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The prognostic values of tumor-infiltrating neutrophils, lymphocytes and neutrophil/lymphocyte rates in bladder urothelial cancer

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ABSTRACT

Tumor-infiltrating neutrophils (TINs) and lymphocytes (TILs) are found to play essential roles in many tumors and associate with the prognosis of patients. But, the prognostic values of TINs, TILs and NLR (neutrophils-lymphocytes ratio) in bladder cancer (BC) are still undefined. The object of our study was to systematically interrogate the associations of these immune cells with clinical outcomes of BC patients. In our study, a total of 102 patients pathologically diagnosed with BC were included. $CD66b^+$ and $CD8^+$ antibodies were used to mark neutrophils and $CD8^+$ lymphocytes by immunohistochemistry. The results found that TINs and NLR were significantly associated with pathological T-stages of tumors ($P < 0.01$), but TILs were not. And TINs were also related to pathological tumor grades ($P = 0.012$). Regarding the prognostic values, TINs was related to the high risk of recurrence in non-muscle invasive BC (NMIBC) patients. Elevated TINs and NLR were associated with poor overall survivals of BC patients, whereas higher TILs were related to longer survivals ($P < 0.01$). Multivariate analysis showed that both of TINs (*HR 2.427, 1.024-5.752, P = 0.044*) and NLR (*HR 3.529, 1.147-10.864, P = 0.028*) were independent unfavorable prognosis markers. In conclusion, Tumor infiltrating immune cells, including TINs, TILs and NLR were important markers in predicting the prognosis of bladder cancer patients. TINs and NLR were more likely to be negative predictors, but TILs were favorable in patients with BC.

1. Introduction

Bladder urothelial cancer (BC) is one of the most common genitourinary malignancies characterized by high prevalence and recurrence rates [1]. It's reported that 5-year recurrence rates of non-muscle invasive bladder cancer (NMIBC) range from 50% to 70% and 5-year rates of progression range from 10% to 20% [2]. Despite the combination of radical cystectomy, chemotherapy, intravesical instillations of BCG and other drugs, only 30–40% of patients with muscle invasive bladder cancers (MIBC) survive 5 years or longer [3]. An essential part of patient management and treatment planning is based on risk stratification. Improving the accuracy of recurrence and progression prediction could help risk-based individuals with a better treatment strategy [4]. Thus, it is important to identify effective markers to predict the prognosis of patients with BC.

In recent years, there is an enhancing awareness in the cancer community that the tumor microenvironment plays a vital role in tumor epigenetics, tumor differentiation, immune escape, and infiltration metastasis [5]. Host immune response and immune cells are crucial factors of the tumor microenvironment [6]. The microenvironment in tumor tissues resembles a status of chronic inflammation, which contains many different inflammatory cells and mediators [7]. Tumor-infiltrating neutrophils (TINs) and lymphocytes (TILs) are main components of inflammatory cells in tumor microenvironment. Neutrophils are considered as the first line of defense during inflammation and infections [8]. Compared with other immune cells in the cancer tissue, neutrophils have received less attention because their lifespan is believed to be too short to influence cancer progression [9]. Actually, it has been reported that the tumor microenvironment may extend the survival of neutrophils both locally and systematically [10]. TILs are a group of lymphocytes located around tumor cells that exhibit diverse functions in various subsets. $CD8^+$ T lymphocytes primarily belong to cytotoxic T lymphocytes (CTLs), which are primarily responsible for the removal of target cells, including tumor cells [11]. Studies have suggested that tumor infiltrating CD8 lymphocytes have antitumor function as judged by their favorable effect on prognosis of many tumors [12–14].

For clinical value, TINs have been reported to be associated with

Abbreviations: TINs, tumor-infiltrating neutrophils; TILs, tumor-infiltrating lymphocytes; NLR, neutrophil/lymphocyte rate; BC, bladder cancer; IHC, immunohistochemistry; OS, overall survival; NMIBC, non-muscle invasive bladder cancer; MIBC, muscle invasive bladder cancer; TURBT, transurethral resection of the bladder tumor

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Table 1
The clinical and pathological characters of included 102 patients.

Clinical and pathological characters		Number	Percent
Age	< = 70y	56	55%
	> 70y	46	45%
Gender	Male	86	84%
	Female	16	16%
Smoking	No	48	48%
	Yes	53	52%
pT	Ta, T1	53	52%
	T2-T4	49	48%
Grade	Low	36	35%
	High	66	65%
Tumor size	< = 2 cm	45	44%
	> 2 cm	57	56%
Tumor number	Single	58	57%
	Multiple	44	43%
Surgical type	TURBT	62	60%
	RC, PC	40	40%
TIN	Low (< 20/Hp)	48	47%
	High (> 20/Hp)	54	53%
TIL	Low (< 12/Hp)	44	43%
	High (> 12/Hp)	58	57%
NLR	Low (< 1)	48	47%
	High (> 1)	54	53%

Note: pT: Pathological T stage; TURBT: Transurethral resection of the bladder tumor; RC: Radical cystectomy; PC: Bladder partial resection.

patient outcome in many tumors, such as renal cell carcinomas [15], esophageal squamous cell carcinoma [16], pancreatic neoplasia [17], and cholangiocarcinoma [18]. A meta-analysis concluded that TINs were typically pro-tumor and were strongly associated with poorer prognosis in the majority of cancers [19]. $CD8^+$ lymphocytes have been found to be associated with better clinical response in carcinomas [20]. In bladder cancer, the number of $CD8^+$ lymphocytes observed in non-recurrent group is lower than that in recurrent patients [21]. Denser TILs are associated with MIBC, but higher prevalence of TILs are associated with a favorable response, even in the setting of a more invasive disease [22]. Although TINs and TILs have been widely researched, the prognostic value of TILs, TINs and NLR in BC is still unconcluded. The main aims of this study are to investigate the associations of TINs and CD8+ TILs with clinical-pathological features, and to analyze the effect of the distribution of TINs and CD8+ TILs on prognosis of BC patients.

2. Materials and methods

2.1. Patients and specimens

102 patients pathologically diagnosed with urothelial bladder cancer in the second hospital of Tianjin medical university (Tianjin, China) from January 2010 to June 2012 were analyzed retrospectively. The tumor tissues of these patients were collected after surgical treatment and used for immunohistochemistry. Clinical and pathological features including ages, genders, smoking states, tumor sizes (maximum diameter of the tumor), numbers, pathological grades and pTNM stages were recorded. The tumors were classified by 2009 UICC TNM staging and 2004 WHO/ISUP classification [23,24]. The patients with any inflammatory signs, concurrent disease, and preoperative/postoperative chemoradiotherapy were excluded. The included patients were followed up every 2–6 months after leaving the hospital. The median follow-up was 50 months (range from 4 to 92 months).

2.2. Immunohistochemistry (IHC)

Bladder tumor samples obtained from the department of pathology were stained by IHC methods. Rabbit anti-$CD8^+$ monoclonal antibody (ZA-0508, Working solution, Zhongshan Golden Bridge Biotechnology Inc, China) and Mouse anti-$CD66b^+$ polyclonal antibodies (555723, 1:100, BD Biosciences, USA) were used to detect tumor infiltrating lymphocytes and neutrophils respectively. Briefly, after routine deparaffinization, rehydration, blocking of endogenous peroxidase activity, and heat-induced epitopes-retrieval, the tissue sections were incubated with primary antibody at 4 °C overnight. Then, the universal secondary antibodies (ZB-2010, 1:150, Zhongshan Golden Bridge Biotechnology Inc, China) were added.

2.3. Evaluation of immunostaining

The IHC staining was evaluated by two experienced pathologists under the microscope (Olympus Company) independently. Four fields were randomly selected under low magnification (×100) and the positive cells were counted at high magnification (×200). Counts were performed in the tumor and the stroma respectively. Intratumoral leukocytes were defined as neutrophils or CD8+ lymphocytes that infiltrated into cancer nests or stroma, whereas peritumoral leukocytes were the cells that distributed along the tumor-connective tissue junction. The median leukocyte count was used as cutoff to categorize each case into either a high or low group. Areas of necrosis, distortion artifacts and cells within the blood vessels were excluded.

2.4. Statistical analysis

All data analyses were performed with SPSS version 20.0. Continuous variables were analyzed with *t* student test and categorical variables were with chi-square tests. Data of stained cell at different locations were expressed as mean and range, and tested with Student *t* tests. The correlation analysis was performed by calculating the Pearson correlation coefficient. We used chi-square test when comparing the clinical pathological features between groups. The Kaplan-Meier estimates and the log-rank test were used to compare the relapse-free survival (RFS) and overall survival (OS). Multivariate analysis was performed using a Cox proportional hazard model with factors found to be consistently meaningful from univariate analysis. $P < 0.05$ for the difference was considered as significance.

3. Results

3.1. Characteristics of patients

A total of 102 patients first diagnosed with bladder cancer were included in our study. The median age of patients was 67 years (range from 33 to 88 years). 84% of patients were male, and 16% were female. 52% of patients had a history of smoking. 52% were NMIBC and 48% had MIBC. The median and mean lengths of tumor size were 3 cm and 2.8 cm, respectively. 57% tumors were solitary, others were multiple. The patients treated by transurethral resection of the bladder tumor (TURBT) accounted for 60%; most of them were diagnosed with NMIBC. The cutoff values of TINs and TILs were 20/Hpf and 12/Hpf respectively according to the distribution of the scores, and the cutoff value of NLR was 1. Besides, the cutoff value of TPN and TPL was determined by median of patients. The details of these patients were summarized in Table 1.

3.2. Association of TIN, TIL and NLR with clinical-pathological features

The levels of TINs and TILs in BC tumor tissues were evaluated by IHC. The typical staining of TINs and TILs in NMIBC and MIBC was shown. (Fig. 1) In our study, chi-square test was performed to investigate the associations of TINs, TILs and NLR with the clinical- pathological characters of BC patients. The results revealed that elevated level TINs were significantly associated with the worse tumor pathological T-stages and grades of patients ($P < 0.05$). NLR was found to be related to pathological T stage ($P = 0.005$). However, no associations