RESEARCH ARTICLE

Editorial Process: Submission:03/08/2023 Acceptance:08/09/2023

Prognostic Significance of *PD-L2* Expression in Association with Neutrophil-to-Lymphocyte Ratio in Urothelial Carcinoma of the Bladder

Ahmad Zulfan Hendri[1], Indrawarman Soerohardjo[1], Didik Setyo Heriyanto[2], Said Alfin Khalilullah[2], Yurisal Akhmad Dany[1], Aria Danurdoro[1]*

Abstract

Background: The prognostic significance of tumoral programmed death-ligand 2 (*PD-L2*) expression in urothelial bladder cancer (UBCs) is under-investigated , although it can potentially to become a regulatory agent of cancer immunity. In the search for supporting biomarkers, the neutrophil-to-lymphocyte ratio (NLR) as a readily available surrogate marker of immune status has been associated with clinical outcomes and other prognostic factors in various types of cancer. Here we evaluate the prognostic ability of baseline NLR in addition to *PD-L2* expression in bladder cancer. **Methods:** We used a retrospective cohort of UBCs patients from the authors' institutions. We classified patients according to their *PD-L2* and NLR levels. We associated the prognostic outcome of each group with disease-free survival (DFS) and overall survival (OS). **Results:** Thirty patients had a tumor with positive *PD-L2* expression. We found no significant correlation between *PD-L2* expression and NLR. *PD-L2* status failed to provide a significant prognostic impact (disease-free survival [DFS] and overall survival [OS] rate at 5 years, 42.85% in *PD-L2*-high versus 65.75% in *PD-L2*-low patients; p = 0.057, 42.85% in *PD-L2*-high patients versus 62.5% in *PD-L2*-low patients; p = 0.112, respectively). NLR status also failed to exhibit a significant prognostic impact (DFS and OS rate at 5 years, 44.44% in *PD-L2*-high versus 66.66% in *PD-L2*-low patients; p = 0.232, 55.55% in *PD-L2*-high versus 71.43% in *PD-L2*-low patients; P = 0.894, respectively). When *PD-L2* status and NLR status were combined, the NLR-low and *PD-L2*-low were significant factors to predict a favorable disease-free survival (hazard ratio, 4.525 [95% confidence interval, 1.020 to 20.080]; P = 0.047. However, the multivariate analysis failed to show it as an independent factor. **Conclusion:** These findings suggest that the prognostic impact of *PD-L2* expression could be affected by the NLR status.

Keywords: Immune checkpoint- prognostic biomarker- programmed cell death-legend 2 (*PD-L2*)

Asian Pac J Cancer Prev, **24 (8)**, 2673-2679

Introduction

Urothelial bladder cancer (UBC) ranks among the most prevalent malignancies, accounting for more than 400,000 new cases and 165,000 deaths each year worldwide (Vartolomei et al., 2018). Although the combination of surgery, chemotherapy, and radiotherapy has improved patient outcomes, the prognosis remains poor despite recent advancements in those multidisciplinary therapeutic strategies (Huang et al., 2018; Siegel et al., 2018). Accordingly, the discovery and validation of novel prognostic markers are urgently needed to improve postoperative survival.

The B7/CD28 pathways have a pivotal role in developing and maintaining immunity by preserving T-cell activity via its positive co-stimulatory signals and providing an inhibitory signal that dictates T-cell responses (Andersen, 2014). The inhibitory molecules are essential in establishing immune evasion in the tumor microenvironment (TME) whose features are comprised of the programmed death-1 receptor (PD-1, CD279) and its ligands, PD-L1 (CD274, B7-H1) and *PD-L2* (CD273, B7-DC) (Ahmad et al., 2017). Currently, only the PD-1/PD-L1 pathway has been extensively studied about its relevance in cancer (Yang et al., 2020). Nevertheless, it has a notable drawback of having only 1-quarter of the tumor cells with a strong or moderate expression of PD-L1 (Wang et al., 2018). This demands us to explore the new possibility of other highly expressed immune molecules, particularly those associated with worse prognosis in bladder cancer to develop better approaches.

Despite several studies elucidating prognostic information of tumoral *PD-L2* expression status in various human malignancies, the evidence concerning the

*[1]Division of Urology, Department of Surgery, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada/Dr. Sardjito Hospital, Yogyakarta, Indonesia. [2]Division of Urology, Department of Surgery, Faculty of Medicine, Universitas Syiah Kuala/ dr. Zainoel Abidin General Hospital, Banda Aceh, Indonesia. *For Correspondence: danurdoroaria@gmail.com*

prognostic role of *PD-L2* in bladder cancer is still lacking (Yang et al., 2020). The neutrophil-to-lymphocyte ratio (NLR) is an easily measured and also highly reproducible test to be incorporated in the daily practice of pre-operative bladder cancer treatment, and is determined by dividing the number of neutrophils by the number of lymphocytes. It is widely considered to be a potential surrogate of systemic inflammatory status and adaptive immunity balances (Tashima et al., 2020). It is suggested that high NLR was correlated with a poor prognosis of bladder cancer (Vartolomei et al., 2018). Lately, several studies mentioned the NLR was associated with the survival benefit of PD-1/PD-L1 inhibitors, suggesting it to be a potential indicator of immunity status (Yang et al., 2020). Here, we investigated the prognostic significance of tumoral *PD-L2* and expression status in association with NLR in patients with bladder cancer.

Materials and Methods

Patients

Patients with bladder cancer treated for urothelial carcinoma of the bladder in our institution from 2014 through 2016 were retrospectively reviewed. Patients who could not or refused to give informed consent for this study were excluded. Subjects were eligible if their specimen contains a tumor percentage of >50% for Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) processing. Thirty patients were finally included in this study (Table 1).

Abdominal computed tomography (CT) and chest x-rays were performed preoperatively. The staging was determined using the current tumor, node, metastases (TNM) classification (UICC TNM staging system, 8th edition) (Paner et al., 2018). Tissue was collected at the time of transurethral resection. The lymphocyte count and neutrophil count were obtained from the routine preoperative blood test. The NLR was measured by dividing the neutrophil count by the lymphocyte count. The institutional review board and the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada/ Dr. Sardjito Hospital, Yogyakarta, Indonesia, approved the present study (KE/FK/0179/EC/2019). Written informed consent was acquired from each subject for participating in this study. The procedures and methods were performed following our institutional guidelines and relevant regulations.

Evaluation of tumoral PD-L2 expression

PD-L2 expression on UBCs was evaluated with a qRT-PCR examination in Pathology Anatomy Laboratory, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada. The FavorPrepTM Tissue Total RNA Mini Kit (FAVORGEN Biotech Corp. Pingtung County, Taiwan) was used to isolate total RNAs according to the manufacturer's protocol. DNA was extracted using FavorPrep Genomic DNA Extraction Mini Kit (Favorgen Biotech Corp.) The thermal cycler was as follows: reverse transcription at 42°C for 5 min, enzyme activation at 95°C for 3 min, followed by 40 cycles of denaturation at 9°C for 1-3 sec and combined annealing and extension steps at 60°C for more than 20 sec. A reaction with an exponential signal increase before cycle 40 was considered positive. These procedures will produce DNA fragments in the length of 185 bp. The amplified products were analyzed using 2% agarose gel electrophoresis with 0.5 µg/mL ethidium bromide staining and visualized under UV transillumination. All amplifications were conducted in triplicate. Glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used as the internal control. PCR products were measured using the Exicycler™ 96 Quantitative Real-Time PCR System (Bioneer, Daejeon, South Korea). We used the Livak method for calculating gene expression.

Statistical analysis

Demographic data were reported as frequencies for categorical variables, the proportions were compared by the chi-square test, and means with standard deviations (SD) for continuous variables were compared using a non-parametric test (Mann-Whitney U-test). Spearman's rank correlation coefficients (two-sided) were used to evaluate correlations between NLR and *PD-L2* expression. The receiver operating characteristic (ROC) curve analyses and Youden Index (Youden Index = sensitivity + specificity − 1) were performed to determine the optimal cut-off scores of NLR and TPS.

The disease-free survival (DFS) was determined as the period from surgery to any presence of regional/ distant recurrence or death from any cause. The overall survival (OS) referred to the time from surgery to death of any cause. Follow-up data were obtained via routine follow-up or telephone calls. The Kaplan-Meier approach was performed to generate survival curves, and survival differences were compared with the log-rank test. Multivariable survival analysis was conducted utilizing the Cox proportional hazards regression model to estimate the hazards ratio (HR) and 95% confidence interval (CI) and identify the independent prognostic factors.

The p-values < 0.05 were considered to be statistically significant. All of the statistical analyses were done under the GraphPad Prism version 8 (GraphPad Software., San Diego, CA) and IBM SPSS 25.0 software (IBM Corp, Armonk, NY, USA).

Results

Distribution of NLR and cut-off value for prognostic analyses

The NLR value of each case is shown in Fig. 1. The ROC curve analysis failed to show a significant diagnostic performance of NLR to death (area under ROC curve [AUC-ROC], 0.604; P = 0.366) (Figure 1). Based on the ROC curve, the NLR value of 3.12 (Youden index 0.7) was employed as the cut-off value to classify each patient into NLR-high (NLR, 3.12 or higher) or NLR-low (NLR, less than 3.12) patient in further survival analyses (Figure 1)

The DFS and OS according to NLR status

The NLR provided no significant prognostic impact for OS (p=0.8941) and DFS (p=0.2322) of bladder cancer (Figure 2).

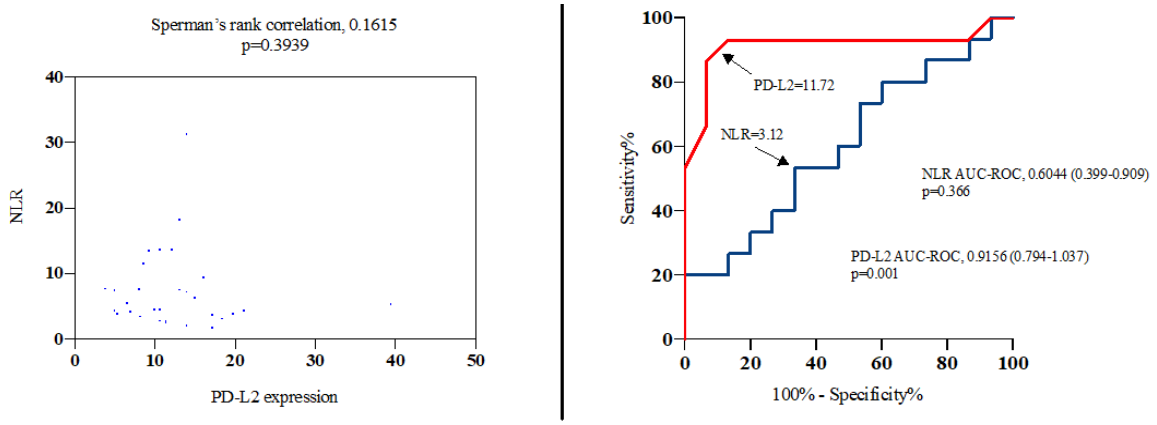

Figure 1. Distribution of Neutrophil-to-Lymphocyte Ratio (NLR) and PD-L2 Expression (left). Receiver operating characteristics (ROC) curves to examine diagnostic performance of NLR (right upper) and PD-L2 expression (right lower) for prediction of death from any cause. AUC-ROC, area under ROC curve.

PD-L2 expression in correlation with other patient characteristics

The distribution of *PD-L2* expression is shown in Figure 1, in which there was no significant correlation between *PD-L2* and NLR. *PD-L2* expression status in correlation with several patient characteristics is shown in Table 2. Some clinical characteristics showed a statistically significant difference according to *PD-L2* expression status ($p<0.005$).

Table 1. Demographics and Baseline Characteristics of Patients with Bladder Cancer

Characteristics		All patients (n=30)
Age, median (years)		62 (41-96)
Sex, n (%)	Male	24 (80)
	Female	6 (20)
Smoking status, n (%)	Never	3 (10)
	Former	18 (60)
	Current	9 (30)
Brinkman index, median (pack-year)		60.0 (0-166)
Tumor stage, n (%)	T1	15 (50)
	T2	14 (46.6)
	T3	0
	T4	1 (3.3)
Node stage, n (%)	Nx	9 (30)
	N0	16 (53.3)
	N1	2 (6.6)
	N2	2 (6.6)
	N3	1 (3.3)
Metastasis stage, n (%)	M0	14 (46.6)
	M1	6 (20)
Tumor grade, n (%)	Low	9 (30)
	High	21 (70)
Histologic variant, n (%)	Yes	5 (16.6)
	No	25 (83.3)
NLR, median (range)		4.9 (1.7-31.2)
PD-L2 expression, median (range)		11.3 (3.7-39.4)

Table 2. *PD-L2* Expression (TPS) in Correlation with Patient Characteristics

Parameter	*PD-L2* expression		
	PD-L2 low	*PD-L2* High	
Age, median (years)	62	62	
≤ 70 (n=20)	10	10	0.709
>70 (n=10?)	6	4	
Gender			
Male (n=24)	11	15	0.631
Female (n=6)	1	3	
Smoking status			
Never (n= 3 (10%))	2	1	
Former (n= 18 (60%))	7	11	>0.999
Current (n= 9 (30%)	5	4	
Brinkman index, median (pack year)	51	60	*0.033
Tumor stage			
T1 (n=15)	14	1	
T2 (n=14)	2	12	>0.999
T3 (n=0)	0	0	
T4 (n=1)	0	1	
Node stage			
Nx (n=9)	5	4	
N0 (n=16)	10	6	
N1 (n=2)	1	1	>0.999
N2 (n=2)	1	1	
N3 (n=1)	0	1	
Metastasis stage			
M0 (n=14)	14	10	0.378
M1 (n=6)	2	4	
Tumor grade			
Low (n=9)	8	1	*0.017
High (n=21)	8	13	
Histological variant			
Yes (n=5)	3	2	>0.999
No (n=25)	17	8	
NLR status, median	4.47	5.79	*0.008
Low (n=)	11	10	>0.999
High (n=)	5	4	

NLR, neutrophil to lymphocyte ratio; Data represented as absolute counts (%) or median.

Table 3. Univariable and Multivariable Cox Model of Prognostic Factors for Disease-Free Survival and Overall Survival.

	UROTHELIAL BLADDER CANCER											
	Disease-free survival						Overall survival					
	Univariable analysis			Multivariable analysis			Univariable analysis			Multivariable analysis		
	HR	95% CI	P-	HR	95% CI	P-	HR	95% CI	P-	HR	95% CI	P-
Age (per 1-year increase)	0.979	0.941-1.018	0.291				0.986	0.943-1.031	0.526			
Gender, male (vs female)	0.593	0.163-2.156	0.427				1.082	0.244-4.803	0.917			
Smoking status, current (vs never smoker)	1.017	1.001-1.033	0.035				1.001	0.985-1.018	0.894			
Tumor number, single (vs multiple)	2.315	1.734-7.299	0.152				0.596	0.215-1.655	0.321			
Tumor size, <3cm (vs ≥3 cm)	1.556	0.543-4.460	0.41				1.892	0.644-5.565	0.246			
Pathologic stage, low (vs high grade)	0.739	0.262-2.061	0.739				1.38	0.439-4.340	0.581			
Clinical stage, Ta-1 (vs T2-4)	0.48	0.170-1.357	0.166				0.726	0.248-2.130	0.56			
Lymph node metastasis, No (vs Yes to metastasis)	1.077	0.379-3.058	0.89				2.967	0.937-9.402	0.065			
NLR-low/PD-L2-low vs others	4.525	1.020-20.080	0.047	7.301	0.712-74.822	0.094	1.058	0.361-3.098	0.918	1.978	0.387-10.098	0.412

NMIBC, non-muscle invasive bladder cancer; MIBC, muscle invasive bladder cancer; HR, hazard ratio; CI, confidence interval. NLR, neutrophil-to-lymphocyte ratio.

Prognostic impact of PD-L2 expression status

The ROC curve analysis showed that *PD-L2* provided a significant and robust diagnostic performance to predict death (AUC-ROC, 0.916; p = 0.001) (Figure 1). Based on the ROC curves, the cut-off value of *PD-L2* expression was 11.72 (Youden index 0.8). Patients in the *PD-L2*-high group showed a worse prognosis of DFS in bladder cancer, but the results did not reach a statistical significance (Figure 2). The overall survival is better in *PD-L2*-low, but the difference did not reach a statistical significance (Figure 2).

Prognostic impact of PD-L2 expression status in combination with NLR status

When *PD-L2* expression and NLR status were stratified, "NLR-low (<3.12) and *PD-L2*-low" patients showed the most favorable prognosis (Figure 3, upper), and the difference was highly significant (Figure 3, lower). Univariate analyses of each risk factor, including age, gender, smoking status, tumor number, tumor size,

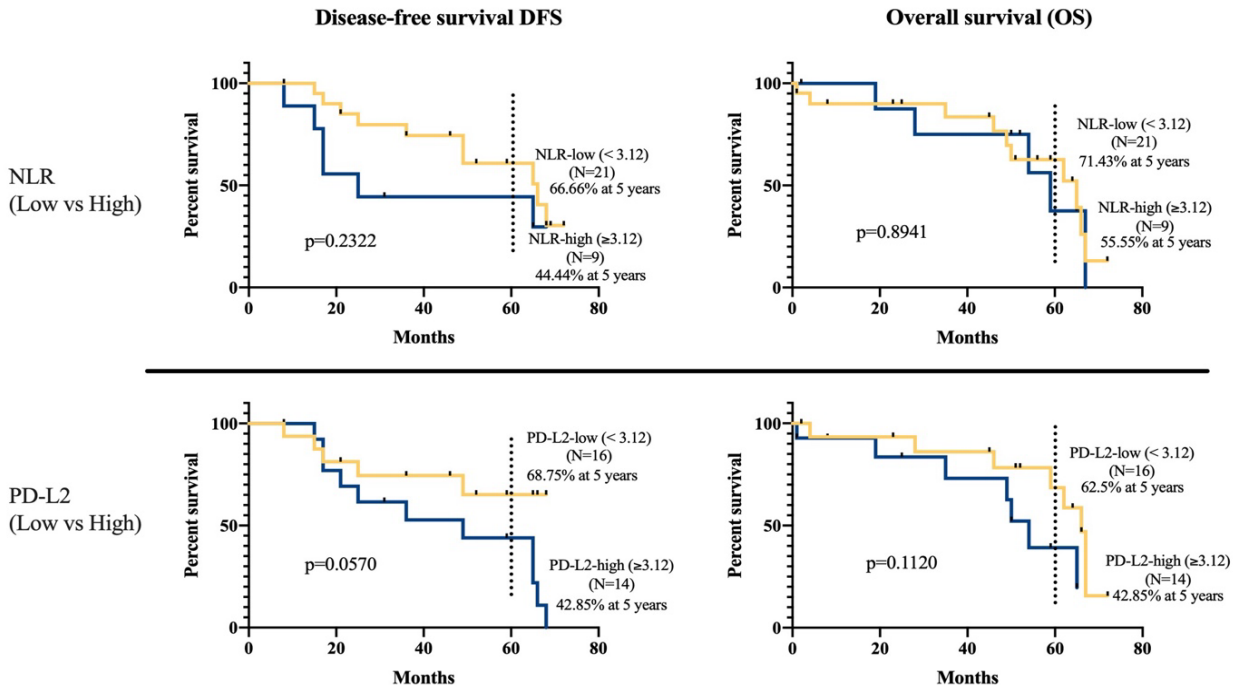

Figure 2. Disease-Free Survival (DFS) and Overall Survival (OS) Curves According to Neutrophil-to- Lymphocyte Ratio (NLR) and Tumoral *PD-L2* Expression Status. The cut-off value for NLR and PD-L2 were 3.12 and 11.72, respectively.

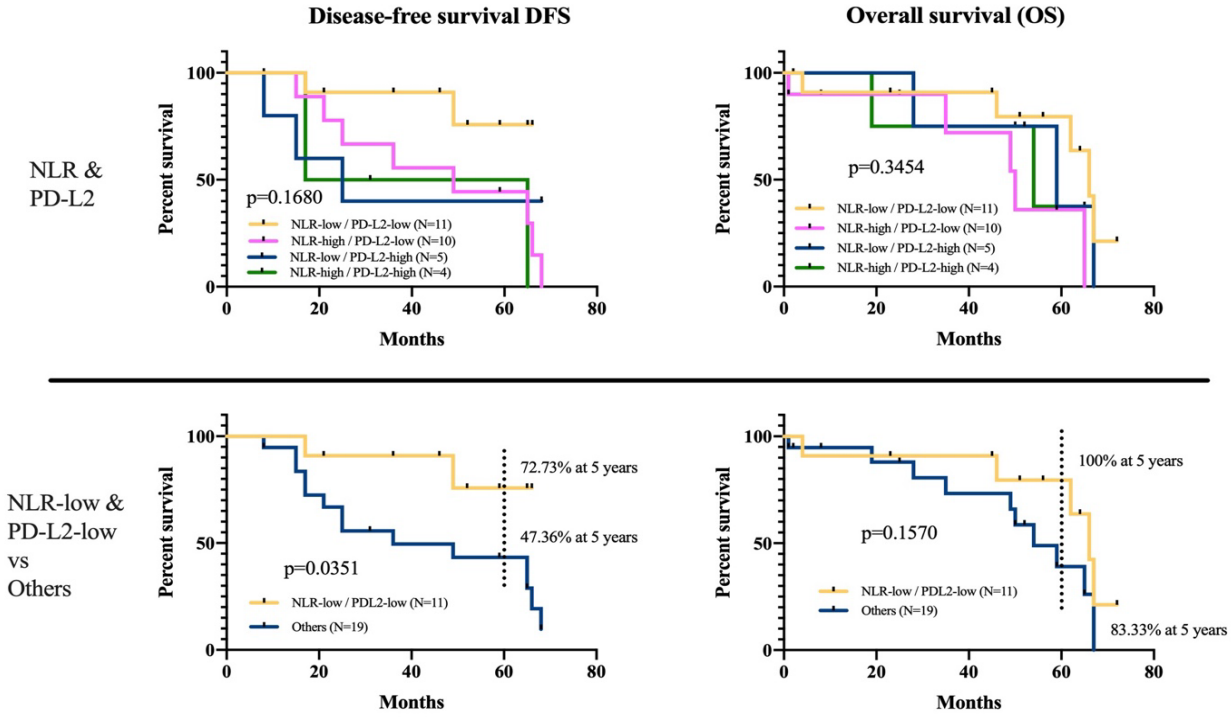

Figure 3. Disease-Free Survival (DFS) and Overall Survival (OS) Curves According to Tumoral PD-L2 Expression Status in Combination with Status of Neutrophil-to-Lymphocyte Ratio (NLR). The cut-off value for NLR and PD-L2 were 3.12 and 11.72, respectively.

pathologic stage, clinical stage, lymph node metastasis, showed that the "NLR-low (<3.12) and *PD-L2*-low (<11.72)" was a significant predictor for better DFS in bladder cancer. However, the multivariate analysis failed to show any role of *PD-L2* and NLR as independent factors to predict a favorable DFS. The univariate and multivariate analyses on OS also showed no statistically significant results (Table 3).

Discussion

The current study first demonstrated that the prognostic impact of *PD-L2* expression on urothelial bladder cancer (UBCs) might be influenced by the NLR. The "NLR-low and *PD-L2*-low" status was associated with a favorable prognosis in the patients with bladder cancer after transurethral resection, especially in terms of DFS.

PD-L1 immunohistochemistry is the only biomarker that has been evaluated for its predictive significance in a prospective randomized trial, where it failed to predict survival (Lavoie et al., 2019). *PD-L2* expression status on tumor cells is recognized lately from several studies as one promising biomarker for an early predictor of the efficacy of PD-1 antibody therapies (Yang et al., 2020). In real-world practice, the utilization of anti-PD-1 antibodies in bladder cancer treatment is suggested in the settings of locally advanced or metastatic urothelial carcinoma, especially for those who are not eligible for any platinum-based chemotherapy or as the second-line therapies after failed chemotherapy (Yang et al., 2020). Nevertheless, the prognostic significance of *PD-L2* expression status in bladder cancer remains uncertain. Although earlier research suggested that *PD-L2* human tumors are generally an independent predictor of responsiveness to PD-1 blockage (Dowell et al., 2021), other studies observed contrasting results. *PD-L2* overexpression was not associated with a favorable prognosis regarding relapse or progression of non-muscle-invasive bladder cancer (NMIBC) or recurrence-free survival of MIBC (Le Goux et al., 2017). The contradictory outcomes may be explained by the retrospective nature of the current study, the relatively small number of patients, and heterogeneous patient characteristics. As shown in our study, the prognostic impact of *PD-L2* expression on bladder cancer cells may be impacted by the state of cancer immunity activity and various factors correlated with cancer immunity.

Although anticancer immunotherapy has emerged as a promising treatment strategy in cancer nowadays, the natural inhibitory feedback loop's involvement following immune activation has become a notable stumbling block for future development. This includes the upregulation of PD-L1 and *PD-L2*, which are imperative to restrict the immune responses in preserving host health while also destroying pathogen and neoplasms (Pitt et al., 2016). Therefore, almost any successful tumor-immunotherapy aims to induce immunological activation and inflammation. Notable progress has been made in knowing how to induce immunological activation in immunotherapies. One example is *PD-L2* as an immune checkpoint receptor ligand, which plays a role in the adaptive immune response's negative regulation. *PD-L2* interacts directly with PD-1 expressed on cytotoxic T lymphocytes (CTLs), further downregulating the CTL-mediated immune responses (Ahmad et al., 2017;

Yang et al., 2020). Hence, when cancer immunity failed to be activated during the tumor cells killing process, tumor cells may escape this barrier no matter the number of *PD-L2* expressed. Once the cancer immunity gets effectively activated, tumor cells with no *PD-L2* expression can be eliminated through the CTL-mediated immune responses, and only *PD-L2*-expressed tumor cells will survive (Teishima et al., 2019). The NLR has been described as a potential marker to predict immune-inflammation status (Tashima et al., 2020). Previous studies demonstrated low NLR correlation with a favorable prognosis in various malignancies, including bladder cancer (Vartolomei et al., 2018). The positive association was also described in patients treated with PD-1/*PD-L2* inhibitors (Cao et al., 2018; Capone et al., 2018; Hasegawa et al., 2019; Ogihara et al., 2020; Yamamoto et al., 2020). Taken together, the *PD-L2* expression status and NLR could be related to each other in the prognosis of patients with bladder cancer. Our study demonstrated that the "NLR-low and *PD-L2*-low" status among patients with bladder cancer carries a favorable prognosis, mainly for DFS.

The results of the present study pointed out that the prognostic benefits of *PD-L2* expression on UBCs could be associated with the NLR status, but this ought to be vigilantly interpreted as several limitations inherent to using a retrospective design are present . First, only 30 patients were included; this may lead to underpowered statistical analysis. For example, high *PD-L2* patients seem to have worse prognostic in several models of comparison, including the OS (Figure 2), but the results failed to achieve statistical significance. More extensive prospective studies are needed, with the adjustment of the minimum sample size that should be calculated to yield adequate statistical power. Second, this was a single-institution investigation; thus, multicenter studies should be conducted to generalized our findings. . Finally, we also concluded the heterogenous case mix existed in our patients' characteristics. A larger clinical trial in a prospective manner should be encouraged to validate our results and get the optimal results.

In conclusion, the prognostic impact of *PD-L2* expression on TCs was distinct according to NLR in bladder cancer. *PD-L2*-high on BCas was associated with a poor prognosis among NLR-low patients, but it provided no prognostic impact among NLR-high patients. "NLR-low and tumoral *PD-L2*-low" patients showed a favorable prognosis.

Author Contribution Statement

AZH, IS and AD conceptualized the study. DSH, SAK, YAD collected the data used for the analysis. AD drafted the manuscript. All authors reviewed and approved the final draft of the manuscript.

Acknowledgements

The authors thank all study participants and their families for providing valuable information in this study.

Study Approval

This study has been granted approval by the research committee of the Faculty of Medicine at Universitas Gadjah Mada.

Availability of Data

Upon a reasonable request, the corresponding author will make the datasets utilized in this study available.

Ethics approval and consent

The Ethics Committee, Medical Research Council of Universitas Gadjah Mada (Nr. KE/FK/0179/EC/2019) accepted the study protocol. The research was carried out in compliance with the Helsinki Declaration. All study participants gave informed consent to participate.

Conflict of Interest

All authors report having no competing interests.

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