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EXPRESSION OF TLR4 AND MAJOR INFLAMMATORY CYTOKINES IN PATIENTS WITH BLADDER CANCER OF DIFFERENT GRADE AND STAGE

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***Background*: The bladder cancer is immunogenic, and neoantigens generated by tumor cells trigger a notable immune response in the host. On the other hand, multiple immune escape mechanisms allow for avoiding the recognition by the host immune system. Toll-like receptor type 4 and inflammatory cytokines play major role in the immune response to bladder cancer. *Aim*: To assess the expression of *TLR4* and the genes of major inflammatory cytokines in tumor cells and in unaffected tissue of the bladder. *Materials and Methods:* The pairs of samples from the urinary bladder tumor and unaffected adjacent tissue were obtained from 50 surgically treated patients with bladder cancer. The level of expression of *TLR4, TGF-β1, INF-γ, TNF-α* genes was evaluated by real-time polymerase chain reaction. *Results:* Bladder cancer cells are characterized by lower expression levels of *TLR4, TGF-β1, INF-γ, TNF-α* as compared to unaffected tissue. In patients with recurrent cancer, expression of *TLR-4* and cytokines does not change both in tumor and in unaffected tissue of the bladder. Expression of *TLR4* is identically low both in low- and high-grade cancer. Expression levels of the *INF-γ* and *TNF-α* are remarkably low in muscle-invasive cancer compared to the unaffected bladder tissue. The level of *TGF-β1* in bladder cancer is comparable to the unaffected tissue of the bladder, while in the intact and metastatic lymph nodes it is significantly upregulated. *Conclusion*: Bladder cancer tissue differs from the unaffected part of the bladder wall in the level of *TLR4, TGF-β1, INF-γ, TNF-α* expression.**

***Key Words*: urinary bladder cancer, *TLR4, TGF-β1, INF-γ, TNF-α*, gene expression.**

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Bladder cancer (BC) is a complex disease to manage due to its high potential for local recurrence and metastatic progression. Urothelial carcinomas show distinctive molecular changes at the chromosome and/or gene expression level [1, 2]. It is estimated that up to 50% of patients with muscle invasive BC (MIBC) would progress to metastatic form of disease within two years after radical surgery, and the cumulative 5-year survival is about 50% [3].

BC is considered to be immunogenic due to high somatic mutational load [4], which generates neoantigens allowing host immunity to rise antitumor response [5]. On the other hand, the immune escape mechanisms employed by BC allow avoiding recognition by the host immune system. Shedding of the soluble form of certain surface molecules by cancer cells results in mimicking normal immune cells. It makes cancer cells invisible for immune cells and immune evasion occurs [6]. Additionally, cancer cell evasion is facilitated by the production of several proteins, including proteinases, inhibitory cytokines, inflammatory factors [7].

The key players in the host immune defense and in cancer immunity are the Toll-like receptors (TLR), which belong to the family of pattern-recognition receptors (PRR) family, being the key players in the innate immunity functioning [8].

Among TLRs the expression of TLR type 4 (TLR4) turned out to be the most prominent and relevant to the progression of BC and its prognosis [9]. TLR4 is present both on immune cells and BC cells [10], which makes it difficult to distinguish the dual role of TLR4 in cancer progression and anticancer immunity [11, 12].

The aim of the study is to assess the expression of genes responsible for TLR4 and major inflammatory cytokines in BC.

MATERIALS AND METHODS

The study included 50 consecutive patients with urothelial BC, aged 64.5 ± 9.4 years, who underwent radical surgery in 2019–2021. During the surgery, we collected the samples of tumor tissue and unaffected tissue of the bladder wall adjacent to the tumor, 30 mm^3 each sample that must contain the muscular layer of the bladder wall. The samples were placed in 1 ml tubes with RNAlater Solution (Ambion, USA) and stored in the freezer at –20 °C.

The tissue samples for the study were collected in the following manner:

- During radical cystectomy the sample of a tumor, and part of the healthy looking bladder wall adjacent to the tumor were cut off the bladder after it was removed from the patient.
- During the partial cystectomy the samples were taken from the resected tumor, and from the healthy resection margin of the bladder.

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Abbreviations used: APC – antigen presenting cells; BC – bladder cancer; DC – dendritic cell; INF-γ – interferon gamma; LN – lymph node; MIBC – muscle invasive BC; NMIBC – non-muscle invasive BC; PRR – pattern recognition receptor; RT-PCR – real-time polymerase chain reaction; TGF-β1 – transforming growth factor beta 1; TLR4 – Toll-like receptor type 4; TNF-α – tumor necrosis factor alpha.

- During the transurethral resection of the bladder tumor the samples were taken as chip of the tumor, and separate bite of the bladder wall adjacent to the tumor resection bed.

All patients enrolled in this prospective study have signed the informed consent on the diagnostics and treatment of their disease in accordance with national treatment protocols, and collection of the biological material during the surgical treatment. Local committee on the medical ethics of the Clinical hospital "Feofaniya" has endorsed the conduction of this study by its protocol № 2 on February 17, 2020 as such, which do not violate the rights of the patients.

TLR4, transforming growth factor beta-1 (TGF-β), interferon γ (INF-γ), tumor necrosis factor α (TNF-α) expression levels were evaluated by real-time polymerase chain reaction (RT-PCR) on 7500 Real-Time PCR Systems (Applied Biosystems, USA) using specific primers and f DOI: 10.32471/exp-oncology. 2312-8852.vol-43-no-2.16102 luorochrome SYBR Green (Applied Biosystems, USA). GADPH was used to normalize levels of mRNA for the relative quantification method of analysis. *TLR4, TGF-β, INF-γ,* and *TNF-α* sequences were constructed by Primer Express® Software v3.0 (Applied Biosystems, USA) as presented in our earlier work [13]. Calculations were performed using the ΔCt relative quantification method. mRNA expression value was calculated by the formula:

$x = 2^{-\Delta Ct}$,

where x — mRNA expression value,

ΔCt = Ct (GAPDH) — Ct (target gene).

All tests were run in triplicate.

Total RNA was isolated by phenol–chloroform extraction and the "Ribo-zol" kit ("AmpliSens"). RNA concentration in all samples was measured by ThermoScientific NanoDrop-1000 (Thermo Fisher Scientific, USA) and samples were diluted to 200 ng/μl. cDNA was obtained from total RNA by RT-PCR using "High Capacity cDNA Reverse Transcription Kit" (Applied Biosystems, USA). The reverse transcription reaction was run under the following conditions: 25 °C — 10 min, 37 °C — 120 min and 85 °C — 5 s. DNA was diluted in half with DNA buffer.

Statistical analysis was performed using Statistica version 10 (StatSoft Inc) and MedCalc 12.1 (MedCalc Software Ltd). Gaussian distribution of the group was checked with Shapiro — Wilk test. The data are presented as Mean ± SE. To compare the data in multiple groups, we used Kruskal — Wallis test for nonparametric data. The H0-hypothesis of variables equality was rejected at $p < 0.05$.

RESULTS

The clinical and pathological data of 50 patients with BC are presented in the Table. As we can see, more than half of patients had non-muscle invasive BC (NMIBC). Majority of patients had organ-confined bladder tumor (stages pTa-2). Metastases into the regional lymph nodes (LN) (stage pN+) were diagnosed and studied for the expression profile in 14% patients. Most aggressive high-grade BC (G3–4) was diagnosed in 36% of patients.

Table. Clinical and pathological data of bladder cancer patients

Characteristics	Number of cases	%
Males	41	82
Females	9	18
Stages of bladder cancer		
Non-muscle invasive cancer:	27	54
pTa	15	
pT1	12	
Muscle-invasive cancer:	23	46
pT2	13	
pT3	9	
pT4	1	
Grades of bladder cancer		
Low grade:	32	64
G1	15	
G2	17	
High grade:	18	36
G3	17	
G4	1	
N+	7	14
Methods of surgical treatment of bladder cancer		
Radical cystectomy	5	10
Partial cystectomy	9	18
Transurethral resection	36	72
Patients with bladder cancer recurrence	8	16
Average time to tumor relapse, years, M ± SE	1.5 ± 0.4	

Most patients (90%) were treated with bladder-sparing surgery, which reflects the current trend in managing BC.

Levels of *TLR4, IFN-γ, TGF-β1* and *TNF-α* expression in the tumor tissue and conventionally normal tissue are presented in Fig. 1. The tumor tissue has lower expression of *TRL4, IFN-γ* and *TNF-α* ($p = 0.038$, 0.0011, and 0.002, respectively) as compared to the paired healthy tissue sample whereas no difference in the expression of *TGF-β1* was noted ($p = 0.368$). The unaffected tissue has distinctively higher expression of *IFN-γ* and *TNF-α* compared to the tumor tissue (Fig. 1).

Expression level of *TLR4, INF-γ, TGF-β1* and *TNF-α* was further analyzed separately in tissue of high grade BC (grade 3–4) and low grade bladder cancer (grade 1–2), as compared with the paired samples of unaffected tissue (Fig. 2).

TLR4 expression was similar both in high- and low-grade BC. In patients with low-grade BC unaffected bladder tissue has higher expression of *TLR4* than the tumor. We found the difference in the *TLR4* expression from the healthy tumor-free bladder wall in patients with low-grade and high-grade BC, the higher *TLR4* expression was noted in patients with low-grade BC ($p = 0.02$).

The expression of *INF-γ* is distinctively higher in the healthy part of the bladder compared to the tumor in patients with low-grade BC ($p = 0.009$). The same trend is observed in patients with high-grade BC, but the difference was not statistically significant. The expression of *INF-γ* is equally low both in high- and low-grade BC.

The expression of *TGF-β1* is equally low both in low- and high-grade BC. In patients with high-grade BC the expression of *TGF-β1* is distinctively lower in tumor compared to the unaffected tissue of the urinary bladder ($p = 0.04$). When considering the expression of *TGF-β1* in conventionally normal tissue of the urinary bladder, it is higher in patients with high-grade BC compared to patients with low-grade BC ($p = 0.046$), and this trend is inverse to the expression of *TLR4*.

The expression of *TNF-α* is remarkably low in tumor tissue compared to the healthy tissue, and this

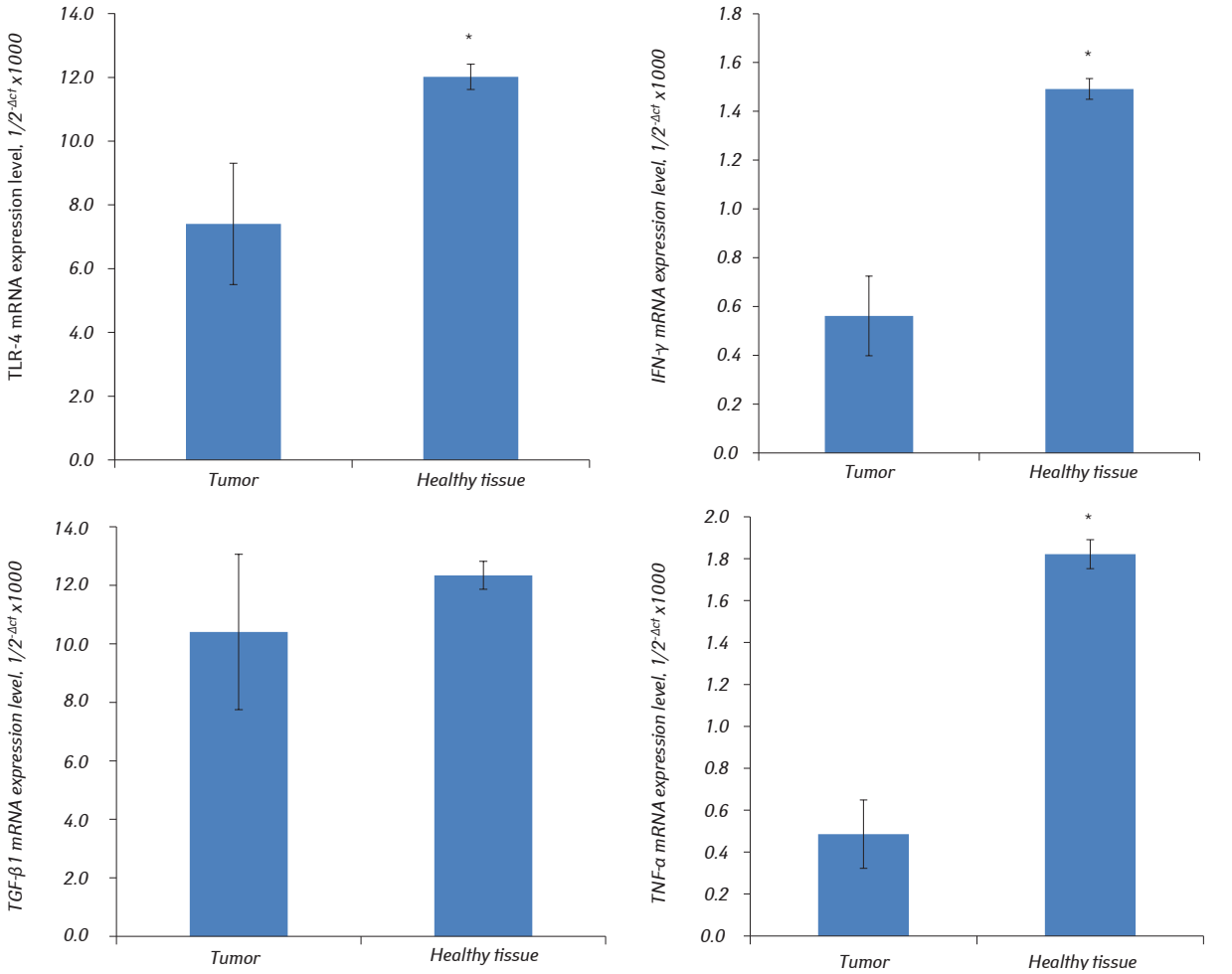

Fig. 1. Expression of *TLR4, TGF-β1, INF-γ, TNF-α* in the urinary bladder from BC patients: tumor *vs* unaffected tissue of the bladder. *$p < 0.05$ compared to the bladder tumor

difference is more pronounced in high-grade BC patients than in low-grade BC patients. In unaffected tissue of bladder, the insignificantly higher expression of *TNF-α* was noted in patients with high-grade BC.

Levels of *INF-γ, TGF-β1* and *TNF-α* expression in tissues of NMIBC (stages Ta, T1) and MIBC (stages 2, 3 and 4), and in the paired samples of normal tissues are presented in Fig. 3.

The *TLR4* demonstrates identical pattern of expression both in NMIBC and MIBC, with higher expression of *TLR4* in unaffected tissue of the bladder, but this difference did not reach the level of statistical significance.

The expression of *INF-γ* is higher in healthy part of the bladder independently on the BC stage; in patients with NMIBC its expression reached the level of significance ($p = 0.042$).

TGF-β1 expression shows no significant alterations, although in patients with high-grade BC it was lower in tumor than in the unaffected tissue.

TNF-α is less expressed in the tumor tissue than in the healthy bladder tissue, in particular, paired samples from patients with MIBC demonstrated significant upregulation of *TNF-α* in the unaffected tissue compared to tumor ($p = 0.01$).

In our study, eight patients underwent repeated surgery for the recurrence of the BC during 1.5 ± 0.4 years after primary treatment. Stages of BC in these patients ranged from 1 to 3. Level of expression of *TLR4, INF-γ, TGF-β1* and *TNF-α* in the tumor, unaffected part of the bladder during primary surgery, and during repeated surgery did not demonstrate statistical significance, which can be attributed to the small group of patients.

Seven patients had BC metastases to the regional lymph nodes (LN), which were removed and sampled during the open radical surgery (radical cystectomy in 2 patients, and partial cystectomy in 5 patients). We tested the level of expression of *TLR4, INF-γ, TGF-β1* and *TNF-α* in a LN with metastases and intact LN, in BC and the unaffected part of the bladder wall. The results are presented in Fig. 4.

Among all studied parameters, in BC patients with metastases to the regional LN only *TRL4* expression is significantly lower in tumor compared to the metastases in the LN ($p = 0.017$). The cytokine *TGF-β1* is significantly upregulated in the tissue of the regional LN affected with metastases, though this difference was insignificant.

DISCUSSION

BC is considered to be an immunogenic tumor, and the major components of the innate and adaptive immunity, which include TLR4 and main inflammatory cytokines, can be changed in the course of the

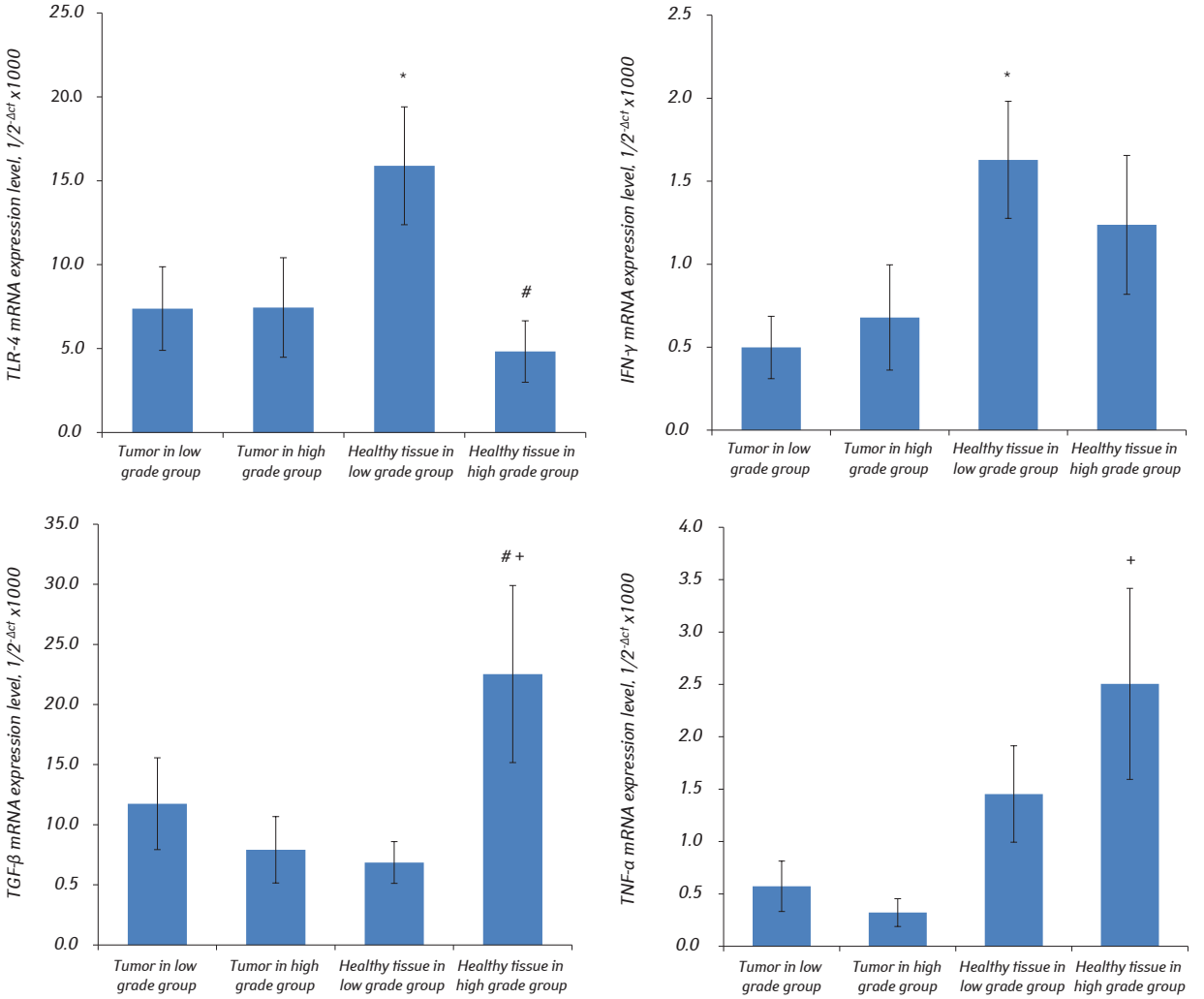

Fig. 2. Expression of *TLR4, TGF-β1, INF-γ, TNF-α* in BC depending on tumor grade (high-grade *vs*. low-grade). *$p < 0.05$ compared to tumor tissue in low grade group, +$p < 0.05$ compared to tumor tissue in high grade group, #$p < 0.05$ compared to unaffected tissue of bladder in low grade group

treatment. On the other hand, the manipulation with these molecules might present an opportunity for the therapeutic impact onto the disease course.

Kusuhara *et al*. [9] arrived at a conclusion that lower expression of TLR4 correlates with poor prognosis of BC, with squamous differentiation of MIBC, lower cell proliferation, but higher invasiveness of cancer cells; depletion of TLR4 in low-stage BC cell lines increased the expression of cell morphology related genes, including keratin proteins and small proline-rich proteins. In our study, we found that in all BC cases tumor tissue had lower expression of TLR4 as compared to the unaffected portions of the urinary bladder.

TLRs possess immunoadjuvant ability and are expressed on antigen-presenting cells (APCs), including macrophages or dendritic cells (DCs). They play an important role in the initiation of innate immune response [14] by playing a role of a first contact with invading microbial pathogens, damage associated molecules, or cancer cells [15]. After TLR binds to specific ligand, it initiates the signaling cascade which leads to activation of transcription factors, expression of inflammatory factors, maturation of APCs, and release of bactericidal peptides, inflammatory cytokines and chemokines, including TNF-α, interleukin-6 and -12, and IFN [11]. This lead to subsequent activation of T cells, both natural killers and cytotoxic lymphocytes, and ensue in proliferation and differentiation of T helper cells types 1 and 2 (Th1 and Th2), which form and regulate adaptive immunity [16]. In our study, we report the downregulation of IFN-γ and TNF-α in the tumor tissue, along with downregulation of the TLR4, which might signify the suppression the immune response in BC.

Tumor cells activated by TLR signals can release cytokines and chemokines, which in turn recruit immune suppressive cells to release further aberrant cytokines and chemokines, with subsequent uncontrolled tumor progression [17]. Nevertheless, in our study we did not report the upregulation of the TLR4, and consequently, no elevation of expression in the inflammatory cytokines was noted, compared to the healthy tissue.

BC is associated with increased levels of regulatory T cells (Tregs) as well as Th1 inhibitory cytokines (e.g, TGF-β and IL-10) to evade immune surveillance [18]. TGF-β causes Th1 activation, leading to release of IFN-γ and IL-2. This subse-

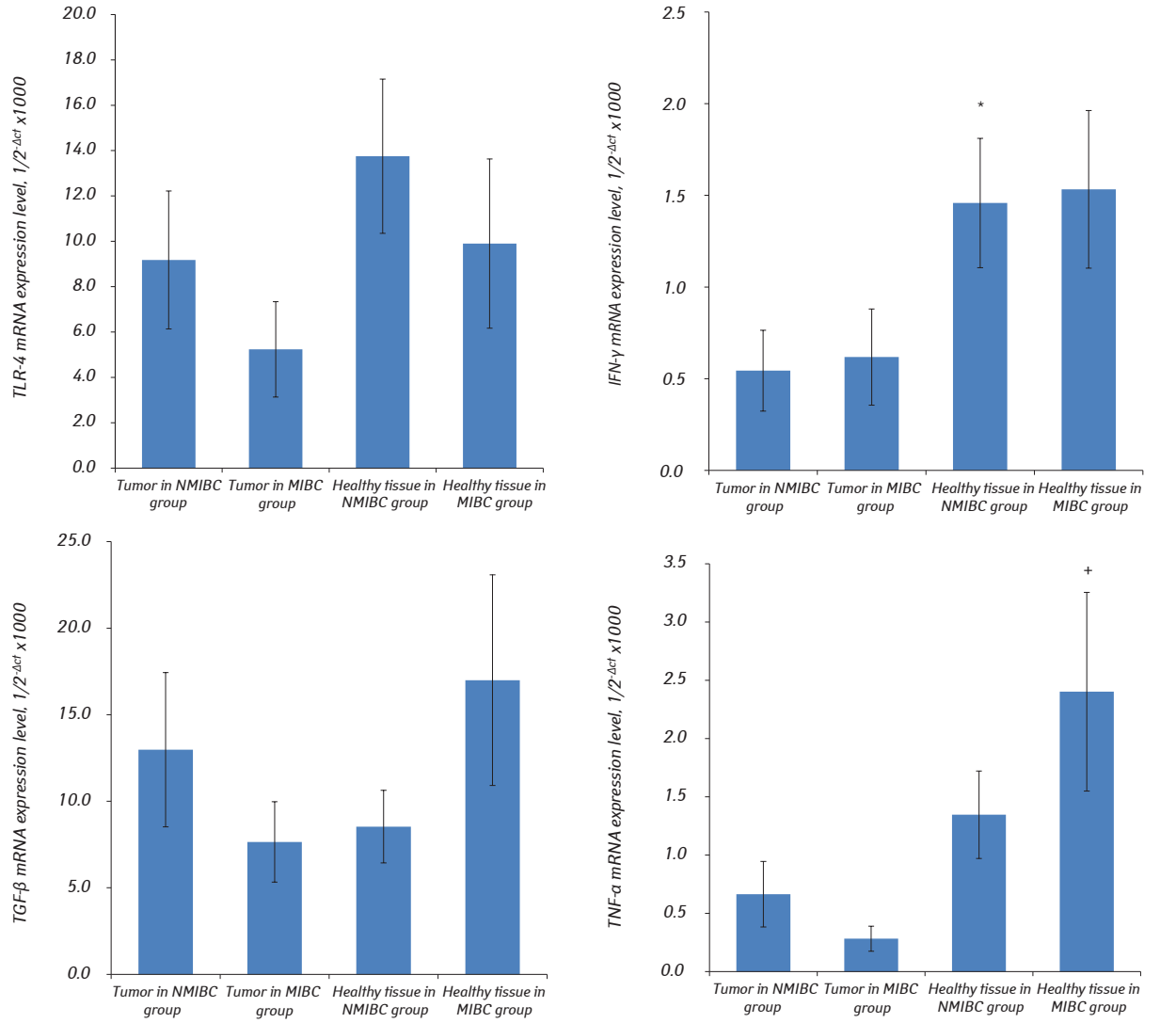

Fig. 3. Expression of *TLR4, TGF-β1, INF-γ, TNF-α* in BC depending on tumor stage: NMIBC *vs* MIBC. $^{*}p < 0.05$ compared to tumor in MIBC group, $^{+}p < 0.05$ compared to tumor in NMIBC group

quently stimulates cytotoxic lymphocytes [19]. In our study, we observed the significant upregulation of TGF-β only in the tissue of the regional LN, and especially in the metastases in those nodes, although invalid statistically. This finding underlines the role of TGF-β in the migration of the cancer cells and establishment of the metastases.

To escape from immune surveillance, BC employs several positive regulation strategies, including inflammation, injury, microbial infection and tissue repair. TLR engagement in tumor cells via ligands leads to activating signal cascades and cytokine and chemokine induction, which consequently lead to tumor invasion, cancer cell survival, chemoresistance, tumor progression and metastasis [6].

Shedding of the soluble form of certain surface molecules by cancer cells results in mimicking normal immune cells. It makes cancer cells invisible by immune cells [6] Additionally, cancer cell evasion is facilitated by the production of several factors, including proteinases, inhibitory cytokines, inflammatory factors, small molecules, such as nitric oxide [7].

Qian *et al*. [20] showed that activation of TLR4 by LPS has led to upregulation of IL-6 expression through p38 and ERK kinase stimulation. Moreover, TLR4 activation stimulates mitogen-activated protein kinases ERK and JNK and leads to upregulated expression of programmed death ligand 1 (also known as B7–H1 or CD274). All these suggest a probable mechanism of tumor evasion via T cell immunity inhibition [21].

TLRs agonists are effective immune stimulators and have important role in the induction of immune responses with immunotherapeutic potential against cancer. BCG (agonist of TLR2 and TLR4) is approved for intravesical BC treatment. Its role in treating MIBC is not clear [22]. In our study, we demonstrated that expression of TLR4 demonstrated the identical pattern of expression, being low in tumor, compared to high in healthy bladder tissue. This might be an argument for testing this TLR4 agonist in the setting of MIBC with curative purposes.

The presented above data point at the dual role of the TLR4 and major inflammatory cytokines in BC, and argue for the further study of the molecular mechanisms of the BC development, progression, and treatment.

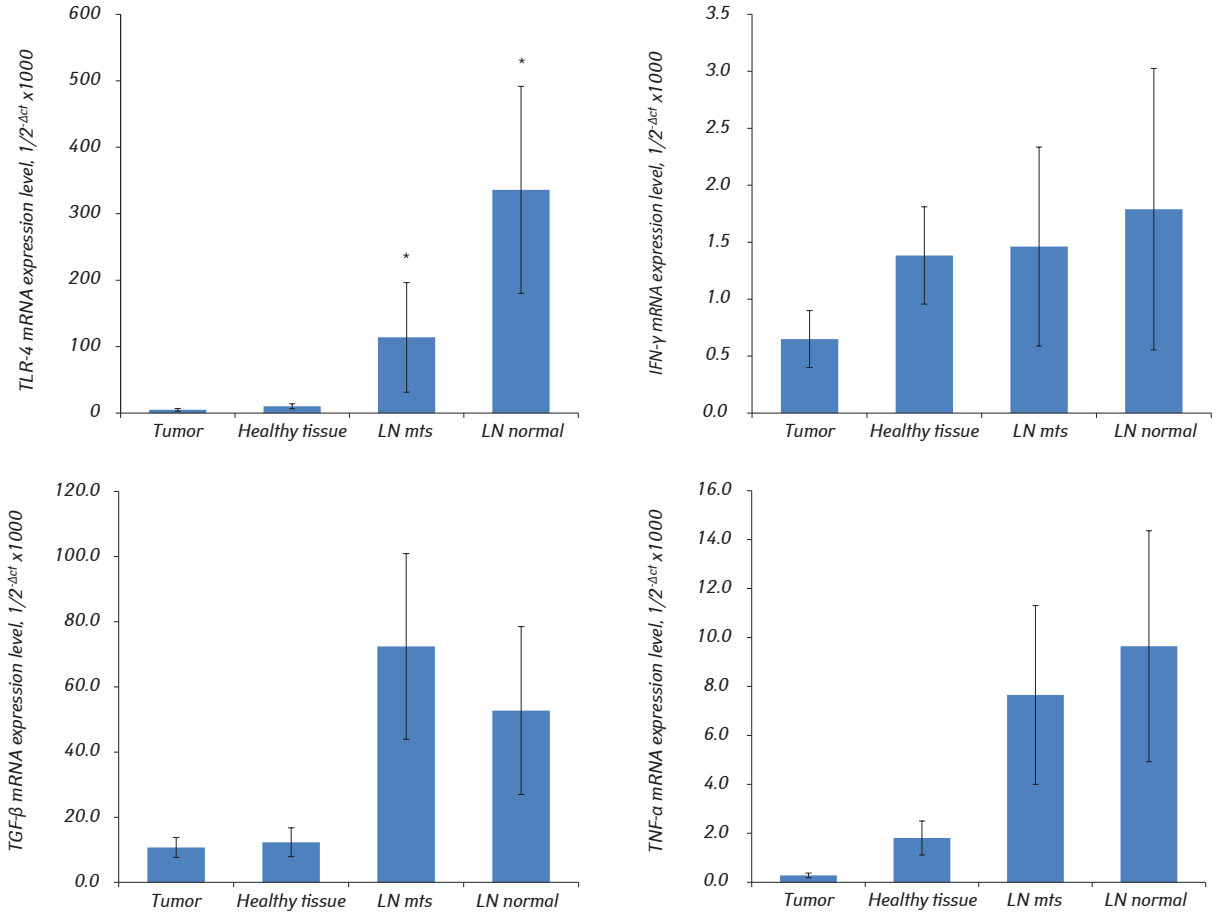

Fig. 4. Expression of *TLR4, TGF-β1, INF-γ, TNF-α* in patients with BC metastases to the regional LN. *$p < 0.05$ compared to tumor

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ЕКСПРЕСІЯ TLR4 І ГОЛОВНИХ ЗАПАЛЬНИХ ЦИТОКІНІВ У ХВОРИХ НА РАК СЕЧОВОГО МІХУРА РІЗНОЇ СТАДІЇ ТА СТУПЕНЯ ЗЛОЯКІСНОСТІ

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Стан питання: Рак сечового міхура є імуногенним. Неоантигени, які продукуються пухлинними клітинами, спричинюють помітну імунну відповідь в організмі хворого. Разом з тим існують численні механізми, які дозволяють пухлині ухилятися від розпізнавання імунною системою організму. Toll-подібні рецептори типу 4 та запальні цитокіни відіграють важливу роль в імунній відповіді на рак сечового міхура. ***Мета***: Визначити експресію *TLR4* та генів головних запальних цитокінів в пухлинних клітинах та умовно нормальній тканині сечового міхура у хворих на рак сечового міхура. ***Матеріали та методи***: В попарних зразках пухлинної тканини та прилеглої до пухлини тканини сечового міхура 50 прооперованих хворих на рак сечового міхура методом полімеразної ланцюгової реакції у реальному часі визначали рівень експресії генів *TLR4, TGF-β1, INF-γ, TNF-α*. ***Результати***: Клітини раку сечового міхура характеризуються низьким рівнем експресії *TLR4, TGF-β1, INF-γ, TNF-α* в порівнянні з клітинами прилеглої до пухлини умовно нормальної тканини сечового міхура. У хворих з рецидивом експресія *TLR4* та досліджуваних цитокінів не змінюється ані в пухлині, ані в прилеглій тканині сечового міхура. Рівні експресії *TLR4* однаково низькі в пухлинах різного ступеня злоякісності. Рівні експресії *INF-γ* та *TNF-α* в пухлинах з інвазією до м'язового шару суттєво знижуються. ***Висновок***: Рівні експресії *TLR4, TGF-β1, INF-γ, TNF-α* в пухлинній тканині відрізняються від таких у прилеглій умовно нормальній тканині сечового міхура.

***Ключові слова*:** рак сечового міхура, *TLR4, TGF-β1, INF-γ, TNF-α*, експресія генів.