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ORIGINAL ARTICLE

PD-L1 Expression in Nonbacterial Chronic Cystitis and Bladder Cancer

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Abstract

Introduction and hypothesis The objective was to assess PD-L1 expression in nonbacterial chronic cystitis (NCC) and bladder cancer (BC).

Methods The present study included 20 NCC and 20 BC patients. The degree of inflammation of the bladder wall was assessed on slides stained with H&E. Viral pathogens (herpes simplex virus, Epstein–Barr virus, cytomegalovirus, and high-risk HPVs) were detected using real-time polymerase chain reaction analyses of the bladder specimens. Immunohistochemistry was performed to assess the PD-L1 expression in bladder tissue.

Results Expression of PD-L1 was detected in 40% of NCC patients and 85% of BC patients. Viral pathogens were found in 50% of NCC patients and 60% of BC patients, with EBV being the most common. In NCC patients the immune cell score correlated strongly with the degree of inflammatory infiltration of the bladder wall ($r=0.867$, $p<0.001$), the presence of lymphoid aggregates in the submucosa ($r=0.804$, $p<0.001$), koilocytosis ($r=0.620$, $p=0.004$), and the presence of viral pathogens ($r=0.784$, $p<0.001$). In BC patients the immune cell score correlated with the degree of inflammatory infiltration of the bladder wall ($r=0.534$, $p=0.015$) and the presence of viral pathogens ($r=0.626$, $p=0.003$), but not with the presence of lymphoid aggregates in the submucosa ($r=0.083$, $p=0.729$), and koilocytosis ($r=0.366$, $p=0.112$).

Conclusions Expression of PD-L1 was detected in a cohort of NCC patients, although the PD-L1 positivity rate was lower than that in BC. Our results demonstrate that the degree of PD-L1 expression in bladder tissue is associated with the presence of viral infections and with the degree of inflammatory infiltration of the bladder wall in both NCC and BC.

Keywords PD-L1 · Epstein–Barr virus · Cystitis · Bladder cancer

Abbreviations

BC	Bladder cancer
CMV	Cytomegalovirus
EBV	Epstein–Barr virus
HPV	Human papillomavirus
HSV	Herpes simplex virus
IC/BPS	Interstitial cystitis/bladder pain syndrome
IUC	Infiltrating urothelial carcinoma
NCC	Nonbacterial chronic cystitis
NPUC	Non-invasive papillary urothelial carcinoma
PCR	Polymerase chain reaction
PD-1	Programmed death-1
PD-L1	Programmed death ligand-1
TC score	Tumor cell score

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Introduction

Nonbacterial chronic cystitis (NCC) is a resumptive term that encompasses nonbacterial infectious and non-infectious chronic cystitis. Nonbacterial infectious cystitis is caused by viruses (e.g., herpes simplex virus [HSV]-1, HSV-2, cytomegalovirus [CMV]) and fungi (*Candida* spp.) [1, 2]. In cases where no distinct etiological factor is found, a diagnosis of interstitial cystitis/bladder pain syndrome is usually considered and cystoscopy is performed. Although the etiology of interstitial cystitis remains unclear, recent studies have demonstrated that Epstein–Barr virus (EBV)

and *Varicella zoster* virus might be implicated in the pathophysiology of interstitial cystitis [3, 4].

The most common pathological findings include epithelial denudation, inflammatory infiltration of the bladder wall, vasodilation, and submucosal edema [5]. Immune-mediated and autoimmune processes are fundamental to the development of NCC; however, the role of the immune checkpoint PD-1/PD-L1 pathway is underestimated. Programmed cell death-1 (CD-279) is one of the key co-inhibitory receptors expressed on immune cells, including T cells, B cells, NK cells, NKT cells, dendritic cells, and monocytes [6]. The interaction between PD-1 and its ligands, primarily PD-L1 (B7-H1), counters stimulatory signaling from T cell receptors, leading to the downregulation and apoptosis of T cells. Hence, the PD-1/PD-L1 pathway is important in inhibiting immune responses and promoting self-tolerance [6, 7].

Tumor cells and viral pathogens (e.g., HSV, CMV, and EBV) take advantage of the PD-1/PD-L1 pathway by inducing PD-L1 expression in lymphoid and peripheral tissues, which, in turn, helps them to evade host immunity. Increased PD-L1 expression in tumor cells is observed in various malignant diseases, such as breast, colorectal, gastric, and bladder cancers (BC) [8]. Recently, it was found that PD-L1 expression in some nonmalignant diseases (e.g., inflammatory bowel disease, Crohn's disease, and inflammatory arthritis) correlates with the degree of inflammation [9, 10].

Thus, in this study, we aimed to investigate PD-L1 expression in bladder tissue in NCC and BC and to assess the role of the PD-1/PD-L1 pathway in the pathophysiology of NCC and BC.

Materials and Methods

A total of 40 bladder specimens were obtained during transurethral resections of the bladder: 20 from patients with NCC and 20 from patients with BC. NCC was defined as a recurrent or persistent condition of cystitis-like symptoms for at least 6 months with a negative urine culture. The NCC patients underwent cystoscopy with hydrodistension for 5 min. The saline was drained at the maximum bladder capacity.

The clinical stage of BC was characterized according to the 2017 TNM classification; for grading, the 1973 WHO classification was used. Among the patients with BC, 25% ($n=5$) had non-muscle invasive BC and 75% ($n=15$) had muscle-invasive BC. For staging, contrast-enhanced computed tomography scans of the chest, abdomen, and pelvis were performed in patients with confirmed muscle-invasive BC.

Histological Evaluation

Formalin-fixed and paraffin-embedded bladder tissue slides stained with hematoxylin and eosin were examined. The degree of inflammation of the bladder wall was graded as follows: mild, with scattered immune cells in the specimen; moderate, with inflammatory infiltration of less than 50% of the bladder wall; and severe, with inflammatory infiltration of more than 50% of the bladder wall. Additionally, the presence of lymphoid aggregates in the submucosa and koilocytosis were assessed.

Real-Time Polymerase Chain Reaction

All bladder samples were stored in liquid nitrogen, then slowly thawed and mechanically homogenized with Lysing Matrix A ceramic beads in FastPrep®-24 Classic (MP Biomedicals, USA). DNA was extracted using Maxwell® RSC 48 (Promega, USA). The amplification and detection were conducted on a CFX96® Touch System (Bio-Rad Laboratories, USA), according to the manufacturer's protocol. The RealBest HSV-1 and HSV-2 polymerase chain reaction (PCR) Kits, RealBest EBV PCR Kits, RealBest CMV PCR Kits, and RealBest HPV screen PCR for types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 (Vector Best, Russia) were used. The presence of viral pathogens was determined by measuring the increase in fluorescence of the FAM fluorophore.

Immunohistochemistry

Paraffin-embedded sections of the bladder specimens 5 μm thick were prepared. After deparaffinization and rehydration of the tissue with xylene and graded ethanol series, antigen retrieval was performed using the Dako Target Retrieval Solution (Dako North America, USA). Endogenous peroxidase was blocked with a 3% hydrogen peroxide solution. PD-L1 expression was detected using the EnVision FLEX visualization system on AutostainerLink 48 (Dako) after the application of the monoclonal mouse anti-PD-L1 clone 22C3 (Dako). The immune cell score, which refers to the percentage of the area covered with PD-L1-positive immune cells, was calculated for both NCC and BC specimens. The tumor cell (TC) score was calculated only for BC specimens. PD-L1 expression was defined based on the immune cell score as follows: none ($<1\%$), mild ($\geq 1\%$ and $<5\%$), moderate ($\geq 5\%$ and $<10\%$), and high ($\geq 10\%$). The pathologist was blinded to the clinical and pathological data.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics (IBM Corp., USA). The Kolmogorov–Smirnov test was used to determine whether the data were normally distributed. Means with 95% confidence intervals were calculated for continuous variables, and percentage frequencies were used to present categorical data. Differences between study groups were tested using Fisher's exact test. The associations between the two variables were evaluated using Spearman's rank correlation coefficient, as either (or both) variables were ordinal. Probability values (p-values) less than 0.05 were considered statistically significant.

Results

Tables 1 and 2 provide clinical information about the patients with NCC (average age: 35.2 ± 4.3 years) and BC (average age: 69.5 ± 4.0 years), accordingly. The results of the pathological examination and real-time PCR of the bladder tissue biopsies are shown in Tables 3 and 4. Viral pathogens were detected in 50% of NCC patients and 60% of BC patients. In both NCC and BC, the EBV was most commonly found (30% and 55% respectively). Severe inflammatory infiltration of the bladder wall was observed more often in BC than in NCC (50% vs 15%, $p = 0.041$). There were no statistically significant differences in the presence of lymphoid aggregates and koilocytosis between NCC and BC.

In NCC, the presence of viral DNA correlated positively with the degree of inflammatory infiltration of the bladder wall (r = 0.624, $p = 0.003$), the presence of lymphoid aggregates in the submucosa (r = 0.577, $p = 0.008$), and koilocytosis (r = 0.816, $p < 0.001$). In BC, the presence of viral DNA also correlated positively with the degree of inflammatory infiltration of the bladder wall (r = 0.791, $p < 0.001$) and the presence of lymphoid aggregates in the submucosa (r = 0.471, $p = 0.036$), but not with koilocytosis (r = 0.375, $p = 0.103$).

PD-L1 Expression in the Bladder Wall

Among NCC patients, PD-L1 expression was positive in 40% of cases (8 out of 20): mild expression was seen in 25% of patients (5 out of 20), moderate expression in none, and high expression in 15% (3 out of 20). Meanwhile, PD-L1 expression was detected significantly less often in NCC patients with cystoscopic findings than in NCC patients without these findings (10% vs 70%, $p = 0.020$). The immune cell score in NCC correlated strongly with the degree of inflammatory infiltration of the bladder wall (r = 0.867, $p < 0.001$), the presence of lymphoid aggregates in the

Table 1 Clinical characteristics of patients with nonbacterial chronic cystitis

Patient	Sex	Age	Inflammatory infiltration	Lymphoid aggregates	Koilocytosis	Cystoscopy	Viral pathogens	Immune cell score, %
1	Female	34	Mild	–	–	–	–	0
2	Female	31	Moderate	–	–	–	EBV, HPV 16	3
3	Female	40	Severe	+	+	–	HSV-2	17
4	Female	23	Moderate	–	–	–	EBV	1
5	Female	25	Mild	–	+	–	EBV	1
6	Female	28	Mild	–	–	–	–	0
7	Female	44	Mild	–	–	–	–	0
8	Female	31	Severe	+	+	–	HPV 31, HSV-2	25
9	Female	25	Severe	+	+	–	HPV 68, HSV-2	18
10	Female	32	Moderate	+	+	–	EBV	4
11	Female	34	Mild	–	–	Hunner ulcers	–	0
12	Female	32	Mild	–	–	Glomerulations	–	0
13	Female	56	Mild	–	–	Hunner ulcers	–	0
14	Female	58	Mild	–	–	Glomerulations	–	0
15	Female	38	Moderate	+	+	Glomerulations	EBV, CMV	1
16	Female	24	Mild	+	+	Glomerulations	HPV 51	0
17	Female	33	Mild	+	+	Glomerulations	–	0
18	Female	48	Moderate	–	–	Glomerulations	EBV	0
19	Female	32	Mild	–	–	Glomerulations	–	0
20	Female	36	Mild	–	–	Hunner ulcers	–	0

Table 2 Clinical characteristics of patients with bladder cancer

Patient	Sex	Age	Stage	Grade	Histology	Inflammatory infiltration	Lymphoid aggregates	Koilocytosis	Viral pathogens	Tumor cell score, %	Immune cell score, %
1	Male	64	T1N0M0	G1	NPUC	Moderate	+	+	EBV, HPV 16	29.5	3
2	Male	73	T1N0M0	G1	NPUC	Mild	+	−	–	1.5	2
3	Male	70	T2aN0M0	G3	IUC	Mild	−	−	–	0	2
4	Male	68	T1N0M0	G1	NPUC	Severe	+	+	EBV	12	7
5	Male	75	T2bN0M0	G2	IUC	Mild	+	+	EBV	5	4
6	Male	81	T2aN0M0	G2	IUC	Mild	−	−	–	0	0.5
7	Male	71	T2aN0M0	G3	IUC	Severe	+	−	HSV-2	0	3
8	Male	67	T1N0M0	G1	NPUC	Mild	+	−	–	0	0
9	Male	84	T3aN0M0	G3	IUC	Moderate	−	+	–	0	3
10	Male	65	T2aN1M0	G3	IUC	Severe	−	+	EBV, HSV-2	0	7
11	Male	77	T4aN0M0	G3	IUC	Moderate	−	+	–	0	2
12	Male	78	T2aN0M0	G2	IUC	Severe	+	+	EBV	1	7
13	Male	65	T3aN2M0	G3	IUC	Severe	+	−	EBV, CMV	2	3
14	Female	77	T3aN0M0	G3	IUC	Severe	+	+	EBV	90	15
15	Male	50	T1N0M0	G2	NPUC	Severe	+	+	EBV, HPV 52, CMV	99	5
16	Male	59	T3aN0M0	G1	IUC	Severe	+	+	EBV, HPV 66	70	1.5
17	Male	67	T3bN1M0	G1	IUC	Moderate	+	+	–	0	1
18	Female	81	T2bN0M0	G3	IUC	Mild	−	−	–	0	2
19	Male	64	T3aN1M0	G3	IUC	Severe	+	+	EBV, CMV	0	0.5
20	Male	53	T2aN1M0	G3	IUC	Severe	−	−	EBV, HPV 39	0	4

IUC infiltrating urothelial carcinoma, *NPUC* non-invasive papillary urothelial carcinoma, *EBV* Epstein–Barr virus, *HPV* human papillomavirus, *HSV* herpes simplex virus, *CMV* cytomegalovirus

Table 3 Pathological examination of bladder specimens, immunohistochemistry, and real-time PCR results in nonbacterial chronic cystitis and bladder cancer patients

	Nonbacterial chronic cystitis ($n=20$)	Bladder cancer ($n=20$)	p-value*
Positive PD-L1 expression, *n* (%)	8 (40)	17 (85)	0.008
Inflammatory infiltration, *n* (%)			
Mild	12 (60)	6 (30)	0.111
Moderate	5 (25)	4 (20)	1
Severe	3 (15)	10 (50)	0.041
Lymphoid aggregates, *n* (%)	7 (35)	13 (65)	0.113
Koilocytosis, *n* (%)	8 (40)	12 (60)	0.343
Viral pathogens, *n* (%)	10 (50)	12 (60)	0.751
HSV	3 (15)	2 (10)	1
EBV	6 (30)	11 (55)	0.200
CMV	1 (5)	4 (20)	0.342
HPV	4 (20)	3 (15)	1

HSV herpes simplex virus, *EBV* Epstein–Barr virus, *CMV* cytomegalovirus, *HPV* human papillomavirus

*Fisher's exact test was used

submucosa ($r=0.804$, $p<0.001$), koilocytosis ($r=0.620$, $p=0.004$), and the presence of viral pathogens ($r=0.784$, $p<0.001$). No statistically significant correlation between the immune cell score and the age of NCC patients was found ($r=-0.379$, $p=0.100$).

Expression of PD-L1 was positive in 85% of BC patients (17 out of 20): mild expression was detected in 60% of patients (12 out of 20), moderate expression in 20% (4 out of 20), and high expression in 5% (1 out of 20). The immune cell score correlated with the degree of inflammatory infiltration of the bladder wall ($r=0.534$, $p=0.015$) and the

Table 4 Pathological examination of bladder specimens, immunohistochemistry, and real-time polymerase chain reaction results in nonbacterial chronic cystitis patients with and without cystoscopic findings

	Without cystoscopic findings ($n=10$)	With cystoscopic findings ($n=10$)	p-value*
Positive PD-L1 expression, *n* (%)	7 (70)	1 (10)	0.020
Inflammatory infiltration, *n* (%)			
Mild	4 (40)	8 (80)	0.170
Moderate	3 (30)	2 (20)	1
Severe	3 (30)	–	–
Lymphoid aggregates, *n* (%)	4 (40)	3 (30)	1
Koilocytosis, *n* (%)	5 (50)	3 (30)	0.650
Viral pathogens, *n* (%)	7 (70)	3 (30)	0.179
HSV	3 (30)	–	–
EBV	4 (40)	2 (20)	0.629
CMV	–	1 (10)	–
HPV	3 (30)	1 (10)	0.582

HSV herpes simplex virus, *EBV* Epstein–Barr virus, *CMV* cytomegalovirus, *HPV* human papillomavirus

*Fisher's exact test was used

presence of viral pathogens (r = 0.626, $p = 0.003$), but not with the presence of lymphoid aggregates in the submucosa (r = 0.083, $p = 0.729$), and koilocytosis (r = 0.366, $p = 0.112$). The TC score correlated with the presence of lymphoid aggregates in the submucosa (r = 0.627, $p = 0.003$) and the presence of viral pathogens (r = 0.562, $p = 0.010$), but not with the degree of inflammatory infiltration of the bladder wall (r = 0.325, $p = 0.163$), and koilocytosis (r = 0.407, $p = 0.075$). There was no correlation between the TC score and the age of BC patients (r = −0.285, $p = 0.223$) and between the immune cell score and the age of BC patients (r = 0.001, $p = 0.997$).

Discussion

Currently, the upregulation of the PD-1/PD-L1 inhibitory pathway in BC is unquestionable [8, 11]. It was proven that high PD-L1 expression in the bladder tissue is associated with more advanced tumors, higher recurrence rates, and lower survival rates [8, 12]. In contrast, PD-L1 (B7-H1) expression is not observed in normal tissues, particularly in the bladder tissue [13–16].

Schistosomiasis and viral infections (high-risk human papilloma viruses [HPVs] and EBV) are known to contribute to the development of BC [17, 18]. According to recent findings, cancer cells and some viral pathogens (e.g., EBV, CMV, HIV) can modulate the PD-1/PD-L1 axis by inducing the overexpression of PD-L1 in tissues [6, 19]. Activation of this inhibitory axis helps viral pathogens to subvert the host antiviral immune response. For instance, PD-L1 is expressed in EBV-positive cancers, such as Hodgkin lymphoma, nasopharyngeal cancer, gastric cancer, as well as chronic active EBV infection [19]. HPV oncoproteins E5 and E6/E7 can upregulate the PD-1/PD-L1 pathway [20]. We verified the PD-L1 overexpression in a cohort of BC patients and its strong association with the presence of viral DNA in bladder tissue. EBV-positive bladder specimens and koilocytosis were seen in more than half of BC patients. Associated with HPV infection, koilocytes are squamous cells with nuclear features of low-grade squamous intraepithelial lesions. HPV infection in BC patients was detected in previous studies; however, clear associations between HPV and urothelial cancer have not yet been established [21]. As we performed PCR analyses for only 14 high-risk HPV types, the HPV-positive bladder specimen count may have been underestimated.

Upregulation of the PD-1/PD-L1 pathway is seen not only in malignant diseases. On the one hand, this signaling pathway plays a substantial role in immunoregulation by limiting immune-mediated tissue damage during infection. On the other hand, virus-induced PD-L1 expression is considered an immune evasion strategy [6]. Chronic bacterial and viral infections cause persistent stimulation of antigen-specific T cells, which additionally induces early T cell exhaustion [19, 20]. Benedict et al. demonstrated that CMV-infected dendritic cells in mice express PD-L1, thereby contributing to antigen-specific T cell anergy [22]. PD-1 blockade can potentially reverse this functional immunodeficiency [23].

Programmed death ligand-1 is usually expressed by T and B cells, epithelial cells, endothelial cells, tumor cells, dendritic cells, and macrophages in areas of dense inflammatory infiltration [8]. It has been reported that CD8 T cells, proinflammatory cytokines (e.g., IFN-γ, TNF-α, IL-4), transcription factors, and microRNAs positively affect the PD-L1 expression in tissues. The results of our study are consistent with those of previous studies; PD-L1 expression correlated significantly with the inflammatory infiltration of the bladder

wall and the presence of lymphoid aggregates in both NCC and BC patients but did not correlate with the age of those patients [15, 24].

Recently, viral infections have been investigated as potential etiological factors of IC. Jhang et al. showed that EBV was present in 87.5% of bladder specimens from ulcerative IC patients and in 17.4% of specimens from non-ulcerative IC patients [3]. In our study, 30% of the bladder specimens from NCC patients were EBV positive. Novel findings included the detection of PD-L1 expression and its association with the presence of viral pathogens in NCC patients. However, the PD-L1 positivity rate in NCC patients is lower than in BC patients, partially due to the lower prevalence of concomitant viral infections. Additionally, the PD-L1 positivity rate in NCC patients with no cystoscopic findings was found to be higher than that in IC patients. Previously Chen et al. detected PD-L1 expression in a cohort of patients with relatively severe IC and found a positive correlation between the degree of PD-L1 expression and the effectiveness of hydrodistension [5].

We acknowledge that the limited number of study participants ($n=40$) led to some results being statistically insignificant. More prospective and retrospective studies at the molecular level are required to confirm the associations between the PD-L1 expression, viral infections, and inflammatory infiltration of the bladder wall found in this study. An in-depth analysis of NCC pathophysiology may lead to the appreciation of the PD-1/PD-L1 pathway as a potential therapeutic target.

Conclusions

Expression of PD-L1 was detected in a cohort of NCC patients, although the PD-L1 positivity rate was lower than that in BC. The degree of PD-L1 expression in bladder tissue is associated with the presence of viral infections and the degree of inflammatory infiltration of the bladder wall in both NCC and BC.

Authors' contributions I.K.: study conception and design, data collection, revision of the manuscript; V.B.: study conception and design, data collection, analysis and interpretation of results, draft manuscript preparation; L.G.: data collection, revision of the manuscript; D.K.: data collection, revision of the manuscript. All authors read and approved the final version of the manuscript.

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Data availability The authors confirm that all data generated or analyzed during this study are available within the article.

Declarations

Ethical approval The study was approved by the Local Ethics Committee of the Russian Medical Academy of Continuous Professional Education (Protocol #15, 16/11/2021) and conducted in accordance with the principles of the Helsinki Declaration. All patients signed an informed consent form before participating in the study.

Conflicts of interest None.

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