CD169-positive sinus macrophages in the lymph nodes determine bladder cancer prognosis

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CD169+ macrophages are suggested to play a pivotal role in establishing anti-tumor immunity. They capture dead tumor cell-associated antigens and transfer their information to lymphocytes, including CD8+ T cells, which is important for successful tumor suppression. This study aimed to determine the prognostic significance of CD169+ macrophages residing in the tumor-draining lymph nodes from cases of bladder cancer. In this retrospective study, 44 bladder cancer patients who received radical cystectomy were examined. The abundance of CD169+ macrophages in the regional lymph nodes and the number of CD8+ T cells in the primary tumor were investigated by immunohistochemistry. A CD169 score was calculated based on the intensity of CD169 staining and the proportion of CD169+ macrophages, and the scores were compared to the patients' clinicopathological parameters. A high CD169 score was significantly associated with low T stage and with a high number of CD8+ T cells infiltrating into the tumor. The group with high CD169 expression had significantly longer cancer-specific survival than the group with low CD169 expression (5-year cancer-specific survival rate: 83.3% vs 31.3%). In a multivariate analysis, the CD169 score was identified as a strong and independent favorable prognostic factor for cancer-specific survival. Our findings suggest that CD169+ macrophages in the lymph nodes enhance anti-tumor immunity by expanding CD8+ T cells in bladder cancer. The CD169 score may serve as a novel marker for the evaluation of bladder cancer prognosis.

KEYWORDS
bladder cancer, CD169, macrophage, prognosis, regional lymph node

1 | INTRODUCTION

Bladder cancer is the second most common form of malignancy in the urinary tract, with over 400 000 new cases diagnosed annually worldwide. Histologically, more than 90% of bladder cancers arise from urothelial cells. Clinically, bladder cancer is highly responsive to immunotherapy. For example, intravesical bacillus Calmette-Guérin (BCG) therapy is highly effective for the treatment of urothelial carcinoma in situ. In addition, intravesical BCG therapy is superior to chemotherapy for preventing the recurrence of non-muscle invasive bladder cancer. Although the detailed mechanism of BCG therapy's effect remains unclear, activation of the immune system by BCG is considered to be crucial for the rejection of bladder cancer. In fact, the depletion of either CD4+ or CD8+ T cells...
eliminates BCG-mediated anti-tumor activity. Natural killer (NK) cells are also considered essential for the induction of anti-tumor immunity by BCG. These studies strongly suggest that the immune response contributes to the inhibition of bladder cancer. However, a pathological marker that allows prediction of bladder cancer prognosis is not well-defined.

Tumor cells escape immune surveillance via various tactics, and macrophages are considered to play a pivotal role in this process. Tumor-associated macrophages (TAM), for example, support tumor progression either by promoting angiogenesis, increasing the number of regulatory T cells, or inducing apoptosis to cytotoxic CD8+ T cells. The number of TAM within the tumor is associated with advanced tumor progression in various cancers both in animal models and human patients. In contrast, macrophages residing in the tumor-draining lymph nodes are important for enhancing anti-tumor immunity. These macrophages are characterized by their surface expression of the CD169 molecule and their localization at the interface between the tissue and circulating fluids. In the lymph nodes of mice, CD169+ macrophages capture lymph-borne dead tumor cells and activate tumor antigen-specific CD8+ T cells. In humans, the number of CD169+ macrophages in the tumor-draining lymph nodes positively correlates with favorable prognosis in patients with colon cancer and malignant melanoma.

In this study, we retrospectively examined the importance of CD169+ macrophages in suppressing bladder cancer. We revealed that the abundance of CD169+ macrophages in the tumor-draining lymph nodes is associated with the amount of CD8+ T cells in the tumor, low T stage, and the favorable cancer-specific survival. Therefore, our findings suggest that CD169+ macrophages in the tumor-draining lymph nodes may be a useful predictor of prognosis in patients with bladder cancer.

2 MATERIALS AND METHODS

2.1 Study design

We conducted a retrospective analysis in accordance with the Declaration of Helsinki with the approval of the ethical committees of Omori Red Cross Hospital (#16-24), Teikyo University Hospital (#16017) and Kumamoto University Hospital (#509). The aim of the present study was to investigate the association between the abundance of CD169+ macrophages in tumor-draining lymph nodes and the prognosis of patients with invasive bladder cancer. After approvals were obtained from institutional review boards, we reviewed patients who underwent radical cystectomy for bladder cancer at Omori Red Cross Hospital or Teikyo University Hospital between March 2003 and March 2015, or at Kumamoto University Hospital between March 2004 and March 2016.

2.2 Patients

We evaluated tumor and regional lymph node specimens that were resected from 44 invasive bladder cancer patients (35 males and 9 females; average age, 70 years; range, 49-85 years) who underwent radical cystectomy. Patients were excluded either if they had received BCG immunotherapy in the past, or if they had distant metastasis before cystectomy. Bladder cancer death was the endpoint for survival analysis.

2.3 Immunohistochemistry

Among the available resected regional lymph nodes, we examined obturator lymph nodes without any metastasis. The portion of the primary tumor with the deepest invasion was also selected for evaluation. The tumor tissues and regional lymph nodes had been routinely fixed in 10% neutral buffered formalin and were embedded in paraffin. Anti-CD169 (clone HSN 7D2; Santa Cruz Biotechnology, CA, USA), anti-CD68 (clone PG-M1; Agilent Technologies, Santa Vlara, CA, USA), anti-CD8 (clone C8/144B; Nichirei, Tokyo, Japan), anti-HLA class I (Hokudo, Sapporo, Japan) and anti-HLA DR (Agilent Technologies) antibodies were used as the first antibody for immunohistochemistry (IHC), and IHC were performed as described previously. Signals were visualized with DAB substrate (Nichirei) or HistoGreen (Linaris, Dossenheim, Germany). CD8+ T cells in the primary tumor were counted in 5 randomly selected high-power fields. CD169 scores for expression in lymph node macrophages were analyzed using the system introduced by Albrecht et al. Cell counting and scoring were conducted by 2 professional pathologists (K. O. and T. S.), who were blinded to the clinico-pathological data.

2.4 Statistical analysis

Statistical analysis was carried out using the JMP 7 software (SAS Institute, IL, USA). Bivariate comparisons of clinico-pathological features between patients with high (n = 18) and low (n = 26) CD169 scores were performed using the χ²-test. The association of multiple prognostic factors with cancer-specific survival was assessed using univariate and multivariate Cox proportional hazard model analysis. Multivariate analysis included pathological T stage, LN metastasis, CD8+ T cell number and CD169 score. Survival curves were calculated using the Kaplan–Meier method, and the difference between survival curves was analyzed using the log-rank test. Regression analysis was used for the assessment of the relationship between 2 variables. Differences were considered statistically significant at P-values of <.05.

3 RESULTS

3.1 CD169 expression in the regional lymph node of patients with bladder cancer

The 44 bladder tumors included T1 disease (5 cases), T2 disease (17 cases), T3 disease (17 cases), and T4 disease (5 cases). Among those, 35 cases were pure urothelial carcinoma, 5 were urothelial carcinoma with squamous differentiation, 2 were urothelial carcinoma...
Pelvic lymph node metastasis was pathologically confirmed in 6 patients (13.6%). The follow-up intervals after radical cystectomy ranged from 2.0 to 133.0 months (average: 33.4 months). During this period, 14 patients died of bladder cancer. The 5-year cancerspecific survival rate was 48.9%. These data are summarized in Table S1. We used immunohistochemistry to investigate the expression of CD169 and CD68 in regional lymph nodes and tumor tissue that were obtained from bladder cancer patients who received radical cystectomy. Consistent with previous reports, the quantity of CD68+ total macrophages in the lymph nodes was similar for all patients (Figure 1A). However, the proportion of CD169+ cells and the intensity of CD169 staining varied widely between the lymph nodes (Figure 1A). We semiquantified the staining intensity for CD169 and the proportion of CD169+ cells among CD68+ total macrophages, as described in the Materials and Methods. The CD169 staining intensity was scored as 0 (no intensity), 1 (weak intensity that was only detectable in high-power fields), 2 (moderate intensity that was detectable in low-power fields) or 3 (strong intensity) (Figure 1B). The proportion of CD169+ cells was scored as 0 (below 1%), 1 (1%-10%), 2 (11%-50%) and 3 (over 50%; Figure 1B). The intensity and proportion scores were added together to provide a CD169 score (range: 0-6) as shown in Figure 1C, with a low CD169 score defined as 0-4 and a high CD169 score defined as 5-6.

**FIGURE 1** Immunohistochemistry (IHC) and scoring system of CD169 in the regional lymph nodes. A, IHC of CD68 and CD169 in lymph nodes, and the figures of a case with high expression of CD169 (upper panels) and a case with low expression of CD169 (lower panels) are presented. Scale bar = 200 μm (left and middle) or 50 μm (right). B, The CD169 intensity score (upper panels) and the proportion score (lower panels) were determined based on the intensity (upper left: weak, middle: moderate, or right: strong) or the proportion of the CD169 staining (lower left, 1%-10%, middle: 11%-50%, or right: above 50%). Scale bar = 100 μm. C, The CD169 score was calculated as 0-6 by adding the intensity (0-3) and the proportion (0-3) score. In the left case, 2 of 10 CD68+ macrophages are weakly positive for CD169, which corresponds to an intensity score of 1, and the proportion score of 2. As a result, this case is defined as the CD169 score of 3. In the right case, 3 of 10 CD68+ macrophages are moderately positive for CD169 and 4 out of 10 CD68+ macrophages are strongly positive for CD169, which corresponds to an intensity score of 3 and a proportion score of 3. As a result, this case is defined as the CD169 score of 6. D, The double IHC for CD169 (brown) and HLA class I (upper panel, green) or HLA DR (lower panel, green) in the regional lymph nodes. Scale bar = 100 μm.
6. The CD169+ macrophages were strongly positive for HLA class I, but had weak expression of HLA DR (Figure 1D).

3.2 | Correlation between CD169 expression in lymph node macrophages and CD8+ T cell infiltration in the primary tumor

We counted the numbers of CD8+ T cells in the primary tumor, and analyzed the correlation with the patients’ clinicopathological factors and CD169 expression in their lymph nodes. It should be noted that CD68+ macrophages infiltrating tumor tissue were negative for CD169 (Figure 2A). Regression analysis revealed a positive correlation between the CD169 score from the patients’ lymph nodes and the density of CD8+ T cells in the tumor (P = .0046, r² = .3110, Figure 2B), and similar results were obtained from bivariate analysis (Table 1). The interactions between the macrophages and T cells were visualized using double immunostaining for CD169 and CD8, which revealed that some CD8+ T cells were adjacent to CD169+ macrophages in the regional lymph nodes (Figure 2C).

3.3 | Correlations between clinicopathological factors and either CD169 expression on lymph node macrophages or CD8+ T cells infiltration in the primary tumor

The CD169 score was not associated with age, gender, the presence of lymph node metastasis, history of chemotherapy, or histological type. However, a high CD169 score was associated with a low T stage (P = .013, Table 1). A Cox proportional hazard model was used to evaluate the 44 bladder cancer patients’ clinicopathological features for associations with cancer-specific survival. The group with a high CD169 intensity score had a higher, albeit not significantly increased, cancer-specific survival rate than the group with a low intensity score (P = .0714, Figure 3A). A similar non-significant trend towards a higher cancer-specific rate was observed in the group with a high CD169 proportion score group (P = .0636, Figure 3B). A high CD169 score, which combined the intensity and proportion score, was associated with better 5-year cancer-specific survival rates (high CD169 score: 83.3% vs low CD169 score: 31.3%, P = .0078, Figure 3C). A high density of CD8+ T cells also tended to positively correlate with a better prognosis; however, the association was not statistically significant (P = .1955, Figure 3D).

The univariate analyses revealed that good cancer-specific survival was associated with a low pathological T stage (P = .042), with absence of a history of adjuvant chemotherapy (P = .012) and with a high CD169 (P = .003; Table 2). The multivariate analysis included pathological T stage, LN metastasis, CD8+ T cell number and CD169 score, and revealed that CD169 score was a strong and independent favorable prognostic factor (P = .021, Table 2).

4 | DISCUSSION

The present study revealed that a high CD169 score was associated with a low T stage and was negatively correlated with low T stage and was strongly associated with favorable cancer-specific survival. Our univariate and multivariate analyses also revealed that the CD169 score was an independent predictor of good prognosis for patients with bladder cancer. The CD169 score was also positively correlated with the quantity of CD8+ T cells infiltrating the primary tumor. In this study, the history of adjuvant chemotherapy was
In conclusion, we demonstrated the prognostic significance of CD169+ macrophages in lymph nodes of patients with bladder cancer. The abundance of CD169+ macrophages in the tumor-draining lymph nodes positively correlated with a favorable prognosis. Thus, pathological examination of CD169+ expression intensity and the proportion of CD169+ macrophages in the tumor-draining lymph node...
FIGURE 3 Kaplan–Meier’s cancer-specific survival curves for patients with bladder cancer. A, The patients were divided into 2 groups according to their intensity score: 0-1 was defined as low intensity and 2-3 was defined as high intensity. IS, intensity score. B, The patients were divided into 2 groups according to their proportion score: 0-2 was defined as a low proportion and 3 was defined as a high proportion. PS, proportion score. C, The patients were divided into 2 groups according to their total CD169 score: 0-4 was defined as a low score and 5-6 was defined as high score. D, The patients were divided into 2 groups according to the amounts of CD8$^+$ T cells in the tumor.

TABLE 2 Associations between clinicopathological features and the bladder cancer-specific survival

| Clinicopathological feature | n | Univariate analysis | | | | Multivariate analysis | | | |
|-----------------------------|---|---------------------|-----|| | | Hazard ratio | $P$-value | Hazard ratio | 95% CI | $P$-value |
| Age (y) | | | | | | | | | | |
| $<$70 | 19 | 1.16 | .784 | ND | ND | ND |
| $\geq$70 | 25 | | | | | | | | |
| Gender | | | | | | | | | |
| Male | 35 | 1.00 | .993 | ND | ND | ND |
| Female | 9 | | | | | | | | |
| Pathological T stage | | | | | | | | | |
| T1, T2 | 22 | 3.11 | .042$^a$ | 1.45 | 0.42-5.54 | .559 |
| T3, T4 | 22 | | | | | | | | |
| LN metastasis | | | | | | | | | |
| No | 38 | 2.65 | .266 | 2.13 | 0.31-9.50 | .393 |
| Yes | 6 | | | | | | | | |
| Adjuvant chemotherapy | | | | | | | | | |
| No | 33 | 3.62 | .012$^a$ | ND | ND | ND |
| Yes | 11 | | | | | | | | |
| CD8$^+$ T cells/mm$^2$ in tumor | | | | | | | | | |
| $<$343 | 25 | 0.44 | .177 | 0.95 | 0.20-3.55 | .947 |
| $\geq$343 | 19 | | | | | | | | |
| CD169 score | | | | | | | | | |
| Low | 26 | 0.10 | .003$^a$ | 0.13 | 0.01-0.76 | .021$^a$ |
| High | 18 | | | | | | | | |

CI, confidence interval; LN, lymph node; ND, not done.

$^a$Statistically significant results.
nodes may help predict the clinical prognosis of bladder cancer patients.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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