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Tumor-infiltrating Immune Cells in H&E-stained Sections of Colorectal Cancer Tissue as a Reasonable Immunological Biomarker

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Abstract. *Background: The density of tumor-infiltrating lymphocytes has been reported to reflect the antitumor immune status, and many reports have shown that tumor-infiltrating CD8$^+$ and total T-lymphocytes may be strong prognostic biomarkers in colorectal cancer. We previously reported that the density of tumor-infiltrating immune cells in hematoxylin and eosin (H&E)-stained sections may be an easily available prognostic biomarker. However, it remains unclear whether the density of tumor-infiltrating immune cells in H&E-stained sections accurately reflects the antitumor immune status. Patients and Methods: A total of 308 patients who underwent curative resection for stage II/III colorectal cancer were enrolled. The density of both tumor-infiltrating immune cells in H&E-stained sections and tumor-infiltrating lymphocyte subsets was assessed by immunohistochemistry. Results: The density of tumor-infiltrating immune cells in H&E-stained sections was significantly and positively correlated with that of tumor-infiltrating CD4$^+$/CD8$^+$/total T-lymphocytes. Conclusion: The density of tumor-infiltrating immune cells in H&E-stained sections may be a reasonable immunological biomarker.*

The density of tumor-infiltrating lymphocytes (TILs), which reflects the antitumor immune status, has been reported to be correlated with tumor progression and the clinical outcome in several malignancies, including non-small cell lung cancer, colorectal, esophageal, and urothelial cancer, and melanoma (1-8). In particular, the density of tumor-infiltrating CD8$^+$ and total T-lymphocytes are reportedly correlated with the antitumor immune status of the host, and these values are used as a component of the 'Immunoscore', which is a powerful immunological biomarker in colorectal cancer (9, 10). On the other hand, an International TILs Working Group made recommendations for the standard methodology for evaluating tumor-infiltrating lymphocytes in hematoxylin and eosin (H&E)-stained sections of breast cancer tissue (11). Based on those recommendations (11), we previously reported that the density of immune cells in the tumor stroma in H&E-stained sections of colorectal cancer tissue was significantly correlated with the survival outcome after curative resection (12). Such a method of evaluating tumor-infiltrating immune cells using H&E-stained sections is easy to apply in the clinical setting due to its convenience. However, it remains unclear whether the density of tumor-infiltrating immune cells in H&E-stained sections accurately reflects the antitumor immune status. The reasons are as follows: Firstly, it is unclear whether the density of immune cells in H&E-stained sections, which are mononuclear immune cells that include not only T-lymphocytes, but also plasma cells and B-cells, is actually correlated with that of CD8$^+$ and total T-lymphocytes. Secondly, there are differences in the area assessed in evaluation using H&E-stained sections and that using immunohistochemistry. Only the TILs in the tumor stroma are evaluated in H&E-stained sections due to technical issues. In contrast, in immunohistochemistry, all TILs in both the tumor stroma and cancer cell nests are evaluated. Whether the density of immune cells in the tumor stroma in H&E-stained sections reflects the antitumor immune status of the whole tumor is not clear. We planned this retrospective cohort study of 308 patients with stage II/III colorectal cancer in order to answer these questions.

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Key Words: Tumor-infiltrating immune cells, H&E, tumor-infiltrating T-lymphocytes, immunological biomarker, colorectal cancer.

Patients and Methods

Patients. A total of 308 patients who underwent curative resection for stage II/III colorectal cancer at the Department of Surgical Oncology of Osaka City University Hospital between 2007 and 2012 were enrolled. The data were analyzed retrospectively. Patients

who underwent neoadjuvant therapy or emergency surgery for perforation or obstruction and those with coexisting inflammatory bowel disease were excluded. The resected sections were pathologically classified according to the seventh edition of the TNM classification of malignant tumors (13).

Evaluation of immune cells in H&E-stained sections. The density of immune cells in H&E-stained sections of colorectal cancer tissue was estimated according to the recommendations by the International TILs Working Group (11) as well as our previous study (12). The method is summarized as follows: we focused on the invasive margin of the tumor at low magnification and evaluated (semi-quantitatively) the percentage of the area of the tumor stroma that was occupied by mononuclear immune cells (at intervals of 10%) at high magnification (×200) (Figure 1). Mononuclear immune cells (including lymphocytes and plasma cells) in the tumor stroma were defined as TILs. The density of TILs was evaluated in five different fields and the average density of TILs was calculated.

Immunohistochemistry. Immunohistochemical staining of CD4 and CD8 was performed as previously described (14, 15). The methods are summarized as follows. Tumor sections from surgically resected specimens from all of the enrolled patients were incubated with primary mouse monoclonal anti-CD4 antibodies (1:80 dilution; Dako, Glostrup, Denmark) at room temperature for 20 minutes, and primary mouse monoclonal anti-CD8 antibodies (1:100 dilution; Dako) at room temperature for 30 min.

The immunohistochemically stained sections were then evaluated by a pathologist who was blinded to the clinical information. The number of $CD4^+$ and $CD8^+$ lymphocytes was counted in each location (*i.e.* in the tumor stroma, cancer cell nests, and in the whole microscopic field) at the invasive margin of the tumor at ×400 magnification (Figure 2). The average number of $CD4^+$ and $CD8^+$ lymphocytes in five randomly selected fields was then estimated. The sum of the number of $CD4^+$ and $CD8^+$ lymphocytes was defined as the total number of T-lymphocytes.

Statistical analysis. Associations between the density of stromal TILs and the number of lymphocytes ($CD4^+$, $CD8^+$ and total T-lymphocytes) were evaluated using Pearson's correlation analysis. The statistical analysis was conducted using the JMP® 13.0.0 (2016 SAS institute Inc., Cary, NC, USA). *p*-Values of less than 0.05 were considered to indicate statistical significance. The R values for the degree of correlation were classed as follows: $0.0 \leq r \leq 0.2$, very weak; $0.2 \leq r \leq 0.4$, weak; $0.4 \leq r \leq 0.7$, moderate; $0.7 \leq r \leq 1.0$, strong.

Ethical considerations. This research conformed to the provisions of the Declaration of Helsinki. All patients were informed of the investigational nature of this research and provided their written informed consent. This research was approved by the Ethics Committee of Osaka City University (approval no. 3853).

Results

Patient characteristics. The patient characteristics are shown in Table I. The median age was 68 years (range=21-96 years). One hundred fifty-eight patients were male (51%) and 150 were female (49%). The tumor depth was T1-3 in the majority of patients (66%). One hundred and sixty-six (54%) patients were negative for lymph node metastasis.

Table I. *Patient characteristics*.

Clinicopathological factor	n=308
Gender, n (%)	
Male	158 (51%)
Female	150 (49%)
Age (years)	
Median (range)	68 (21-96)
Location of primary tumor, n (%)	
Colon	169 (55%)
Rectum	139 (45%)
Tumor depth, n (%)*	
T1-3	204 (66%)
T4	104 (34%)
Tumor diameter (cm)	
Median (range)	4.5 (0.8-12.0)
Histological type, n (%)	
Well-, moderately differentiated	284 (92%)
Poorly differentiated, mucinous	24 (7.8%)
Lymphatic involvement, n (%)	
Negative	83 (27%)
Positive	225 (73%)
Venous involvement, n (%)	
Negative	242 (79%)
Positive	66 (21%)
Lymph node metastasis, n (%)	
Negative	166 (54%)
Positive	142 (46%)

*According to TNM Classification of Malignant Tumors (seventh edition) (13).

The distribution of tumor-infiltrating T-lymphocytes. Regardless of the lymphocyte subsets, the number of lymphocytes infiltrating the cancer cell nests was extremely low and most T-lymphocytes were present in the tumor stroma (Table II).

Associations between the density of immune cells in H&E-stained sections and the density of tumor-infiltrating T lymphocytes subsets. The density of tumor-infiltrating immune cells in H&E-stained sections was weakly positively associated with the density of CD4+ T-lymphocytes in the whole microscopic field and in the tumor stroma (whole microscopic field, r=0.26, $p<0.001$; tumor stroma, r=0.26, $p<0.001$) (Figure 3).The density of immune cells in the H&E-stained sections showed a relatively strong positive association with the density of $CD8^+$ T-lymphocytes in the whole microscopic field and in the tumor stroma (whole microscopic field, r=0.50, $p<0.001$; tumor stroma, r=0.46, $p<0.001$) (Figure 3). The density of immune cells in H&E-stained sections was relatively strongly positively associated with the density of total T-lymphocytes in the whole microscopic field and in the tumor stroma (whole microscopic field, r=0.50, $p<0.001$; tumor stroma, r=0.48, $p<0.001$) (Figure 3).

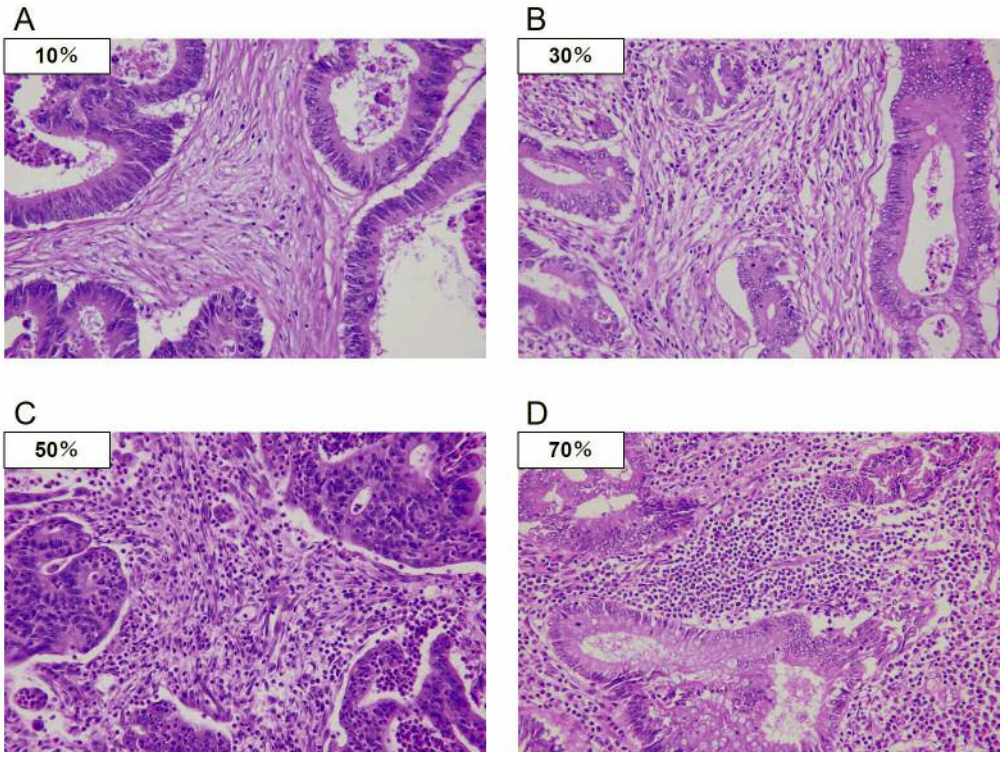

Figure 1. *The evaluation of the density of tumor-infiltrating immune cells in the tumor stroma in hematoxylin and eosin-stained sections of colorectal cancer tissue. A: 10%. B: 30%. C: 50%. D: 70%. Magnification, ×200.*

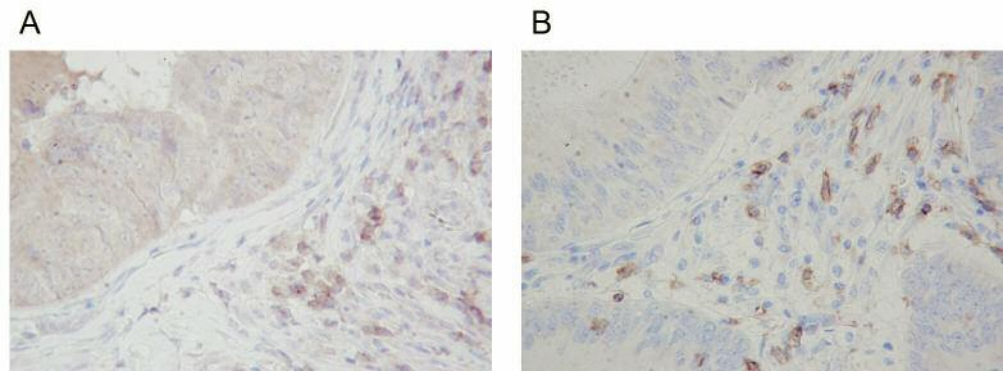

Figure 2. *The immune marker expression of tumor-infiltrating T-lymphocytes in colorectal cancer. A: CD4. B: CD8. Magnification, ×400.*

Discussion

In this study, we made two important observations. Firstly, the density of the tumor-infiltrating immune cells in H&E-stained sections was positively associated with the density of tumor-infiltrating $CD8^+$ and total T-lymphocytes, which has been reported to be significantly correlated with the survival outcome (1, 2, 16, 17). Secondly, as the number of T-lymphocytes infiltrating cancer cell nests was extremely low, the density of TILs in the tumor stroma alone in H&E-stained sections was significantly associated with the density of tumor-infiltrating T-lymphocytes in the whole tumor.

The current study showed a significant positive association between the density of tumor-infiltrating immune

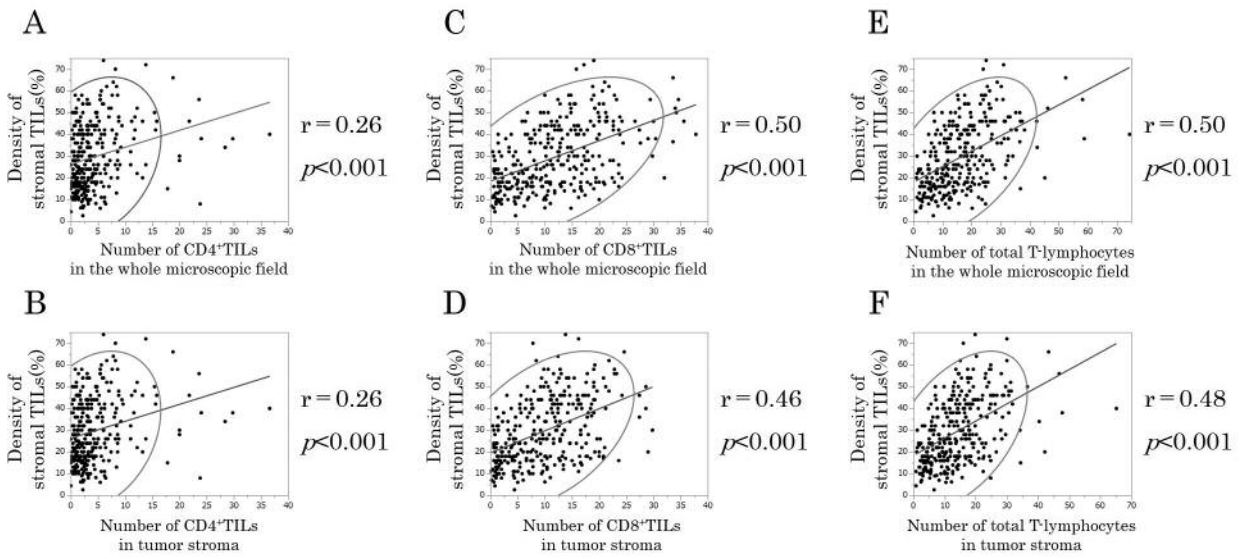

Figure 3. *The associations between the density of hematoxylin and eosin-stained stromal TILs and the number of T- lymphocytes (CD4+, CD8+ and total CD4+CD8+ T-lymphocytes).*

Table II. *The distribution of the T-lymphocytes in tumors.*

	Median number of TILs/field (range)		
Subgroup	Tumor stroma	Cancer cell nests	Whole field (tumor stroma+cancer cell nests)
CD4	2.9 (0-36.6)	0 (0-2.2)	3.0 (0-36.6)
CD8	9.6 (0-29.8)	0.6 (0-16.4)	11.1 (0.2-37.8)
Total T-lymphocytes	13.3 (0.8-65.2)	0.6 (0-16.4)	15.0 (0.8-74.4)

TILs: Tumor-infiltrating lymphocytes.

cells in H&E-stained sections and the density of tumor-infiltrating $CD8^+$ and total T-lymphocytes. Some previous reports, including our previous own, demonstrated that the density of tumor-infiltrating immune cells in H&E-stained sections of colorectal cancer tissue was an immunological biomarker that was associated with the survival outcome (8, 12, 18-20). However, it remains unclear whether the density of mononuclear immune cells, which includes not only T-lymphocytes, but also plasma cells and B-lymphocytes, in H&E-stained sections of colorectal cancer tissue accurately reflects the antitumor immune status. Many authors have reported that the density of tumor-infiltrating $CD8^+$ and total T-lymphocytes was significantly associated with the antitumor immune status and the survival outcome (1, 2, 16, 17). In this background, the density of tumor-infiltrating $CD8^+$ and $CD3^+$ T-lymphocytes is used as a component of the Immunoscore, an immunological prognostic scoring system (10, 21). On the basis of the associations between the density of immune cells in H&E-stained sections and the number of $CD8^+$ and total T-lymphocytes, we concluded that the density of immune cells in H&E-stained sections may accurately reflect the antitumor immune status. Regarding $CD4^+$ T-cells, the functions of each $CD4^+$ T-cell subset in antitumor immunity differ. In concrete terms, T-helper 1 cells have been reported to enhance antitumor immunity (16, 22, 23). However, the functions of T-helper 2 cells, T-helper 17 cells and regulatory T-cells have differed among reports, being described as immunostimulatory, immunosuppressive and neutral depending on the type and stage of cancer (1, 22, 24-26). In fact, some previous reports, including our own, showed that the density of tumor-infiltrating $CD4^+$ T-cells was not associated with the survival outcome (1, 14). Moreover, mononuclear immune cells in cancer stroma in H&E sections, which were evaluated in the current study,

include not only T-cells but also B-cells and plasma cells. Some studies have reported that tumor-infiltrating B-cells may enhance the antitumor immunity and be associated with a favorable clinical outcome (27, 28), while others have reported that B-cells may be a heterogeneous population with several functionally discrete subsets (28-30), and still others have reported that the functions of tumor-infiltrating B-cells and plasma cells remain unclear (11). Thus, there is no consensus concerning the functions of tumor-infiltrating B-cells and plasma cells. As described above, the mononuclear immune cells evaluated in the tumor stroma in H&E sections included several types of immune cells. However, we concluded that the density of the tumor-infiltrating immune cells in H&E sections might be an immunologically reasonable biomarker, as the density of the tumor-infiltrating immune cells in H&E sections was associated with that of the tumor-infiltrating $CD8^+$ and total T-cells, which are components of the Immunoscore (10).

The current study showed that the density of immune cells only in the tumor stroma (other than the immune cells infiltrating cancer cell nests) in H&E-stained sections was significantly associated with the density of infiltrating T-lymphocytes in the whole tumor, because the number of T-lymphocytes infiltrating the cancer cell nests was extremely low. The recommendations on the evaluation of TILs in H&E-stained sections of breast cancer tissue by the International TILs Working Group reported that the density of TILs in the tumor stroma alone may be a more useful and reproducible biomarker than the density of TILs in cancer cell nests (11). The reason for this was believed to be that the TILs in cancer cell nests are fewer and more heterogeneous than those in the tumor stroma alone, and it is difficult to count the number of TILs in cancer cell nests in H&E-stained sections because it is difficult to distinguish the nuclei of cancer cells from those of mononuclear immune cells. Moreover, the scoring of TILs in cancer cell nests has been reported to produce no more information than that which can be gleaned from the TILs in the tumor stroma because the number of TILs in cancer cell nests is usually associated with the number of TILs in the tumor stroma. It was also reported that scoring TILs in the tumor stroma alone had a clear advantage because the density of tumor cells and their growth pattern do not affect the score. The number of T-lymphocytes in cancer cell nests was extremely low in colorectal cancer as well as breast cancer; it was reported that most tumor-infiltrating T-lymphocytes exist in the tumor stroma (31).

The current study is associated with certain limitations. Firstly, in cases of diffuse infiltrative colorectal cancer (such as signet ring cell carcinoma and undifferentiated carcinoma), which were quite rare, it was difficult to evaluate the area occupied by immune cells in the tumor stroma in H&E sections. The density of TILs may need to be evaluated by immunohistochemistry in such types of colorectal cancer. Secondly, although we estimated the average density of TILs in H&E-stained sections from five different fields in order to allow the evaluation to be performed easily, evaluating the density of TILs over the whole tumor at a low magnification (*e.g.* ×100) might be a more suitable method for resolving the issue of heterogeneity in the tumor. Thirdly, although we evaluated the density of TILs in H&E-stained sections semi-quantitatively (every 10%), according to the recommendations by the International TILs Working Group (11), the evaluation of the density of TILs in four levels as well as the Klintrup-Makinen criteria (18) might be easier to perform and might be more useful in the clinical setting. Fourthly, although a previous report showed acceptable interobserver agreement between pathologists assessing TILs (32), the extent of the interobserver variability remains unclear.

The current study demonstrated that the density of tumor-infiltrating immune cells in H&E-stained sections was positively associated with the density of $CD8^+$ and total T-lymphocytes, which have been reported to be the strong prognostic biomarkers. The current study also demonstrated that the density of tumor-infiltrating immune cells in the tumor stroma alone in H&E-stained sections was associated with the density of tumor-infiltrating T-lymphocytes in the whole tumor because the number of T-lymphocytes infiltrating the cancer cell nests was extremely low. Thus, we concluded that the density of tumor-infiltrating immune cells in the tumor stroma in H&E-stained sections may be a reasonable immunological biomarker.

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