

Original Article

Expression of tumoral GSK3- β , PD-L1, and CD8 cell density in urothelial carcinomas, association with tumor grade and overall survival

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Abstract: Bladder cancer is the most common malignancy in the urinary tract, and is biologically and clinically quite heterogeneous. Around 90% of diagnoses are made in the 6th decade, being more prevalent in males. The programmed cell death 1 (PD-1) and programmed cell death ligand 1 (PD-L1) axis play a putative role in immune checkpoint and as a means through which cancer evades the immune system. Inhibition of the glycogen synthase kinase (GSK) 3 leads to the downregulation of PD-1 via upregulation of the transcription factor Tbet. The use of biomarkers PD-L1 and GSK-3 β and evaluation of the immune infiltrate have very promising correlations with urothelial carcinoma prognosis and treatment prediction. Objective: To investigate the protein expression of PD-L1 and GSK-3 β and the CD8-positive immune infiltrates in bladder carcinomas. Materials and methods: This was a cross-sectional study of 140 samples of urothelial carcinomas from 2015 to 2018. Automated digitally assisted scoring and conventional analyses of the markers of GSK-3 β (27C10), CD8 (7103 β) and PDL-1 (22c3), were reviewed by two pathologists independently and a histologic score was calculated. The density of CD8 was also measured. Results: The immunoreexpression of GSK-3 β (91%) was presented in most samples, PD-L1 in 62.9% and CD8 cells present in 46.3% of cases. When analyzed in conjunction, the levels of GSK-3 β and PD-L1 ($P = 0.033$), and CD8 and PD-L1 ($P < 0.002$) showed significant correlations. No significant associations were observed between GSK-3 β and CD8. The positivity of GSK-3 β and PD-L1 was predominant in high-grade tumors. Conclusion: Despite the tumor microenvironment heterogeneity, the expression of CD8, GSK-3 β and PDL1 could be valuable and GSK-3 β could be a potential target in advanced bladder cancer, especially in the context of immunotherapy.

Keywords: Bladder cancer, urothelial carcinoma, immunotherapy, programmed cell death ligand 1 (PD-L1), glycogen synthase kinase (GSK) 3, CD8, immunohistochemistry

Introduction

Bladder cancer is the most common neoplasia of the urinary tract and is the first cause of death among human urologic cancers. More than 575 thousand new cases of bladder cancer are estimated worldwide, varying according to specific regions [1]. Indices from the Brazilian National Cancer Institute (INCA) showed that the rate of new cases of bladder cancer in Brazil from 2020 to 2022 would be 7,590 cases for men and 3,050 for women [2, 3]. The most common histologic subtype is urothelial carcinoma and its variants, followed by squa-

mous cell carcinoma and adenocarcinoma. Urothelial carcinoma is more frequent in men than in women, with proportions of 3:1, accompanied by proportionally high incidence rates at ages 60 to 70 years and by high rates of recurrence after resection [3].

Glycogen synthase kinase (GSK) 3 is a serine/threonine kinase that was originally identified as a regulator of glycogen metabolism, composed of the two isoforms GSK- α and GSK- β (GSK-3 β), and both encoded by different genes located on chromosome/7, chromosome/19, chromosome/16, chromosome/3 [4-6].

Recently, several studies have observed that GSK-3 β is directly linked to the processes of neoplastic transformation, tumor growth, and metastasis [7, 8]. GSK-3 β has been identified as a promising new therapeutic target in bladder cancer [9, 10]. More recently, a new molecule that acts as a GSK-3 β inhibitor, 9-ING-41, has shown antitumor activity in bladder cancer cell lines [9]. Clinically, this drug has shown antitumor effects in neuroblastoma, B-cell lymphoma, glioblastoma, ovarian, pancreatic, renal, and breast cancer [10-12].

GSK-3 β has been proven to act in cell cycle regulation, apoptosis, and immune response in several cancers, with its nuclear labeling linked to high tumor grade [13, 14]. It is constitutively active in resting T cells [15]. Programmed cell death protein 1 (PD-1) is an inhibitory receptor also present in T cells, and its ligand causes the induction and maintenance of the immune response. Thus, the inhibition of this interaction (PD1/PD-L1) increases the activity of T cells, causing an antitumor reaction, and the inactivation of GSK- β blocks the expression of PD1 in CD8 lymphocytes [16]. Expression of active GSK-3 β may also inhibit the proliferation of T cells [17]. In this study, we aimed to evaluate the immunostaining of GSK-3 β in bladder cancer and its association with PD-L1 tumor proportion score and CD8 positive infiltrating cells in the tumor microenvironment e series of urothelial carcinomas.

Methods

Patient samples

This was an observational, cross-sectional and analytical study of a population diagnosed with bladder cancer in a single institution between 2015 and 2018.

One hundred and forty (140) sequential patients were analyzed and included according to inclusion criteria. Clinical and laboratory data were collected from electronic medical records in the respective Hospital Units where each patient was being followed up. All samples from patients diagnosed with invasive and non-invasive bladder cancer confirmed by histological analysis, with sufficient material for the construction of Tissue Microarray (TMA), were included. Samples that did not have materials available to construct the TMA were

excluded. The blocks and slides were stained with hematoxylin and eosin (HE). The Research Ethics Committee approved this study at the Federal University of Ceará (Protocol Number: 3.997.304).

Tissue microarray and immunohistochemistry

The preparation of the TMA was described according to Filho et al. (2021). Briefly, the histologic slides were reviewed by an experienced genitourinary pathologist, and areas from the invasive front of the tumor, and areas with inflammation, were selected. Paraffin blocks were submitted to 3 μ histological sections, subsequently fixed on FLEX IHC Microscope Slides (Agilent®). GSK-3 β (27C10, 1:100, Cell-Signaling®), CD8 (m7103, Dako®), and PDL-1 (22c3, Dako®) immunostaining were performed according to individual protocols for each antibody in Ventana BenchMarck GX machines, Agilent Autostainer, and manual reaction.

Briefly, GSK-3 β followed the antigen recovery process on the PT Link equipment with a pH 6.0 buffer solution, use of the Envision Flex kit (Agilent) for blocking endogenous peroxidase, overnight incubation with primary antibody (corresponding to 12 hours of reaction), immunological amplification and counterstaining with EnVision FLEX (Dako® K4065) + DAB (Dako® K3469).

Slides were scanned in the 3DHitech equipment (Pannoramic Desk®). The files were then imported to Qupath® software, which allowed ample analysis. The files were loaded onto a project in QuPath software (QuPath source code, documentation, and links to the software download are available at <https://qupath.github.io>). QuPath's segmentation feature can detect thousands of cells, identify them as objects in a hierarchical manner below the annotation, TMA cores, or cases, and measure cell morphology and biomarker expression at the same time.

CD8 density was calculated by running a script on QuPath with an output of CD8 positive cells/mm² of tissue (for each core). For PD-L1 and GSK-3 β scoring, analyses were run based on Positive Cell Detection and membrane and cytoplasm staining. Quantification of cytoplasmic positive (mean DAB staining) was measured only in cells detected as tumor cells and

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Table 1. Clinical and histopathological profile of patients with bladder cancer

	n (%)
Sex	
Female	31 (22.1%)
Male	109 (77.9%)
Age (74.9±13.1; 24-108)	
Below 70	47 (33.6%)
>70	93 (66.4%)
Risk factors	
Smoking	21 (15.1%)
Hypertension	21 (15.0%)
DM	4 (2.9%)
Treatment	
Surgery	73 (88.0%)
Adjuvant chemotherapy	27 (32.5%)
Adjuvant radiation therapy	8 (9.6%)
Histological Features	
Muscle Invasion	83 (59.3%)
Necrosis	24 (17.1%)
Invasion/Low-grade	14 (43.8%)
Invasion	66 (65.3%)
Final_pathological grade	
Low-grade	32 (24.1%)
High-grade	101 (75.9%)

not stroma and other cells. QuPath output was able to identify numbers of GSK-3B positive cells/mm² and, in addition, the H-score method was applied by the software, based on the extent and intensity of cytoplasmic staining (1-3), multiplied by the percentage of cells positive (proportion score), with a potential score ranging from 0-300.

Pathologist analysis

All cases evaluated by software analyses on QuPath were confirmed by two experienced (>10 years of experience) pathologists (FT and CDN). The evaluation slides were digitized at 400× magnification and scanned in the 3D-Histech equipment (Pannoramic Desk®). These analyses were calibrated with an excellent agreement between them (correlation coefficient interclass = 0.810, 0.991 and 0.920 for CD8, PDL1 and GSK-3β, respectively). The number of CD8+ cells/mm² counting was performed by each pathologist and the percent and intensity of GSK-3β immunostaining was equally performed to calculate H-score. A

pathologist score PD-L1 on each core using the established Tumor Proportion Score (TPS) method (0-100) [34]. It was defined as the percentage of viable tumor cells showing partial or complete membrane PD-L1 staining at any intensity.

Statistical approach

The mean ± SD of immunostaining was calculated by each method and analyzed by Spearman correlation and Mann-Whitney test (both nonparametric data). Clinic data was showed as absolute and percentual frequencies and immunostaining of each immunomarker was categorized by (GSK-3β H-score cutoff point = 100; CD8 = cutoff point = 20; PDL1 cutoff point = 0) and associated with clinic data by chi-square test. Kaplan-Meier curves were used to calculate recurrence free time and overall survival and Log-Rank Mantel-Cox and Cox regression models were used to determine risk factors for recurrence and death. All analyses were performed in SPSS v20.0 for Windows (IMB®). Significance level was 5% for all analyses.

Additionally, ROC curves were used for markers having death as an outcome in order to estimate immunostaining points suggestive of a worse prognosis.

Results

Clinical and epidemiological characterization of patients with urothelial carcinoma

A total of 140 patients were included, with samples obtained from the transurethral resection or total cystectomies. The final cohort consisted of 140 urothelial carcinoma tissue samples, most of which were from men (n = 109, 77.9%). The mean age of patients was 74.9±13.1, ranging from 24-108 years of age. Most patients were over 70 years old (n = 93, 66.4%). Smoking and systemic arterial hypertension were described in 21 patients and diabetes mellitus in only four (**Table 1**).

The majority underwent surgery, either transurethral resection of the bladder or cystectomy (n = 73, 88.0%), 27 (32.5%) patients underwent adjuvant chemotherapy and 8 (9.6%) underwent adjuvant radiotherapy. Histologically, invasion into the detrusor muscle was present in 83 (59.3%) patients and necrosis in 24 (17.1%).

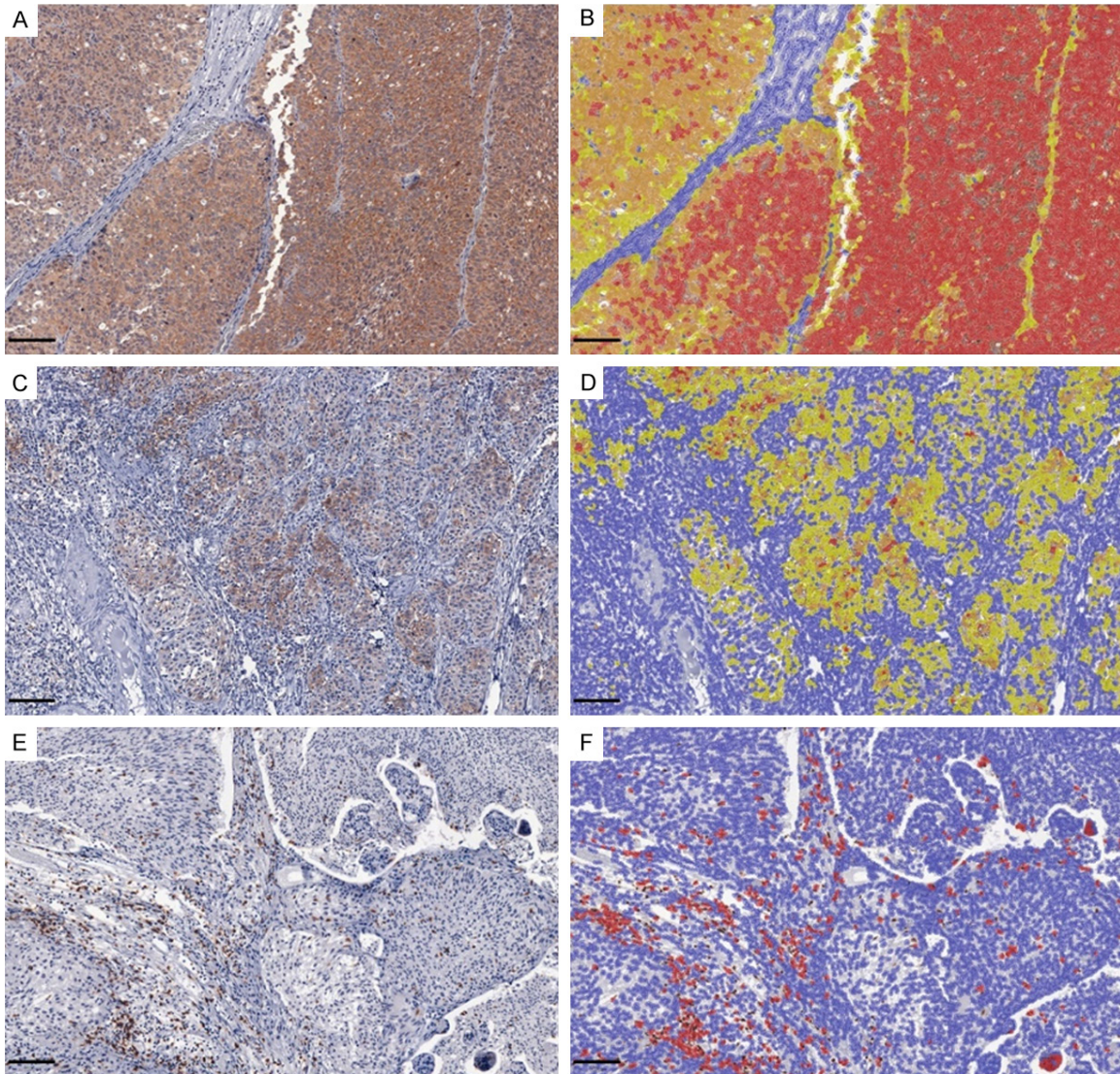


Figure 1. Schematic panel showing immunohistochemical reaction results for GSK-3 β (A and B), PD-L1 (C and D), and CD8 (E and F). (B, D and F) represent Qupath's color outputs. The color outputs for PD-L1 and GSK are blue for negative, yellow for weak positivity, orange for moderate positivity and red for strong positivity. These scores are used in calculator the H-score. The color output for CD8 positive detection used a single threshold (red) for positive cells. Bar = 100 μ m.

Most tumors were classified as high grade (n = 101, 75.9%). All invasive tumors were high-grade (n = 66, 65.3%) (**Table 1**).

ROC curves were determined as prognostically suggestive cutoffs (>0%) for PDL1, (>200) for GSK and (>20) for CD8.

Immunoexpression profile for GSK-3 β in patients with bladder cancer and its relationship with CD8 and PDL1 immunoexpression

High GSK-3 β immunoexpression (H-score above 200) was observed in 12 patients (9.0%)

(**Figure 1A** and **1B**) and six samples could not be evaluated due to loss of tissue in the sections. Overall, the mean GSK-3 β H-score was 71.98 ± 73.98 , ranging from 0 to 300. Nuclear GSK-3 β was seen in 25 cases (18.8%), of which 20 (80.0%) cases were high-grade (P = 0.598). Tumors with nuclear GSK-3 β had PD-L1 TPS of 60.7%, compared to 63.4% in tumors without nuclear GSK-3 β (P = 0.793) (**Table 2**).

Regarding PDL1, 52 (37.1%) patients did not show tumor immunoexpression for PDL1, and 88 (62.9%) showed at least focal marking. The mean PDL1 tumor proportion score (TPS) was

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Table 2. Influence of sociodemographic variables on CD8 positive cell density, PD-L1 and GSK-3 β immunoexpression, recurrence-free time and overall survival of patients with urothelial bladder carcinoma

	GSK-3 β H-score		<i>p</i> Value ^a	PDL1 TPS		<i>p</i> - Value ^a	CD8 (cells/mm ²)			RFT (%)	Relapse-free time		<i>p</i> - Value ^c	SG (%)	Overall survival		<i>p</i> - Value ^c
	Below 200	>200		0%	>0%		Below 20	>20	<i>p</i> - Value ^a		Average \pm SD (CI 95%) ^b	Median (CI 95%)			Average \pm SD (CI 95%) ^b	Median (CI 95%)	
All	122 (91.0%)	12 (9.0%)	-	52 (37.1%)	88 (62.9%)	-	75 (54.0%)	64 (46.0%)	0	68 (80.0%)	50.63 \pm 7.66 (35.63-65.64)	47 (40.26-53.74)	-	77 (89.5%)	76.06 \pm 4.69 (66.86-85.26)	(-)	
Sex																	
Female	20 (21.3%)	10 (25.0%)	0.636	10 (19.2%)	21 (23.9%)	0.524	16 (21.3%)	15 (23.4%)	0.766	12 (75.0%)	41.05 \pm 3.61 (33.98-48.12)	47 (40.51-53.49)	0.947	15 (93.8%)	46.00 \pm 2.85 (40.42-51.58)	(-)	0.441
Male	74 (78.7%)	30 (75.0%)		42 (80.8%)	67 (76.1%)		59 (78.7%)	49 (76.6%)		56 (81.2%)	53.75 \pm 9.37 (35.39-72.11)	52 (36.26-67.74)		62 (88.6%)	74.11 \pm 5.91 (62.54-85.69)	(-)	
Age																	
Below 70	34 (36.2%)	10 (25.0%)	0.208	22 (42.3%)	25 (28.4%)	0.092	28 (37.3%)	18 (28.1%)	0.250	26 (81.3%)	44.34 \pm 3.17 (38.13-50.55)	47 (38.89-55.11)	0.470	32 (100.0%)	(-)	(-)	0.014 [*]
>70	60 (63.8%)	30 (75.0%)		30 (57.7%)	63 (71.6%)		47 (62.7%)	46 (71.9%)		42 (79.2%)	59.08 \pm 7.97 (43.46-74.69)	(-)		45 (83.3%)	68.58 \pm 6.81 (55.22-81.93)	(-)	
Smoking																	
No	77 (82.8%)	35 (87.5%)	0.495	41 (80.4%)	77 (87.5%)	0.259	64 (86.5%)	53 (82.8%)	0.549	51 (78.5%)	51.16 \pm 8.36 (34.77-67.55)	52 (43.08-60.92)	0.699	59 (90.8%)	79.76 \pm 3.61 (72.69-86.84)	(-)	0.529
Yes	16 (17.2%)	5 (12.5%)		10 (19.6%)	11 (12.5%)		10 (13.5%)	11 (17.2%)		17 (85.0%)	39.14 \pm 2.19 (34.85-43.42)	41 (36.63-45.37)		18 (85.7%)	38.04 \pm 2.72 (32.71-43.38)	41 (0.00-88.42)	
Hypertension																	
No	80 (85.1%)	34 (85.0%)	0.987	47 (90.4%)	72 (81.8%)	0.170	64 (85.3%)	54 (84.4%)	0.875	53 (81.5%)	53.03 \pm 8.31 (36.75-69.32)	52 (43.06-60.94)	0.170	60 (92.3%)	79.09 \pm 4.71 (69.86-88.32)	(-)	0.084
Yes	14 (14.9%)	6 (15.0%)		5 (9.6%)	16 (18.2%)		11 (14.7%)	10 (15.6%)		15 (75.0%)	32.23 \pm 3.19 (25.96-38.49)	(-)		17 (81.0%)	32.90 \pm 3.18 (26.65-39.14)	(-)	
DM																	
No	92 (97.9%)	38 (95.0%)	0.371	51 (98.1%)	85 (96.6%)	0.610	73 (97.3%)	62 (96.9%)	0.872	65 (80.2%)	50.89 \pm 7.68 (35.83-65.96)	47 (40.25-53.75)	0.477	74 (90.2%)	76.68 \pm 4.71 (67.46-85.91)	(-)	0.198
Yes	2 (2.1%)	2 (5.0%)		1 (1.9%)	3 (3.4%)		2 (2.7%)	2 (3.1%)		3 (75.0%)	26.50 \pm 7.36 (12.07-40.93)	(-)		3 (75.0%)	26.50 \pm 7.36 (12.07-40.93)	(-)	

^{*}P<0.05, ^aChi-square test or Fisher's exact test (n, %); ^bMann-Whitney Test (Average \pm SD); ^cLog-Rank Mantel-Cox Test (median time \pm SD and median time of recurrence-free survival and overall survival calculated using Kaplan-Meier curves). CI 95% = 95% confidence interval; SLR = Relapse-free survival; GS = global survival; H-score = HistoScore.

9.00±23.60%, ranging from 0 to 100 (Tables 2-4; Figure 1C and 1D).

CD8+ positive lymphocytes were entirely absent in only four samples (2.9%). The average CD8 density was 61.10±101.94 cells/mm², ranging from 0 to 538 cells/mm². Seventy-five (54.0%) samples had less than 20 cells/mm² and 64 (46.0%) exhibited more than 20 cells/mm² (Figure 1E and 1F). Only one sample was not evaluated due to tissue loss in the CD8 immunohistochemistry stain.

There was a significant association between GSK-3β and PDL1 (P = 0.033), but not GSK-3β with CD8 (P = 0.760). However, CD8 immunorexpression was significantly associated with PDL1 tumor positivity (P<0.002) (Table 4).

GSK-3β immunorexpression was not significantly influenced by any clinical, therapeutic, or histopathological characteristics. PDL1 positivity was associated with high-grade tumors (P = 0.030), and CD8+ lymphocyte density was inversely associated with prior RT treatment (P = 0.021) (Table 4).

Analysis of global and disease-free survival in bladder cancer with invasion of the muscle layer

Overall the mean recurrence-free survival was 50.63±7.66 months, with 80.0% of the patients showing no recurrence in 2021. The mean overall survival was 76.06±4.69, with 89.5% of patients alive during the evaluated period. In univariate analysis, not a single variable affected relapse-free time. PDL1 positivity, however, had a 5.81 times higher risk in patients with recurrence (95% CI, 1.06-31.80). High-grade tumors, as expected, increase the risk of recurrent 9.09 (95% CI, 1.11-100.00) times, independent of the other variables (Table 5).

Discussion

The study investigated 140 cases of bladder cancer, a cohort predominant in males with a ratio of 3:1 and more than half of the patients were over 75 years old, similar to the recent literature [1, 18, 19]. The incidence of bladder cancer in the world has differences according to geographic patterns, with the highest prevalence in smokers. Over time, some countries, such as Spain, the Netherlands, Germany, and

Russia, increased women with bladder cancer due to the rise in tobacco consumption [1]. Unfortunately, we did not have a smoking history in all patients, as one of the weaknesses of the study.

Immune checkpoint blockade with anti-PD1 or anti-PDL1 is a highly promising treatment for urothelial carcinomas. A recent phase III trial for muscle-invasive bladder carcinoma compared adjuvant nivolumab with placebo showed a more prolonged disease-free survival, especially in patients with more than 1% PD-L1 expression [20]. In our study, more than 60% of patients showed at least 1% positivity for PD-L1, with a strong association with CD8-positive immune infiltrates and a less strong association with GSK-3β expression. Other studies have evaluated pembrolizumab and atezolizumab in combination or monotherapy, with a better overall response in patients with positive PD-L1 tumors [21, 22]. Currently, these drugs are approved in some countries as monotherapy for PD-L1 positive patients (score above 5%) considered cisplatin-ineligible [23].

The expression of GSK-3β has already been described in breast, lung, pancreas, colorectal cancer and leukemia patients [24, 25]. However, the specific role of this protein in bladder cancer is still unknown [9, 10, 26]. Association of PD-L1 and GSK-3β has been studied in other neoplasms, and our findings that patients with PD-L1 had a higher frequency of strong expression of GSK-3β has been reported before [27]. Despite not finding a significant correlation of GSK-3β with CD8 infiltrating lymphocytes, it has been postulated that inactivation by GSK-3β blocks the expression of PD1 in CD8+ cytotoxic T lymphocytes, increasing cellular immunity through this negative regulation of PD-1 [28, 29].

Our data showed a significant correlation between CD8 lymphocyte density and with tumoral PD-L1 expression. In addition, patients with higher GSK-3β showed positivity to PDL1, suggesting deregulation of cellular behavior, causing greater expression of PD-L1, and possibly causing evasion in immunity mechanisms [30]. Based on this assumption, when this association was evaluated in patients treated with radiotherapy, T cells, which can also overexpress PDL-1, contribute to the prevention of

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Table 3. Influence of therapeutic modalities on GSK-3 β immunoexpression, recurrence-free time and overall survival of patients with urothelial bladder carcinoma

	GSK-3 β H-score		<i>p</i> -value ^a	PDL1 TPS		<i>p</i> -value ^a	CD8 (cells/mm ²)		<i>p</i> -Value ^a	RFT (%)	Recurrence-free time		<i>p</i> -Value ^c	Overall survival		<i>p</i> -Value ^c	
	Below 200	>200		0%	>0%		Below 20	>20			Average \pm SD (CI 95%)	Median (CI 95%) ^b		SG (%)	Average \pm SD (CI 95%)		Median (CI 95%) ^b
Radical surgery																	
No	7 (12.7%)	3 (11.5%)	0.879	9 (12.0%)	1 (16.7%)	0.738	5 (16.1%)	5 (9.6%)	0.378	9 (90.0%)	39.90 \pm 3.89 (32.28-47.52)	(-)	0.620	9 (90.0%)	39.90 \pm 3.89 (32.28-47.52)	(-)	0.946
Yes	48 (87.3%)	23 (88.5%)		66 (88.0%)	5 (83.3%)		26 (83.9%)	47 (90.4%)		55 (78.6%)	41.16 \pm 2.39 (36.47-45.85)	47 (38.41-55.59)		64 (90.1%)	48.01 \pm 2.15 (43.80-52.22)	(-)	
Adjuvant chemo																	
No	35 (63.6%)	20 (76.9%)	0.232	50 (66.7%)	5 (83.3%)	0.400	20 (64.5%)	36 (69.2%)	0.657	42 (79.2%)	41.36 \pm 2.88 (35.73-47.00)	47 (36.45-57.55)	0.813	48 (88.9%)	47.11 \pm 2.62 (41.98-52.24)	(-)	0.575
Yes	20 (36.4%)	6 (23.1%)		25 (33.3%)	1 (16.7%)		11 (35.5%)	16 (30.8%)		22 (81.5%)	40.88 \pm 3.07 (34.86-46.91)	43 (-)		25 (92.6%)	45.60 \pm 2.31 (41.07-50.13)	(-)	
Radiation therapy																	
No	50 (90.9%)	23 (88.5%)	0.730	67 (89.3%)	6 (100.0%)	0.399	25 (80.6%)	50 (96.2%)*	0.021	57 (79.2%)	41.39 \pm 2.37 (36.75-46.03)	47 (38.73-55.27)	0.810	66 (90.4%)	48.23 \pm 2.06 (44.19-52.28)	(-)	0.730
Yes	5 (9.1%)	3 (11.5%)		8 (10.7%)	0 (0.0%)		6 (19.4%)*	2 (3.8%)		7 (87.5%)	30.71 \pm 3.97 (22.94-38.49)	(-)		7 (87.5%)	30.71 \pm 3.97 (22.94-38.49)	(-)	

*P<0.05, ^aQui-quadrado ou exato de Fisher Test (n, %); ^bMann-Whitney Test (median \pm SD); ^cLog-Rank Mantel-Cox Test (median time \pm SD and mediated recurrence-free survival and overall survival calculated using Kaplan-Meier curves). CI 95% = confidence interval 95%; SLR = Relapse-free survival; GS = global survival; H-score = Histoscore.

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Table 4. Influence CD8-positive lymphocyte density and PDL1 immunoeexpression on GSK immunoeexpression, recurrence-free time and overall survival of patients with urothelial carcinoma

	GSK-3β H-score		p-value ^a	PDL1 TPS		p-value ^a	CD8 (cells/mm ²)		p-value ^a	SLR (%)	Recurrence free time		p-Value ^c	Overall survival		p-Value ^c	
	Below 200	>200		0%	>0%		Até 20	>20			Average ± SD (CI 95%)	Median (CI 95%) ^b		SG (%)	Average ± SD (CI 95%)		Median (CI 95%) ^b
Pathologic grade																	
Low-grade	25 (28.1%)	6 (15.4%)	0.123	16 (33.3%)*	16 (18.8%)	0.030	19 (26.0%)	13 (22.0%)	0.595	14 (70.0%)	38.73±3.66 (31.55-45.91)	47 (-)	0.500	19 (95.0%)	44.70±2.24 (40.31-49.09)	(-)	0.271
High-grade	64 (71.9%)	33 (84.6%)		32 (66.7%)	69 (81.2%)*		54 (74.0%)	46 (78.0%)		51 (82.3%)	54.94±9.91 (35.52-74.37)	52 (36.28-67.72)		55 (87.3%)	71.80±6.58 (58.90-84.70)	(-)	
Muscle invasion																	
No	37 (39.4%)	17 (42.5%)	0.735	18 (34.6%)	39 (44.3%)	0.259	31 (41.3%)	26 (40.6%)	0.933	25 (75.8%)	41.19±3.26 (34.80-47.59)	47 (40.94-53.06)	0.692	30 (88.2%)	46.92±3.21 (40.63-53.22)	(-)	0.805
Yes	57 (60.6%)	23 (57.5%)		34 (65.4%)	49 (55.7%)		44 (58.7%)	38 (59.4%)		43 (82.7%)	66.86±7.63 (51.90-81.82)	(-)		47 (90.4%)	79.72±3.96 (71.95-87.48)	(-)	
Necrosis																	
No	77 (81.9%)	34 (85.0%)	0.665	41 (78.8%)	75 (85.2%)	0.333	62 (82.7%)	54 (84.4%)	0.787	54 (78.3%)	40.53±2.47 (35.68-45.38)	47 (39.61-54.39)	0.245	62 (88.6%)	46.95±2.35 (42.34-51.56)	(-)	0.472
Yes	17 (18.1%)	6 (15.0%)		11 (21.2%)	13 (14.8%)		13 (17.3%)	10 (15.6%)		14 (87.5%)	78.69±6.82 (65.32-92.06)	(-)		15 (93.8%)	83.75±5.08 (73.79-93.71)	(-)	
CD8 (cells/mm²)																	
Up to 20	65 (53.7%)	7 (58.3%)	0.760	37 (71.2%)*	38 (43.7%)	0.002	-	-	-	33 (78.6%)	51.08±9.09 (33.26-68.90)	52 (39.08-64.92)	0.889	37 (88.1%)	77.83±4.71 (68.61-87.05)	(-)	0.692
>20	56 (46.3%)	5 (41.7%)		15 (28.8%)	49 (56.3%)*		-	-		34 (81.0%)	41.03±2.72 (35.70-46.35)	47 (-)		39 (90.7%)	45.10±2.27 (40.64-49.56)	(-)	
PDL1																	
0	48 (39.3%)*	1 (8.3%)	0.033	-	-	-	-	-	-	24 (88.9%)	43.61±2.81 (38.11-49.11)	47 (-)	0.204	25 (92.6%)	43.61±2.29 (39.12-48.10)	(-)	0.497
>0	74 (60.7%)	11 (91.7%)*		-	-		-	-		44 (75.9%)	48.06±7.90 (32.56-63.55)	43 (34.18-51.82)		52 (88.1%)	73.21±6.58 (60.31-86.12)	(-)	
GSK-3β H-score																	
<100	-	-	-	-	-	-	-	-	-	61 (80.3%)	57.23±7.30 (42.92-71.54)	47 (38.74-55.26)	0.902	69 (89.6%)	75.89±5.05 (65.95-85.76)	(-)	0.633
>100	-	-		-	-		-	-		4 (66.7%)	43.67±10.76 (22.58-6.75)	52 (-)		5 (83.3%)	45.33±7.91 (29.83-60.84)	(-)	

*P<0.05, ^aChi-squared Fisher Test (n, %); ^bMann-Whitney Test (median ± SD); ^cLog-Rank Mantel-Cox Test (median time ± SD and mediated recurrence-free survival and overall survival calculated using Kaplan-Meier curves). CI 95% = confidence interval 95%; SLR = Relapse-free survival; GS = global survival; H-score = Histocore.

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Table 5. Multivariate analysis of relapse-free time risk factors and overall survival in patients with urothelial bladder carcinoma

Variable	Relapse-free time		Overall survival	
	p-value	Adjusted HR (IC 95%)	p-value	Adjusted HR (IC 95%)
Sex	0.404	2.03 (0.39-10.62)	0.919	0.85 (0.04-17.36)
Age	0.207	2.59 (0.59-11.36)	0.966	2.37 (0.55-1.03)
Smoking	0.160	3.74 (0.60-23.50)	0.995	0.99 (0.09-10.97)
Hypertension	0.094	4.00 (0.79-20.30)	0.641	1.88 (0.13-26.48)
DM	0.809	1.38 (0.10-18.37)	0.417	3.77 (0.15-92.75)
Chemotherapy	0.485	0.59 (0.13-2.63)	0.771	1.36 (0.17-11.02)
Radiation	0.456	2.65 (0.20-34.59)	0.737	1.58 (0.11-22.35)
Final grade	0.040	0.11 (0.01-0.90)	0.969	2.29 (0.70-7.50)
Muscle invasion	0.917	1.08 (0.24-4.85)	0.861	1.24 (0.11-14.25)
Necrosis	0.971	1.04 (0.16-6.92)	0.908	1.19 (0.06-23.03)
PD-L1	0.043*	5.81 (1.06-31.80)	0.441	2.72 (0.21-34.55)
CD8	0.966	0.97 (0.28-3.34)	0.842	0.79 (0.07-8.34)
GSK	0.352	1.98 (0.47-8.38)	0.446	2.08 (0.32-13.60)

*P<0.05, Cox regression; HR = hazard risk for recurrence or death; CI 95% = Confidence interval 95%.

tumor cell recognition. Thus, our data showed that tumors treated with radiotherapy had lower expression of CD8, unlike non-radiated cases. In an in vitro study by Lhuillier et al., it was possible to evaluate the immunogenicity of neoepitopes that were upregulated by radiation, noting that radiotherapy increases the presence of genes encoding immunogenic neoepitopes from the MHC-I and MHC-II, resulting in a TCD8, CD4 cellular response [31].

While we found no significant association in the analysis of overall survival, PD-L1 expression alone was linked with recurrence, as Gu et al. did in gastric cancer [32]. These are preliminary results on a retrospective cohort of a heterogeneous treatment scenario. Regulation of PD-L1 expression may affect the therapeutic effect of immune checkpoint inhibitors. One study suggested that GSK-3 β interacts with and phosphorylates PD-L1, thus allowing PD-L1 stabilization and suppressing T-cell activity [33]. In a melanoma study, for example, eEF2K, which promotes cancer cell proliferation by regulating aerobic glycolysis, was positively correlated with PD-L1 and phospho-GSK-3 β expression, suggesting a molecular mechanism underlying tumor PD-L1 regulation and uncovered an inhibitory role of eEF2K and GSK-3 β in antitumor immunity [28]. Our studies suggest the presence of GSK-3 β as a new prognostic mark-

er in bladder cancer, allowing the proliferation and survival of urothelial cancer cells, allowing GSK-3 as a potential therapeutic target in bladder cancer, as well as PD-L1 and CD8.

The main limitation of the article is the lack of follow-up and treatment information is not available in detailed electronic medical records for our cohort and loss of some tissues during processing.

Is required the development of new studies with confirmatory cohorts that make it possible to correlate patterns of expression of several inflammatory markers associated with the diagnosis of bladder cancer and which are responsive to the treatment employed.

In this study, we used a powerful digital pathology tool to measure PD-L1, and GSK-3 β expression along with CD8-positive cell infiltrates, with confirmation by a standard pathologist's evaluation. GSK-3 β could be a potential target in advanced bladder cancer, and inhibitors may serve as adjuvant therapy, especially in immunotherapy, for patients who are not candidates for conventional therapy or surgery.

Disclosure of conflict of interest

None.

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