inflammatory environment. The inflammasomes' role in tumor development is not yet well known [5].

Bladder cancer is the 7th most commonly diagnosed cancer in males worldwide and the 11th when both genders are considered. Seventy five per cent of bladder cancer cases are non-muscle invasive bladder cancer (NMIBC). Transurethral resection of bladder tumor (TURBT) is the gold standard initial diagnostic intervention with therapeutic and prognostic roles. Adjuvant intravesical therapy with immunotherapy or

chemotherapy is used together with TURBT to reduce the recurrence and/or progression [3].

Bacillus Calmette–Gu rin (BCG) immunotherapy remains the standard intra-vesical agent for NMIBC. The exact mechanism by which BCG prevents recurrence is unknown. However, the bacteria are taken up by the cancer cells. Infection of these cells may trigger a localized immune reaction, clearing residual cancer cells [2, 31]. The cytokine profile of IL-2, IL-12, and Interferon γ is seen after BCG exposure due to a T-helper 1 response [24].

The production of cytokines from tumor cells was demonstrated in urothelial tumor cell lines, in contrast to the initial hypothesis, where macrophages and lymphocytes infiltrating the bladder wall were the primary source of cytokines after instillation of BCG. In this scenario, NLRs are responsible for the release of several cytokines, particularly IL-1β [23].

BCG is delivered as an induction course, consisting of 6-weeks course, followed by a maintenance course. Side effects of BCG therapy include cystitis, prostatitis, epididymo-orchitis, ureteral obstruction, bladder contraction, myco-bacterial osteomyelitis, reactive arthritis, mycobacterial pneumonia, nephritis, infectious vasculitis and disseminated infection [11, 25].

Despite its efficiency, BCG treatment failure may occur in 50% of the cases and would require further treatment [12, 30].Discovering a prognostic marker that can efficiently predict failure is mandatory to spare the patient the passing through a BCG treatment schedule with possible burdens on health and to aid an early decision of radical surgery.

The aim of this study was to evaluate NLR4 gene expression and IL-1β as possible prognostic indicators for NMIBC recurrence and BCG treatment failure, and to detect the difference in their levels among muscle invasive bladder cancer (MIBC) and NMIBC that may aid in primary differentiation between cases.

Material and methods

This study was conducted on 30 patients who had NMIBC and 17 patients who had MIBC, admitted to the Urology departments, Ain Shams University and Tanta University Hospitals during the period from March 2018 to March 2019. The study was approved by the ethical and moral committee of both hospitals. An informed consent was obtained from each patient after explaining the steps of the study.

Inclusion criteria

Patients suspected of having NMIBC, subjected to TURBT for histo-pathologic diagnosis, and indicated for intravesical BCG instillation, according to European Association of Urology (EAU) guidelines [30] (e.g. T1 tumor of any grade, high grade tumor, multiple diffuse Ta disease, large tumors more than 2 cm in diameter, primary treatment of carcinoma in situ, etc.), and having no contraindication for BCG therapy. Patients having MIBC were also included.

Exclusion criteria

Different intravesical instillations, contraindications to BCG therapy (e.g. active autoimmune disease, gross hematuria, total incontinence, urinary tract infection, active tuberculosis, liver disease, pregnancy, lactation, and immune suppression) [15].

Patients were subjected to: Full history taking, pelvi-abdominal examination to detect palpable masses and diagnosis and staging of the tumor by urine analysis and urine cytology for exfoliated cancer cells, ultrasonography to assess the urinary tract and CT urography to evaluate the bladder, lymph nodes and the adjacent organs. Diagnosticcystoscopy and TURBT were performed for patients whom imaging studies revealed a bladder tumor and tissues were subjected to pathological analysis. Cases diagnosed as MIBC were subjected to radical cystectomy. Tumor grading and staging were done according to EAU guidelines [4].

Urine collection and processing

Urine samples were obtained from all patients (NMIBC and MIBC cases) in sterile cups before operation. From NMIBC cases, four more samples were obtained as mentioned below.

Evaluation of NLR4 gene expression was performed in pre-surgical sample for MIBC and in 4 samples for NMIBC: pre-surgical sample, sample collected 4 hours after the 3rd dose of BCG instillation, and samples collected during follow up (3 and 6 months post-surgically).

IL-1β level was estimated in pre-surgical sample for MIBC cases and in 4 samples from NMIBC cases: pre-surgical sample, samples collected before the third dose of BCG instillation and four hours after, and at three months follow up.

Samples were kept at 4 °C and processed within 4 hours. Each sample was centrifuged at 3000 rpm for 10 minutes. Deposits were washed in 10 ml phosphate buffered saline (Oxoid®, UK), centrifuged and both deposit (for detection of NLR4 expression) and supernatant (for IL-1β level) were preserved in -80 °C until experiment pursuance.

Estimation of NLR-4 gene expression by RT-PCR

Primers used:

1) B actin:

Forward (ATCGTGCGTGACATTAAGGAGAAG),

Reverse (AGGAAGGAAGGCTGGAAGAGTG),

2) NLR4:

Forward (AGACTCGTCACGAAGGGAGA),

Reverse (ATAAAACCTCATCCCTGTCTATGT)

(PrimerPCRTM Bio-Rad's assays, USA)

Reverse transcription of mRNA to complementary DNA was done by using Biosystems® TaqMan® MicroRNA Reverse Transcription Kit (Thermofisher scientific, America). RT master mix was prepared, mixed gently, centrifuged, then placed on ice. For each 15 μl RT reaction, total RNA was combined (1

to 10 ng of total RNA per 15 μl reaction) with the RT master mix in a ratio of 5 μl RNA: 7μl RT master mix. Twelve μL of the RT master mix containing total RNA were dispensed into labeled tubes to which 3μL of the RT primers were added.

Reverse transcription was done using the 9600 emulation mode. Expression levels of NLR4 gene in relation to expression of β actin as a reference housekeeping gene were evaluated by means of RT-PCR (Step One, Applied Biosystems) with SYBR Green-ER qPCR SuperMix Universal. ROX was used as passive reference dye.

Each sample was run in triplicate. Specificity was verified by melt curve analysis run automatically.

Estimation of IL-1β level in urine samples

IL-1β Human ELISA Kit (Bioassay technology laboratory, China) was used as instructed. One hundred and twenty μl of the standard solution (8000 pg/L) was constituted with 120μl of standard diluent to generate 4000 pg/L standard stock solution. Duplicate standard points were prepared 1:2 with standard diluent to produce 2000 pg/L, 1000 pg/L, 500 pg/L and 250 pg/L solutions. Standard diluent served as the zero standard (0 pg/L). Samples, anti-IL-1β antibody, substrates and stop solution were added and the plate was incubated following manufacturer's instructions. After washing, the optical density (OD) was determined using a microplate reader at 450 nm.

Statistical analysis

GraphPad prism 8.0 (GraphPad Software, Inc, San Diego, CA) was used to perform statistical analysis. For each relation, normality test was done first by Tick D'Agostino-pearson. In case of normal distribution parametric, T test was done. In case of unconfirmed normal distribution, non-parametric, Mann–Whitney test was done. In both cases, a P value > 0.05 was considered statistically significant. NLRP4 gene expression level was presented in two different ways, normalized CT values (CT target gene-CT reference gene) in case of comparing unpaired data from different patients' groups, while presented as fold changes when comparing paired data like values obtained from each patient at different time intervals. CT values are inversely proportional to the actual gene expression level i.e. the lowest the CT values are, the higher the gene expression level is.

Results

Demographic data of the patients

The Demographic data of the patients, staging and grading of tumors and number of patients who developed tumor recurrence are shown in table 1.

Estimation of NLRP4 gene expression levels

1. NLRP4 gene expression in pre-surgical samples.

There was a highly significant statistical difference (P value = 0.0004) between NLRP4 gene expression

TABLE 1. DEMOGRAPHIC DATA OF THE PATIENTS, TUMOR GRADING AND STAGING

Variables			Number	%
Gender				
Male			40	85
Female			7	15
Age (mean = 62.1±11.9 SD)				
35-55			14	29.8
55-75			29	61.7
75-82			4	8.5
Smoking				
Smoker			21	44.7
Non-smoker			11	23.4
Ex-smoker			15	31.9
Tumor size				
5:> 10 mm			7	15
10:> 20 mm			24	51
20:30 mm			16	34
Tumor type				
NMIBC*			30	63.8
MIBC**			17	36.2
***Tumor staging and tumor grade				
NMIBC (30)	TaG3 n = 14 (46.6%)	Recurrent	3	21.4%
		Non-recurrent	11	78.6%
	T1G3 n = 9 (30%)	Recurrent	0	0
		Non-recurrent	9	100%
	CIS n = 2 (6.7%)	Recurrent	2	100%
		Non-recurrent	0	0
	TaG3 + CIS n = 2 (6.7%)	Recurrent	2	100%
		Non-recurrent	0	0
	T1G3 + CIS n = 3 (10%)	Recurrent	3	100%
		Non-recurrent	0	0
MIBC (17)	T2G3		5	29.4%
	T3G3		9	52.9%
	T4G3		3	17.6%

Note.*, NMIBC: non-muscle invasive bladder cancer; **, MIBC: muscle invasive bladder cancer; ***, Tumor staging was done according to TNM staging system described by European Association of Urology (16): Ta: Not infiltrating the submucosa cassock, T1: Infiltrating the submucosa cassock, T2a: Infiltrating the first half of the muscular tunic, T2b: Infiltrating the second half of the muscular tunic, T3a: Microscopic infiltration of peri-bladder fat, T3b: Macroscopic infiltration of peri-bladder fat, T4a: Infiltration of neighboring organs: prostate, seminal vesicles, uterus, vagina, T4b: Infiltration of the pelvic and / or abdominal wall, G1: low grade, G2: intermediate grade, G3: high grade, CIS: carcinoma in situ.

TABLE 2. COMPARISON BETWEEN NLRP4 GENE EXPRESSION IN PRE-SURGICAL SAMPLES IN NMIBC AND MIBC PATIENTS GROUPS

CT values*	Number	Mean	SD**	SEM***	Coefficient of variation	K2 (D'Agostino and Pearson test)	P value (Mann–Whitney test)
NMIBC	30	0.8667	1.479	0.2701	170.7%	6.153	0.0004
MIBC	17	2.824	2.069	0.5017	73.27%	16.36	

TABLE 3. NLRP4 GENE EXPRESSION IN NMIBC PATIENTS GROUP IN PRE-SURGICAL SAMPLES IN RECURRENT AND NON-RECURRENT CASES OF NMIBC

CT* values	Number	Mean	S.D**	SEM***	Co-efficient of variation	K2 (D'Agostino and Pearson test)	P value
Before surgery in recurrent cases	10	-0.5000	1.650	0.5217	330.0%	1.229	0.0001
Before surgery in non-reccurent cases	20	1.550	0.7592	0.1698	48.98%	3.877	

levels in NMIBC and MIBC patients when comparing normalized CT values. The level was much higher in NMIBC patients (Table 2, Figure 1A).

Recurrent cases showed much increased levels when compared to non-recurrent cases of NMIBC (P value = 0.0001) (Table 3, Figure 1B).

2. NLRP4 gene expression level in pre-surgical samples compared to levels after the 3rd dose of BCG in NMIBC group.

When comparing gene expression in NMIBC cases in pre-surgical urine samples and samples taken after 3rd dose of BCG instillation, the expression level showed significant increase after BCG (P value = 0.0001) (Figure 1C), and higher fold changes encountered in recurrent cases when compared to non-recurrent cases (p value = 0.001), represented by fold changes in relation to pre-surgical levels (Table 4, Figure 1D). Expression levels were 21±9.28 folds higher than pre-surgical levels in recurrent cases, while was only 11±4.02 folds higher in non-recurrent ones.

3. NLRP4 gene expression levels at follow up.

Regarding the expression level in samples taken at 3rd month post-surgically at the follow up cystoscopy in NMIBC non-recurrent and recurrent cases, 5.6±2.01 and 1±0.71 folds higher were encountered compared to pre-surgical levels, respectively (P value = 0.0001). Close results were found in samples obtained at 6th month follow up, where non recurrent and recurrent cases showed 4.15±2.2 and 0.92±0.58 folds higher than pre-surgical samples respectively, represented by fold changes in relation to the pre-surgical level (P value = 0.001) (Table 5, Figure 1E, 1F).

Estimation of IL-1β levels

1. IL-1β level in NMIBC and MIBC.

IL-1β levels were higher in pre-surgical samples in case of NMIBC, ranging from 0.384 to 0.834 pg/ml, than MIBC, ranging from 0.342 to 0.641 pg/ml (P value < 0.0001) (Figure 2A).

2. IL-1β level before surgery and before the 3rd dose BCG in NMIBC cases.

Regarding NMIBC cases, there was high statistical significant difference between IL-1β level before surgery and before the 3rd dose BCG instillation (ranging from 0.38 to 0.677 pg/ml), being higher before surgery (P value = 0.0006) (Figure 2B)

3. IL-1β level before and after 4 hours of the 3rd dose BCG.

Levels shouted four hours after the 3rd dose of BCG instillation (ranging from 0.877 to 2.947 pg/ml) compared to before the 3rd dose instillation (P value = 0.0001) (Figure 2C).

4. IL-1β levels at 3rd month follow up of NMIBC group.

IL-1β levels at the 3rd month follow up were lower than the pre-surgical values (P value = 0.058) (Figure 2D), and lower than levels obtained 4 hours af-

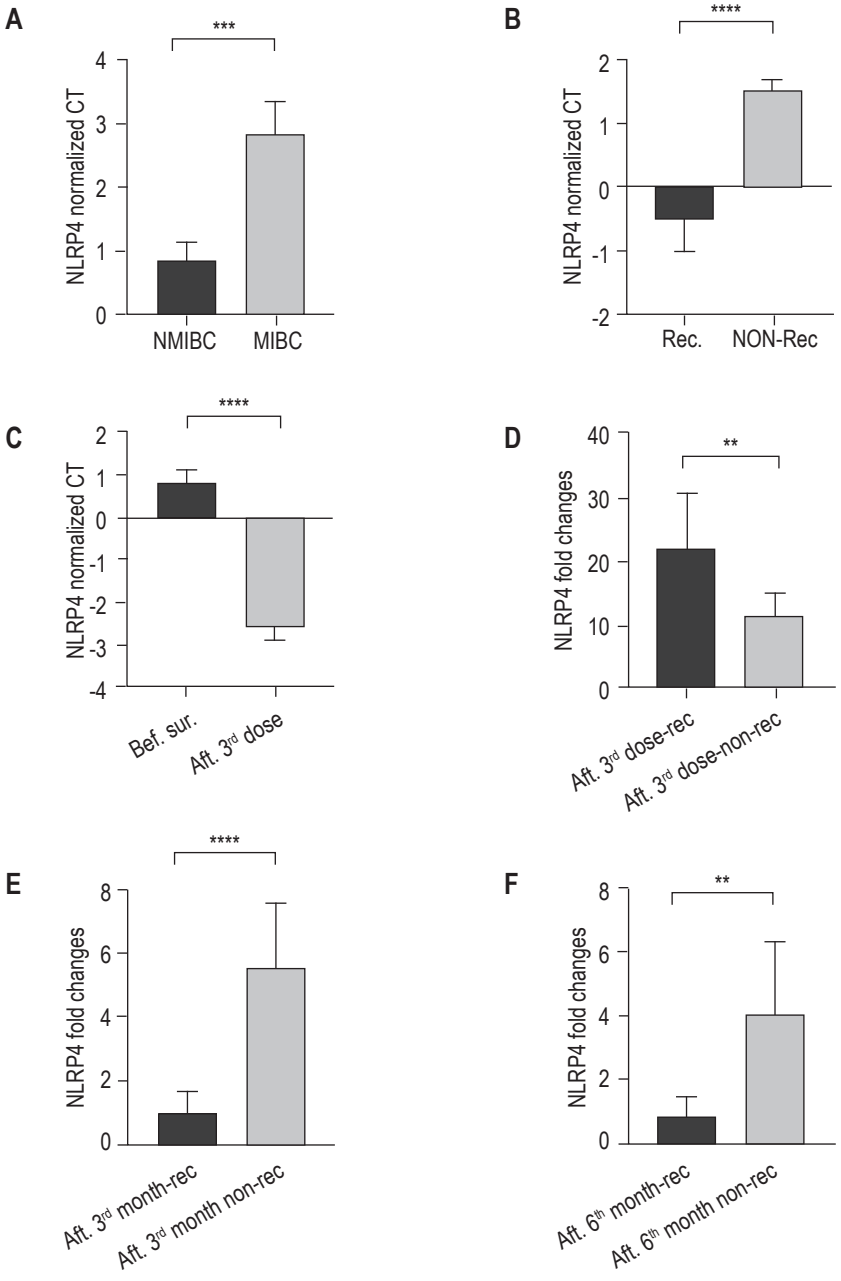

Figure 1A. NLRP4 gene expression levels were estimated by RT-PCR and expressed as mean values of CT values (±SD)
Note. Comparison between NLRP4 gene expression in urine samples pre-surgically (before TURBT) in NMIBC and MIBC patients groups.

Figure 2B. NLRP4 gene expression levels were estimated by RT-PCR and expressed as mean values of CT values (±SD)
Note. Comparison between NLRP4 gene expression in urine samples pre-surgically in recurrent and non-recurrent cases of NMIBC.

Figure 1C. NLRP4 gene expression levels were estimated by RT-PCR and expressed as mean values of CT values (±SD)
Note. Comparison between NLRP4 gene expression in urine samples pre-surgical levels compared to levels in samples taken after the 3rd dose of BCG intravesical instillation of the induction therapy.

Figure 1D. NLRP4 gene expression levels were estimated by RT-PCR and expressed as mean values of CT values (±SD)
Note. Comparison between NLRP4 gene expression in urine samples after the 3rd dose of BCG in recurrent and non-recurrent cases.

Figure 1E. NLRP4 gene expression levels were estimated by RT-PCR and expressed as mean values of CT values (±SD)
Note. Comparison between NLRP4 gene expression in urine samples after the 3rd month post-surgically in recurrent and non-recurrent cases.

Figure 1F. NLRP4 gene expression levels were estimated by RT-PCR and expressed as mean values of CT values (±SD)
Note. Comparison between NLRP4 gene expression in urine samples at 6th month follow up post-surgically in recurrent and non-recurrent cases.

TABLE 4. NLRP4 GENE EXPRESSION LEVEL AFTER THE 3rd DOSE OF BCG IN NMIBC RECURRENT AND NON-RECURRENT CASES

Fold changes regarding pre-surgical CT* value	Number	Mean	S.D**	SEM***	Coefficient of variation	K2 (D'Agostino and Pearson test)	P value
After 3rd dose BCG in recurrent cases	10	21.600	9.276	2.933	42.94%	2.546	0.001
After 3rd dose BCG in non-recurrent cases	20	11.20	4.021	0.8991	35.90%	26.70	

TABLE 5. NLRP4 GENE EXPRESSION LEVEL AT THE FOLLOW UP CYSTOSCOPIES (3 AND 6 MONTHS AFTER TURBT) IN NMIBC RECURRENT AND NON-RECURRENT CASES

Fold changes in comparison to pre-surgical CT* value	Number	Mean	S.D**	SEM***	Coefficient of variation	K2	P value (Mann–Whitney)
After 3rd month in recurrent cases	4	1.000	0.7071	0.3536	70.71%	No. too small	> 0.0001
After 3rd month in non-recurrent cases	20	5.600	2.010	0.4496	35.90%	26.70	
After 6th month in recurrent cases	6	0.9167	0.5845	0.2386	63.77%	No. too small	0.0018
After 6th month in non-recurrent cases	20	4.150	2.207	0.4935	53.18%	2.507	

ter the 3rd dose of BCG instillation (P value < 0.0001) (Figure 2E).

5. IL-1β levels in recurrent and non-recurrent cases.

When comparing IL-1β levels in recurrent and non-recurrent cases after the 3rd dose BCG instillation, there was high statistical significant difference, being significantly higher in recurrent cases than non-recurrent ones (P value > 0.0001) (Table 6, Figure 2F), while levels at the 3rd month follow up were higher in non-recurrent cases than recurrent ones (P value > 0.0001) (Table 6, Figure 2G).

Discussion

BCG intravesical immunotherapy has an appreciated role in both controlling NMIBC, as well as minimizing recurrence rates [7]. The exact mechanism of its beneficial immune-stimulation is still unclear [1, 26]. Hereby, we studied NLRP4 gene expression and IL-1β level in urine samples in patients suffering from bladder cancer after intravesical instillation of the 3rd dose of BCG induction therapy and compared it to the pre-surgical levels in both recurrent and non-recurrent cases. We also evaluated the change in the gene expression and IL-1β levels in MIBC and NMIBC cases for its possible prognostic indicator for invasiveness and recurrence.

Out of the 47 patients enrolled in the current study, 24 (51%) were presented with tumor size ranging from 10: > 20 mm, 16 patients (34%) were presented with tumors ranging in size between 20:30 mm and 7 (15%) patients were presented with tumor size less than 10 mm. Thirty mm was the largest size encountered in this study.

The study was conducted on 30 NMIBC cases and 17 MIBC cases. Rregarding NMIBC, 14 cases (46.6%) were TaG3 of whom 3 (21.4%) showed recurrence within the follow-up period. Nine cases (30%) were T1G3 of whom no recurrence was detected. Three cases (10%) were T1G3 + CIS and two cases (6.7%) were CIS, all of them showed recurrence within the follow up period. Regarding MIBC, 9 cases (52.9%) were T3G3, 5 cases (29.4%) were T2G3, and 3 cases (17.6%) were T4G3.

The Immune system exact role in tumorigenesis is still not totally well known. The role of persistent

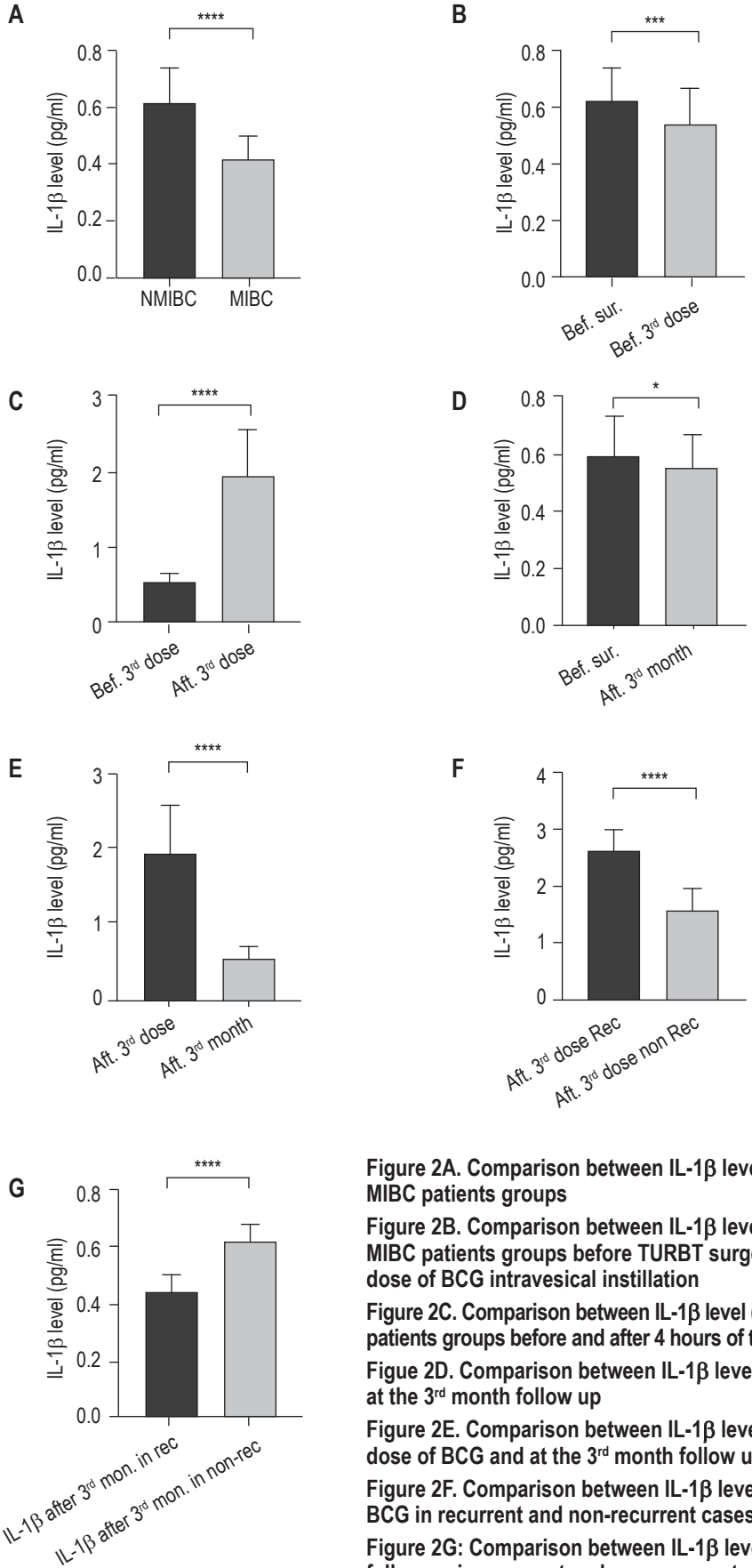

Figure 2A. Comparison between IL-1β level (A) in NMIBC and MIBC patients groups

Figure 2B. Comparison between IL-1β level (A) in NMIBC and MIBC patients groups before TURBT surgery and before the 3rd dose of BCG intravesical instillation

Figure 2C. Comparison between IL-1β level (A) in NMIBC and MIBC patients groups before and after 4 hours of the 3rd dose BCG

Figue 2D. Comparison between IL-1β level before surgery and at the 3rd month follow up

Figure 2E. Comparison between IL-1β level 4 hours after the 3rd dose of BCG and at the 3rd month follow up

Figure 2F. Comparison between IL-1β level after the 3rd dose BCG in recurrent and non-recurrent cases.

Figure 2G: Comparison between IL-1β level at the 3rd month follow up in recurrent and non-recurrent cases

TABLE 6. IL-1β LEVELS IN RECURRENT AND NON-RECURRENT CASES AFTER 3rd DOSE BCG INSTILLATION AND AT 3rd MONTH FOLLOW UP

IL*-1β level	Number	Range of values (pg/ml)	Mean	SD**	P value (un paired T test)
After 3rd dose BCG in recurrent cases	10	2.218:2.947	2.671	0.3158	> 0.0001
After 3rd month in non-recurrent cases	20	0.8767:1.098	1.596	0.3611	
After 3rd month follow up in recurrent cases	10	0.3797:0.5137	0.4536	0.04945	> 0.0001
After 3rd month follow up in non-recurrent cases	20	0.5843:0.09255	0.6352	0.04724	

infection as well as inflammation in various tumors stages has been well documented. A characterized immune response usually follows inflammation cascade, which involves neutrophils, macrophages, dendritic cells, T lymphocyte, B lymphocyte and natural killer cells. Inflammasomes induce maturation of inflammatory cytokines as IL-1β, which has a confirmed role in carcinogenesis when over expressed [20].

Several inflammasomes can affect carcinogenesis by influencing differentiation, apoptosis and adaptive immunity. Inflammasome inhibitors are expected to be novel anti cancerous agents, however their application is much limited being still under clinical trials and being cancer type specific [32].

According to the current study, there was statistical significant increase in NLRP4 expression levels in NMIBC (CT = 0.87±1.48) compared to MIBC (CT = 2.82±2.07). As far as we searched, no published results were found regarding comparative gene expression levels between NMIBC and MIBC cases. One published study indirectly related invasive bladder cancer to inflammasome was done by Mearini et al. [19] , who reported urinary levels of different miRNA targeting inflammasomes in bladder cancer cases, assuming that miR-185-5p was higher in MIBC than in NMIBC. This miRNA targeting nod-like receptor anti apoptosis protein causes silencing of corresponding mRNA.

Gene expression in recurrent cases was higher in pre-surgical urine samples than in non-recurrent cases. The expression level further increased up to 21 fold than the pre-surgical level in the sample taken after injection of the 3rd dose of BCG. This level decreased distinctly to become 1 fold increase over pre-surgical level at the 3rd month follow up then to only 0.9 fold at the 6th month.

In non- recurrent cases, gene expression level started pre-surgically in much lower levels than those encountered in recurrent cases. There were 11 folds increase in expression level after 3rd dose of BCG instillation and then decreased to be 5.6 folds higher in the sample taken at 3rd month follow up than in pre-surgical samples. Gene expression further decreased to become 4.1 fold higher in samples taken at 6 month follow up than the pre-surgical levels.

In accordance to our results, Poli et al. [23] reported that there were high levels of NLR4 expression in bladder cancer, and recurrent cases showed higher NLR4 expression levels pre-surgically when compared to non-recurrent cases. In another study by Poli et al [22], they found that NLR4 expression levels were only higher in pre-BCG period in recurrent cases when compared to non-recurrent ones, in contrast to NLRP6 which showed higher level in post induction together with pre induction period.

Depending on these findings, higher levels of expression in pre-surgical sample and the sample after 3rd dose of BCG may raise suspicion that the case may be complicated later on by recurrence, i.e. could be considered as a possible prognostic factor for recurrence. A cut off value for gene expression level is hence required to be estimated.

IL-1β is a member of the interleukin 1 family of cytokines. This cytokine is produced by activated macrophages. It is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis [6, 29].

Several types of inflammasomes are suggested to play role in tumorigenesis due to their immune-modulatory properties, modulation of gut microbiota, cell differentiation and apoptosis. Over-expression of IL-1β caused by inflammasomes may result in carcinogenesis. NLRP3 inflammasome polymorphisms are suggested to be connected to malignancies such as colon cancer and melanoma. IL-1β secretion was elevated in the lung adenocarcinoma cell line A549. Inhibition of inflammasome and IL-1β expression de-

creased development of cancer cells in melanoma [21, 33].

IL-1β levels were estimated for NMIBC and MIBC cases in urine samples pre-surgically and during BCG therapy in case of NMIBC before and 4 hours after the 3rd dose and during 3rd month follow-up of those cases for searching its possible use of for primary differentiation between NMIBC and MIBC, and also as a prognostic factor for possible recurrence in case of NMIBC cases.

IL-1β levels in NMIBC pre-surgical urine samples were higher (0.62 pg/ml ± 0.12) when compared to MIBC samples (0.42±0.08). No published results have been found regarding IL-1β levels in NMIBC and MIBC cases. Xue et al. [33] reported that IL-1β together with programmed death ligand 1 and tumor necrosis factor-α antagonize the effect of IL-21/IL-21R axis, which inhibits Wnt/β catenin, so inhibiting tumor growth and invasion in non-small cell lung cancer. These findings come in contrary to the suggestion introduced by Zhang and Hwang [34] who worked on oral transitional cell carcinoma. They reported that IL-1β has an important role in tumor invasiveness.

The level of IL-1β was generally higher in pre-surgical samples (0.62 pg/ml ± 0.12) when compared to its level before the 3rd dose of BCG induction therapy (0.53 pg/ml ± 0.13). Its level was distinctly higher four hours after administration of the 3rd dose BCG (1.96 pg/ml ± 0.62) than both previous levels. Levels decreased bellow pre-surgical level at 3rd month follow up (0.57 pg/ml ± 0.099).

The levels of IL-1β estimated in samples collected four hours after the 3rd dose BCG was higher in cases that showed recurrence later on than non-recurrent cases. The levels decreased in both cases and became higher in non-recurrent cases (0.64 pg/ml ± 0.05) than in cases already developed recurrence at the 3rd month diagnosed during follow-up (0.45 pg/ml ± 0.05).

Shintani et al. [28] also followed urinary cytokine profiles (including IL-1β level) in urine samples from 25 patients underwent TURBT for NMIBC. Cytokines levels were evaluated 4, 8 and 24 hours after injection of the 1st and 6th dose of BCG intravesical instillation. They reported that the level peaked 4 hours after the 6th instillation dose. The level in non-recurrent cases was higher (1.81 pg/ml ± 1.46) than in recurrent cases (1.59 pg/ml ± 1.82). This comes in discordance to the results we observed, as when comparing IL-1β levels in samples taken 4 hours after 3rd dose of BCG among recurrent and non-recurrent cases, levels were higher in recurrent cases (2.67 pg/ml ± 0.32) than in non-recurrent ones (1.59 pg/ml ± 0.36).

Videira et al. [9] found opposite results to what was reported by Shintani et al. [28], as the formers studied the systemic molecular IL-1 β gene expression in blood cells obtained from 58 cases subjected to BCG treatment weekly during induction phase and at fixed follow-up intervals (3, 6, 9, and 12 months). They compared results obtained from non-recurrent and recurrent cases. They reported significantly less expression of IL-1β (18.54%) in non-recurrent cases than in recurrent ones (25.61%, P = 0.018) 24 hours after the 6th BCG induction dose. This comes in the same venue to the finding in the current study.

In the same context, Salmasi et al. [27] followed urinary cytokines profile (IL-1β level was included) from fifty patients suffering from intermediate or high-risk NMIBC for evaluation of intravesical BCG ± intradermal vesigenurtacel-1 therapy. Samples were collected at fixed interval at baseline, week 7, week 13, week 28, and at the end of treatment. They observed that low levels of IL-1β encountered at the 13th weak were usually associated with high recurrence rate.

Increased IL-1β level is an expected inflammatory response to BCG treatment, which has an appreciable role in enhancing the immune response in bladder tissue. However, inflammatory cytokines stimulate tumor proliferation and angiogenesis in case of prolonged exposure. Prolonged exposure acts as a favorable condition for occurrence of tumor metastasis as consequence of generated reactive oxygen and nitrogen species secondary to DNA damage. This can be an explanation for the relation between increased urinary cytokine level and the increased probability to develop recurrence [8, 16].

It deserves to be mentioned that basal levels of the immune-modulators are supposed to be enough for influencing the BCG treatment response. We found that there is increased level of IL-1β during BCG treatment (after 3rd dose BCG), regardless whether the patient developed relapse later on or not, with the level being significantly higher in relapsing cases. This can be considered an agreement with the suggestion presented by Masson et al. [18] that specific cytokines are necessary for BCG treatment feedback, although moderate values are still wished to avoid possible drawback.

Also, patients most prone to develop recurrence mostly have genetic predisposition to face exacerbation of inflammatory responses to BCG treatment or to the disease itself with exaggerated cytotoxic responses, which may add to the disease burden. An example for that was reported by Hawkyard et al. [13] regarding interferon-γ, being essential for BCG therapy immunological response as it has inhibitory effect on cancer cells, but it can stimulate novel cellular inflammatory mechanisms that can promote tumor initiation and progression related to micro environmental factors and signaling intensity.

To conclude, on following NLRP4 gene expression and IL-1β levels during BCG administration among recurrent and non-recurrent cases of thirty NMIBC

cases, there was a significant statistical difference in both levels in samples collected after the third dose BCG, being higher in patients showed subsequent recurrence at the 3rd and 6th month follow-up. If these preliminary reported findings will be confirmed in upcoming larger cohort's studies, it could be promising in prognosis of such cases, with the possibility of early manipulation of individualized treatment schedule, keeping patients most probably prone to encounter recurrence safe from possible side-effects of BCG therapy. The assessment of NLRP4 expression and IL-1β levels could help predict failure of BCG therapy, playing an appreciable role in early deciding radical surgery. When comparing NLRP4 expression and IL-1β levels between MIBC and NMIBC cases, increased values were noted among non-invasive ones. This finding may serve as a possible diagnostic tool, which represents a challenging issue. A cut off value for gene expression and cytokine level is hence required to be estimated.

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