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ORIGINAL ARTICLE – UROLOGIC ONCOLOGY

Prognostic Role of the Immunoscore for Patients with Urothelial Carcinoma of the Bladder Who Underwent Radical Cystectomy

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ABSTRACT

Background. Increasing evidence suggests that cancer progression is strongly influenced by the host immune response, which is represented by immune cell infiltrates. The T-lymphocyte-based Immunoscore is reported to be a reliable prognostic factor in colon cancer, but its significance in urothelial carcinoma of the bladder (UCB) is at an early stage of exploration. This study aimed to determine whether the tumor immune infiltrate, as evaluated by the Immunoscore, could act as a useful prognostic marker for UCB patients who have undergone radical cystectomy (RC).

Methods. In this study, immunohistochemistry was used to examine the Immunoscore of 221 UCB patients who underwent RC. The Immunoscore of the patients was determined by the densities of CD3+ and CD8+ T cells at the tumor center and the invasive margin.

Results. A highly significant association between a low Immunoscore and a shortened patient survival ($P < 0.001$, log-rank test) was demonstrated. In different subsets of UCB patients, a low Immunoscore also was a prognostic indicator of pT $\leq$ 2, pN(–)-status tumors, negative vascular invasion, or both ($P < 0.05$). Importantly, the Immunoscore together with the patient's pT status provided significant independent prognostic parameters in the multivariate analysis ($P < 0.05$). Furthermore, a significant correlation ($P = 0.003$) of a low Immunoscore with an increased UCB labeling index of Ki-67 (a cell proliferation marker) was observed in this UCB cohort.

Conclusions. The findings suggest that the Immunoscore, as examined by immunohistochemistry, might serve as a novel prognostic marker for UCB patients who have undergone RC.

Urothelial carcinoma of the bladder (UCB) is among the most common and lethal urothelial malignancies diagnosed in the United States.[1] For patients with muscle-invasive UCB and non-muscle-invasive disease at high risk of recurrence and progression, radical cystectomy (RC) plus pelvic lymphadenectomy remains the standard treatment.[2,3] Approximately half of the patients who have UCB treated with RC will experience lethal metastases despite advancements in prognostic tools and novel drugs.[4] Conventionally, the assignment of predicting recurrence and disease progression in UCB patients is primarily based on the Union for International Cancer Control (UICC) tumor-node-metastasis (TNM) classification system.[5–7] In clinical settings, however, the anatomy-based system provides useful, but incomplete prognostic information, and the selection of patients for multimodal forms of treatment remains a significant challenge.[8] Thus, identification of specific prognostic biomarkers would allow for a better

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understanding of the underlying nature of UCB, thereby enabling the development of targeted therapeutic interventions.

Immunotherapy is emerging as a powerful weapon for combatting UCB, although the Bacillus Calmette-Guerin has been used to treat non–muscle-invasive UCB for more than 30 years.[9] Extensive efforts toward the identification of immune biomarkers have focused on associations between pre- and posttreatment levels of soluble antigens such as antibody titers, immune cell populations in serum, and assessment of tumor-infiltrating leukocytes (TILs).[10] Findings have shown that TILs influence the treatment of UCB patients positively,[11–13] suggesting that TIL counts may be a good indicator for the progression and recurrence of UCB. Although these studies have shown important roles for TILs, no convenient and effective scoring system is in place for UCB patients who have undergone RC.

The Immunoscore is a standardized scoring system derived from a measure of CD3+ and CD8+ cell densities at the tumor center (CT) and invasive margin (IM).[14] Findings have shown it to be a reliable and statistically significant prognostic tool for predicting the survival of patients with colorectal cancer,[15] gastric cancer,[16] rectal cancer,[17] or pancreatic cancer.[18] In colorectal cancer, for example, prognostication by the conventional TNM classification system has been improved and found to be more accurate with the addition of the Immunoscore.[19] A previous study reported that higher densities of CD3+ and CD8+ TILs are associated with the improved survival of UCB patients.[20] Recently, Yu et al.[21] demonstrated that the Immunoscore is a potential predictor of clinical outcomes after cystectomy. However, more than 40% of the patients in their cohort received perioperative chemotherapy, which potentially contributed to selection biases. In addition, these two studies arrived at conclusions based on limited sample sizes.

The current study aimed to determine whether the Immunoscore, initially defined to quantify in situ immune infiltrates, can be applied to predict the prognosis of UCB patients after RC. Immunohistochemistry (IHC) was used to examine the Immunoscore in a large UCB cohort, and the clinicopathologic/prognostic significance of the Immunoscore in UCB patients also was assessed. Furthermore, immune infiltrates have been shown to associate with cell proliferation.[22,23] Hence, we examined both the Immunoscore and cell proliferation using Ki-67 as an assessment marker.

METHODS

Patient Tissue Specimens

Patients with primary urothelial cell carcinoma of the bladder who underwent cystectomy from February 2000 to January 2014 at the Sun Yat-sen University Cancer Center (Guangzhou, China) were included in this study. Patients who had previously presented with any other forms of tumor were excluded from the study. Patient medical records were retrospectively reviewed, with the primary goal of assessing cancer-specific survival as defined by the period between cystectomy and UCB-related death or last follow-up visit.

All the patients gave written informed consent for study participation. We ultimately obtained 221 archived formalin-fixed, paraffin-embedded (FFPE) tissue samples from the Department of Pathology at the Sun Yat-sen University Cancer Center. The Institutional Research Ethics Committee approved the use of these samples for this purpose. Two urologic pathologists (JWC and DX) independently reviewed all the histologic specimens to assign pathologic stages according to the criteria of the TNM Classification of the International Union Against Cancer, 8th edition (UICC, 2017).

Immunohistochemistry (IHC)

In this analysis, IHC studies were performed using a standard streptavidin–biotin–peroxidase complex method. In brief, tissue sections were first de-paraffinized and rehydrated. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 20 min. Antigen retrieval then was performed on the tissue by boiling the tissue slides in 10 mmol of citrate buffer (pH 6.0) in a pressure cooker for 10 min. Nonspecific binding was blocked with 10% normal rabbit serum for 20 min at room temperature. The tissue slides then were incubated with anti-CD3 (Abcam, Cambridge, MA, USA, diluted 1:500 in PBS), anti-CD8 (Abcam, diluted 1:500 in PBS), and anti-Ki-67 (Abcam, diluted 1:100 in PBS).

All the tissue sections were incubated in their respective primary antibodies in a humidified chamber overnight at 4 °C. Subsequently, the sections were incubated with biotinylated rabbit anti-mouse immunoglobulin G (IgG, diluted 1:100) for 30 min at 37 °C. The sections then were incubated with a streptavidin-peroxidase conjugate for 30 min at 37 °C, and the color was developed with 3′-3′ diaminobenzidine. Finally, the nucleus was counterstained with Meyer's hematoxylin.

For the negative control, normal mouse IgG was used in place of the primary antibody. The densities of CD3+ and CD8+ T cells both at the CT and IM of UCB were determined using ImageJ software (National Institute of Health, Bethesda, MD, USA).[24] The cell-counting method included watershed cell segmentation.[25] The IM area was defined as 0.5 mm extending into the tumor core and 1 mm extending beyond the tumor.[26] For evaluation of the results, only slides that incorporated the IM area of the tumor were selected. Three noncontiguous areas of highest lymphocyte density were selected for both CT and IM.

To eliminate sampling errors, an average of three noncontiguous areas was used. Immunoscore assessment was performed according to the densities of CD3+ and CD8+ T cells at both the CT and IM, with a cutoff value set at the median of each index. High Immunoscore values for each index were scored 1, whereas low Immunoscore values were scored 0. All the calculated scores were considered as the final Immunoscore value. A final score of 0 indicated low densities of CD3+ and CD8+ T cells at both the CT and IM, whereas a score of 1 indicated a high density of either cell type at either the CT or IM. A maximum score of 4 indicated high densities of both cell types in both regions. Furthermore, patients with Immunoscores higher than 2 were defined as having high Immunoscores, whereas those with an Immunoscore of 2 or below were defined as having a low Immunoscore.

Nuclear Ki-67 expression was determined by counting the number of positively stained cells per section, which then was expressed as a percentage.[27] For all the samples, at least 400 epithelial cells were counted. Scoring of these samples was independently performed by two pathologists (JWC and DX) who had been blinded to patient clinicopathologic values. Any disagreements (∼ 6% of the total informative cases) were reviewed a second time, followed by a conclusive verdict by both pathologists.

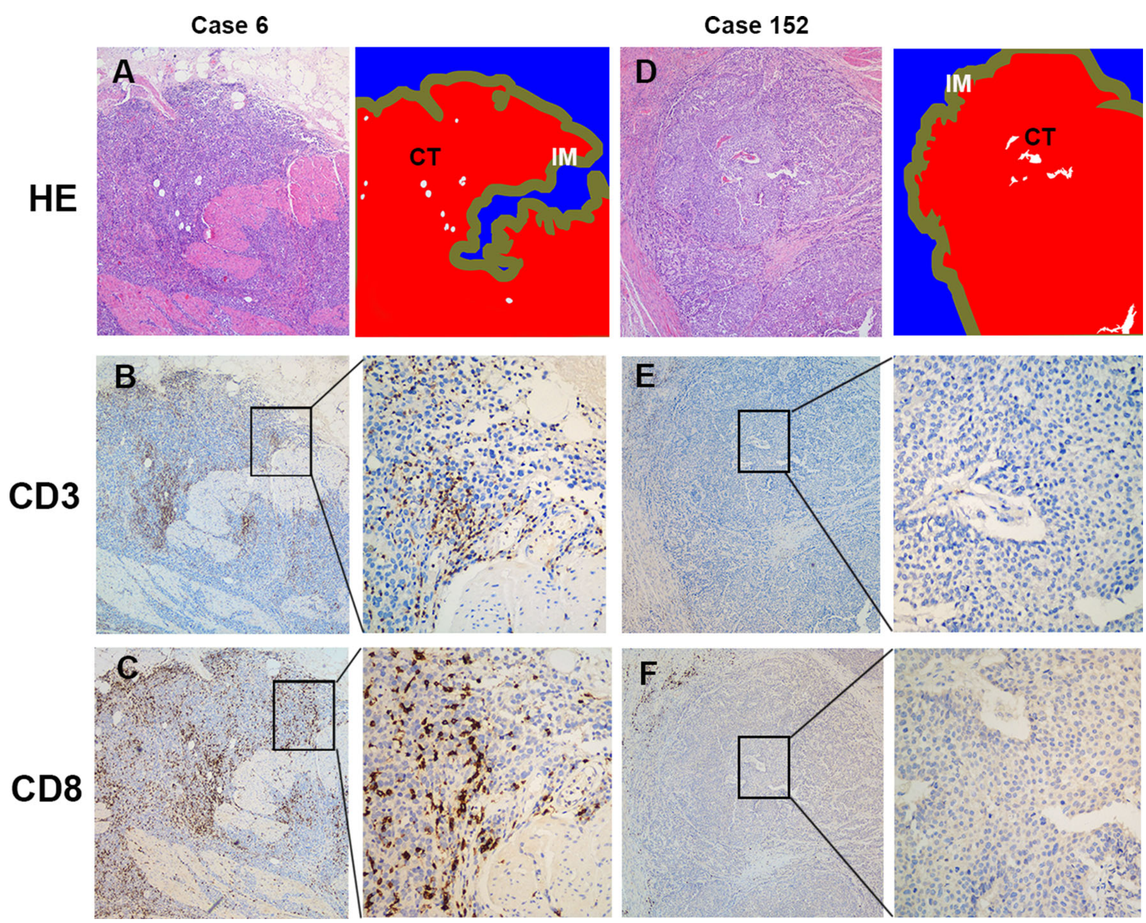

FIG. 1 Representative immunohistochemical images of CD3+ and CD8+ T cells at the center tumor (CT) and invasive margin (IM) of urothelial carcinoma of the bladder (UCB). **a** *Left:* Hematoxylin and eosin-stained section of a UCB specimen (case 6) showing the tumor regions of interest (CT and IM, original magnification, × 40). *Right:* Bladder tissue is divided into tiles, with the CT area highlighted in red and the IM area highlighted in brown. **b**, **c** Representative images showing high densities of CD3+ and CD8+ cells in IM. Original magnification: × 40 (*left*) and × 200 (*right*). **d** Hematoxylin and eosin-stained section of another UCB specimen (case 152) showing the tumor regions of interest (CT and IM). **e**, **f** Representative images showing low densities of CD3+ and CD8+ cells in CT. Original magnification: × 40 (*left*) and × 200 (*right*)

Statistical Analysis

All statistical analyses were performed using SPSS software (SPSS standard version 17.0, SPSS Inc., Chicago, IL, USA). To determine the association between the Immunoscore and the clinicopathologic features of the patients as well as the association between the Immunoscore and the molecular features of UCB, a Chi square test was used. For survival analysis, the UCB patients were subjected to Kaplan–Meier analysis. The log-rank test was used to compare the different survival curves. Multivariate survival analysis was performed on all parameters found to be significant, with univariate analysis using the Cox regression model. All *P* values lower than 0.05 were considered statistically significant.

RESULTS

Patient Characteristics

The study enrolled 221 UCB patients who underwent RC between February 2000 and January 2014 at the Sun Yat-sen University Cancer Center (Guangzhou, China). All the patients underwent RC, bilateral pelvic lymphadenectomy, and urinary diversion, and none of the patients had received pelvic irradiation or systemic chemotherapy before surgery. The median follow-up time was 59 months (range, 2–178 months). The densities of CD3+ and CD8+ immune infiltrates in CT and IM regions were assessed by immunohistochemical-based analyses using ImageJ software (Fig. 1). The observed median densities of CD3+ cells were 227.3 cells/mm^2 at the CT and 198.6 cells/mm^2 at the IM. The median densities of CD8+ cells were 1127.3 cells/mm^2 at CT and 644.7 cells/mm^2 at IM.

We further analyzed the outcomes according to the definitions of a high Immunoscore (> 2) and a low

TABLE 1 Association of the Immunoscore with the patient's clinicopathologic features in urothelial carcinoma of the bladder (UCB)

Variables	All cases (*n* = 221)	Immunoscore (%)		*P* value[a]
		Low (*n* = 117) *n* (%)	High (*n* = 104) *n* (%)	
Age (years)				0.101
≤ 60	121	58 (47.9)	63 (52.1)	
> 60	100	59 (59.0)	41 (41.0)	
Gender				0.260
Female	32	14 (43.8)	18 (56.3)	
Male	189	103 (54.5)	86 (45.5)	
Tumor multiplicity				0.504
Unifocal	120	66 (55.0)	54 (45.0)	
Multifocal	101	51 (50.5)	50 (49.5)	
Smoking history				0.111
No	98	46 (46.9)	52 (53.1)	
Yes	123	71 (57.7)	52 (42.3)	
Vascular invasion				0.188
Negative	185	96 (51.9)	89 (48.1)	
Positive	36	21 (58.3)	15 (41.7)	
WHO grade				
Low	44	28 (63.6)	16 (36.4)	0.112
High	177	89 (50.3)	88 (49.7)	
pT status				0.186
pT1,Ta, Tis	43	23 (53.5)	20 (46.5)	
pT2	74	32 (43.2)	42 (56.8)	
pT3	57	33 (57.9)	24 (42.1)	
pT4	47	29 (61.7)	18 (38.3)	
pN status				0.985
pN–	172	91 (52.9)	81 (47.1)	
pN+	49	17 (53.1)	19 (46.9)	

[a]Chi square test

TABLE 2 Prognostic value of the Immunoscore in urothelial carcinoma of the bladder (UCB)

Immunoscore	Cases	Overall survival		
		HR	95% CI	*P* value[a]
Total				0.001
Low	117	2.260	1.357–3.765	
High	104	1		
pT status				
pT1,Ta,Tis				0.014
Low	23	8.723	1.089–69.847	
High	20	1		
pT2				0.111
Low	32	2.265	0.805–6.378	
High	42	1		
pT3–pT4				0.261
Low	62	1.426	0.763–2.664	
High	42	1		
pN status				< 0.001
pN–				
Low	91	3.052	1.610–5.785	
High	81	1		
pN+				0.842
Low	26	1.094	0.451–2.649	
High	23	1		
Vascular invasion				
Negative				0.002
Low	96	2.520	1.390–4.567	
High	89	1		
Positive				0.967
Low	21	0.978	0.344–2.779	
High	15	1		

HR hazard ratio, *CI* confidence interval

[a]Chi square test

Immunoscore (≤ 2). Of the 221 patients, 117 (52.9%) were given a low Immunoscore, with the remaining 104 patients (47.1%) receiving a high Immunoscore. Furthermore, the potential association between the Immunoscore and the clinicopathologic features of the UCB patients was further evaluated. We did not detect a significant relationship between Immunoscore and UCB patient clinicopathologic features including age, gender, smoking history, vascular invasion, World Health Organization (WHO) grade, pT status, pN status, and multiplicity ($P > 0.05$; Table 1).

Relationship Between the Clinicopathologic Variables, Immunoscore, and UCB Patient Survival

Using univariate analysis, we found a low Immunoscore to correlate strongly with a poor OS for the entire UCB cohort. Furthermore, the Immunoscore could stratify patient survival into pT $\leq$ 2, pN(–) and negative vascular invasion (Table 2, Fig. 2). Kaplan–Meier analysis also demonstrated a significant impact on patient survival using certain clinicopathologic prognostic parameters such as tumor pT status, pN status, and vascular invasion status ($P < 0.05$). Multivariate analysis demonstrated that both the Immunoscore and the pT status were independent predictors of OS for the entire UCB cohort (Table 3).

Correlation Between Immunoscore and Cell Proliferation in UCBs

The expression of Ki-67 was assessed as a labeling index (LI), defined as the percentage of Ki-67-positive cells in each tumor. We found the median LI value for the entire UCB cohort to be 27.6%. Next, we used this result as the cutoff value to define a low LI of Ki-67 as below the median value and a high LI of Ki-67 as equal to or above the median value. Interestingly, we observed an inverse correlation between the Immunoscore and LI of Ki-67 (Fig. 3). We also detected a higher Ki-67 LI frequency in tumors assigned a low Immunoscore (32.5%, 38 of 117 cases) than in those assigned a high Immunoscore (15.4%, 16 of 104 cases, Chi square test; $P = 0.003$).

DISCUSSION

Urothelial carcinoma of the bladder is one of the most common forms of cancer, with approximately 429,800 new cases every year and more than 165,000 deaths worldwide.[28] For patients with muscle-invasive UCB, RC is the standard, potentially curative, surgical treatment. However, the 5-year overall survival (OS) rate for patients with pT3–4 pN– or pN+ M0 UCB after RC still is only approximately 50%.[29] Cancer recurrence is thought to be due mainly to systemic disease occult at the time of surgery. Findings have shown tumor stage, lymph node status, and vascular invasion to be good prognostic factors for progression and patient survival.[5,6,30] However, these factors focus entirely on the differences among the different UCB tumors and do not account for interactions between the tumor and the host immune response. Improved prognostic and predictive markers for UCB recurrence after RC that incorporate these interactions are urgently needed.

In the current study, we observed the intra-tumoral immune cell densities of both CD3+ and CD8+ cells with their associated Immunoscored and found that they were

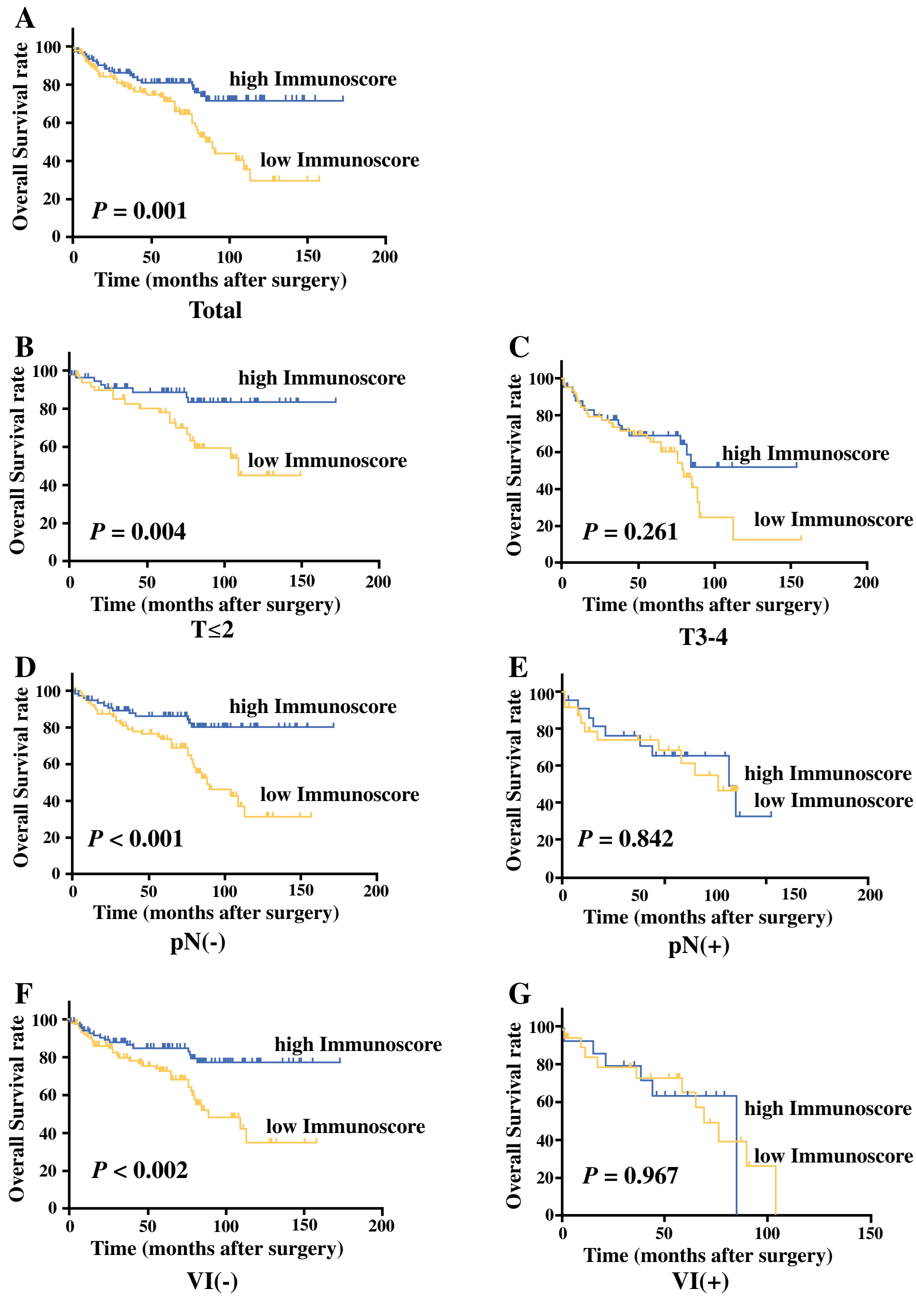

FIG. 2 Survival analysis of the Immunoscore in the entire cohort and different subsets of patients with urothelial carcinoma of the bladder (UCB). **a** Entire cohort. **b** pT $\leq$ 2. **c** pT3/4. **d** pN(–). **e** pN(+). **f** Negative vascular invasion. **g** Positive vascular invasion

TABLE 3 Multivariate cox regression analysis for overall patient survival

Factors	HR	95% CI	*P* value
Immunoscore[a]	2.005	1.195–3.363	0.008
pT status[b]	1.730	1.099–2.723	0.018
pN status[c]	1.451	0.755–2.788	0.264
Vascular invasion[d]	1.429	0.766–2.664	0.262

[a]Low versus high, [b]pT1 versus T2 versus pT3–pT4, [c]pN– versus pN+, [d]Negative versus positive

significantly associated with the cancer-specific survival of UCB patients. These findings show a potentially important role of the host immune response in UCB progression. The Immunoscore was not significantly linked to factors such as clinical stage or tumor grade, but lower Immunoscores were strongly and independently predictive of lower OS, as confirmed via a multivariate analysis.

When we performed a stratified survival analysis of UCB patients, we further found that a lower Immunoscore was significantly associated with the survival of specific UCB patient subsets. Specifically, a low Immunoscore was of better prognostic value for patients who exhibited a lack

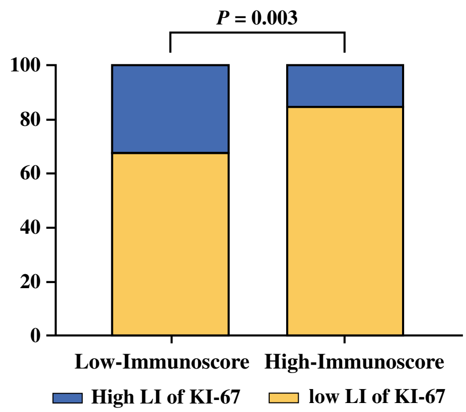

FIG. 3 Association of the Immunoscore (*high/low*) with Ki-67 expression in primary urothelial carcinoma of the bladder (UCB) specimens. Correlation analysis demonstrates that the frequency of cases with a high labeling index of Ki-67 is significantly greater in tumors with a low Immunoscore than in those with a high Immunoscore

of lymph node involvement or a localized tumor stage. For patients with more advanced disease not meeting either of these criteria, a low Immunoscore was not able to predict OS accurately. Similar findings regarding the predictive value of a low Immunoscore also were found to be a function of whether patients presented with vascular invasion or not. Taken together, these findings suggest that among patients with UCB, the Immunoscores achieves the highest prognostic value for the individuals with localized disease.

Consistent with previous reports,[15,16,19] our study indicated that a high density of immune cell infiltrates is associated with a decreased risk of cancer recurrence. However, the reason behind a high density of immune cell infiltrates in some patients but not in others remains unclear. Increasing evidence highlights the intricate interplay between the host innate immunity and cancer cells.[31]

In melanoma cells, the activation of the Wnt/β-catenin pathway has been shown to exclude the anti-tumor immune response via a mechanism of Batf3-lineage dendritic cell recruitment failure.[32] Analysis of specimens from xenograft models has indicated that BRAF inhibition increases tumor infiltration by T cells and enhances antitumor activity.[33]

In hepatocellular carcinoma cells and homozygous PTEN-deleted tumors, tumor-infiltrating lymphocytes were completely depleted of the EGFR amplified.[34] The heterogeneity of immune cell infiltration in UCB patients may be a consequence of genomic differences, or it may differ depending on the type of tumor. Recently, Yu et al.[21] described unusual differences in disease-free survival and OS outcomes with CD3- or CD8-specific outcomes for UCB patients.

Furthermore, Yoon et al.[26] reported significantly greater inter-tumoral heterogeneity in the densities of CD3+ and CD8+ tumor-infiltrating T-lymphocytes in DNA mismatch repair-deficient colon cancers. These findings clearly underscore the clinical importance of heterogeneity in T cell markers in various tumor microenvironments. Unfortunately, limited studies have addressed the roles of the immune system in tumor heterogeneity.

In a recent study, Angelova et al.[35] showed that the immune system can influence tumor heterogeneity by immune selection. These authors demonstrated that neoantigen depletion, which results from active immune selection, was observed at the sites with a high Immunoscore and at those with a spatial proximity between Ki-67+ tumor cells and CD3+ cells.[35]

Although we have shown a positive association between the Immunoscore and the expression of Ki-67, an important marker for cell proliferation in a number of our UCB patients, the precise signaling pathways ultimately involved in these processes remain to be determined. Nevertheless, our results suggest an important role of the host immune response in the control of UCB cell proliferation, which may be at least partially responsible for the tumorigenesis and/or progression of UCB.

Addressing the limitations of this study is important. This study was retrospective, meaning that all the data were obtained from a single tertiary center. Thus, it would be ideal for future studies to be based on multiple centers or for community-based prospective studies to be conducted. Further multicenter prospective studies could support the rationale of this study. That is, the Immunoscore provides an accurate prediction of the UCB patient's prognosis and clinical response, which can result in the application of new immunotherapy approaches.

CONCLUSIONS

The findings of this study suggest that the Immunoscore is a reliable indicator of an improved prognosis for UCB patients who have undergone RC. Particularly, it was more predictive of oncologic outcomes for patients with early T stage, node-negative and negative vascular invasions. Equally important, the Immunoscore has biologic meaning for quantifying infiltrates, and it may provide a tool for novel immunotherapy approaches.

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AUTHOR CONTRIBUTIONS HML, ZWL and XDL designed the research and wrote the paper. XDL, CWH, ZFL, LJJ, HML and FJZ analyzed data and prepared figures. CWH, LJJ, JWC and DX advised on the research design and collected the clinical information. ZWL finalized the paper for submission. All authors read and approved the final manuscript.

AVAILABILITY OF DATA AND MATERIALS The key raw data have been deposited into the Research Data Deposit (http://www.researchdata.org.cn), with the approval number of RDDB2019000530.

DISCLOSURE The authors declare that they have no conflict of interest.

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