

## CD8<sup>+</sup> T Cells Infiltrated within Cancer Cell Nests as a Prognostic Factor in Human Colorectal Cancer

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### Abstract

The pathophysiological significance of tumor infiltrating lymphocytes remains controversial. To clarify their role, we performed clinicopathological analysis of CD8<sup>+</sup> T cells in 131 cases of human colorectal cancer. CD8<sup>+</sup> T cells were classified into three groups by their localization: (a) those infiltrated within cancer cell nests; (b) those distributed in the cancer stroma; and (c) those present along the invasive margin (tumor-host interface). Of these, CD8<sup>+</sup> T cells within cancer cell nests were most significantly associated with a better survival of patients by both mono- and multivariate analyses. The impact on survival was similar to that of Dukes' staging. Granzyme B<sup>+</sup> cytoplasmic granules were detected in lymphocytes within cancer cell nests, confirming their activated, cytotoxic phenotype. CD8 and Ki-67 double immunohistochemistry confirmed higher proliferative activity of CD8<sup>+</sup> T cells within cancer cell nests. Our data suggested that human colorectal cancer tissue was infiltrated by various numbers of T cells that had cytotoxic phenotype, contributing to a better survival of patients. This infiltration of colorectal cancer cell nests by CD8<sup>+</sup> T cells could be a novel prognostic factor.

### Introduction

Human cancer tissue is infiltrated by TILs<sup>2</sup> (1-3). TILs have been considered to be a manifestation of host immune reactions to cancer cells (4). However, its pathophysiological significance in human cancer tissue has remained controversial. One of the major reasons for this was a premise that human cancer arises through evading the host immune surveillance, either as a result of weak immunogenicity of tumor cells or by certain immunosuppressive effects from tumor cells (5-8).

We previously studied host immune responses in EBV-associated gastric cancer, revealing that infiltration of the tumor by CD8<sup>+</sup> T cells was the most prominent reaction characterizing this type of cancer compared with usual gastric cancer (9). Infiltrating CD8<sup>+</sup> T cells in EBV-associated gastric cancer possessed significantly higher levels of proliferative activity and perforin granules, suggesting their immunologically activated state. These conspicuous reactions could be induced by immunological recognition of certain antigen(s) associated with EBV (9). This study has taught us that CD8<sup>+</sup> T cells infiltrated into cancer cell nests could be representative of host immune reactions against cancer cell growth. In the present study, we expanded this concept to human colorectal cancers, which are one of the most common malignancies in the world. In human colon or rectum cancer, previous reports described the following immune-related prognostic factors: (a) continuous lymphocytic reactions along the invasive margin (10); (b) Crohn's-like lymphoid aggregates (11, 12); (c) TILs

along the invasive margin and in the stroma (3); and (d) follicular or paracortical (T-zone) hyperplasia in regional lymph nodes (13). These studies suggest that colorectal cancers represent tumors in which certain immune reactions can work to diminish the aggressiveness of cancer cells, despite presumptive immunosuppressive environments. However, the impact of these factors may not be so high, or the assessment method is not so specific from the immunological viewpoint because they were based on conventional histology. We need more simple and reliable methods to assess the host immune reactions. Based on our previous study on EBV-associated gastric cancer, we show here that infiltration of CD8<sup>+</sup> T cells within cancer cell nests is a new, reliable prognostic indicator in human colorectal cancer, bearing a similar impact as that of Dukes' staging.

### Materials and Methods

**Tissue Samples.** One hundred thirty-one surgically resected cases of colorectal cancer were randomly selected from the files of Tohoku Rosai Hospital (colon cancer) and Department of Surgery I, Tohoku University Hospital (rectum cancer) with operations performed during 1986 to 1989. These cases had follow-up data for at least 5 years. No cases received radiation or chemotherapy before operation. All cases were histopathologically classified into well, moderately, or poorly differentiated adenocarcinomas according to the WHO classification (14). To stage cancer, we adopted Dukes' classification: A, cancer invasion confined within the submucosa or the muscularis propria without metastasis; B, cancer invading into the subserosa or the adventitia without metastasis; C, with lymph node metastasis; and D, with simultaneous hematogenous metastasis. The number of cases of each stage was 21, 48, 54, and 8 for Dukes' A, B, C, and D, respectively. Metastasis in lymph nodes was checked by histopathological examination in all cases. The approximate number of lymph nodes examined was 10-20 per one case. Liver metastasis was diagnosed either by histopathological examination of metastatic foci or by computed tomography.

**Immunohistochemistry.** A biotin-streptavidin-peroxidase method using Histofine kit (Nichirei, Tokyo, Japan) was adopted on formalin-fixed, paraffin-embedded sections as recommended by the manufacturer. The primary antibodies used were mouse monoclonal anti-CD8 (clone C8/144B; DAKO, Glostrup, Denmark; 1:100) and anti-granzyme B (clone GrB-7; Kamiya Biomedical Co., Seattle, WA; 1:40). The pretreatment condition of specimens was autoclave heating (120°C for 5 min) and microwave heating (95°C for 15 min) for CD8 and granzyme B, respectively. The positive control of granzyme B staining was a case of natural killer cell lymphoma. We counted cells positive for granzyme B with a sparsely granulated pattern as activated cytotoxic T cells using an oil-immersion lens (×1000) in representative 23 cases. Three areas were chosen in each case. Cells strongly positive for granzyme B were excluded because we judged them to be natural killer cells.

**Classification of CD8<sup>+</sup> T Cells by Location and Their Quantification.** By immunohistochemistry for CD8, we classified CD8<sup>+</sup> T cells into three groups: (a) those distributed along the invasive margin of cancer; (b) those infiltrated in cancer stroma; and (c) those infiltrated within cancer cell nests (Fig. 1A). For (a) and (b), we semiquantitatively scored the degrees of infiltration into four groups: 0, nil; I, mild; II, moderate; and III, severe. For CD8<sup>+</sup> T cells within cancer cell nests, we counted the number of immunoreactive cells with a microscopic field of ×200 (0.933 mm<sup>2</sup>). Three areas with most abundant distribution were selected, and the average numbers of 0, 1-19,

Received 6/3/98; accepted 7/13/98.

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<sup>2</sup> The abbreviation used is: TIL, tumor-infiltrating lymphocyte.

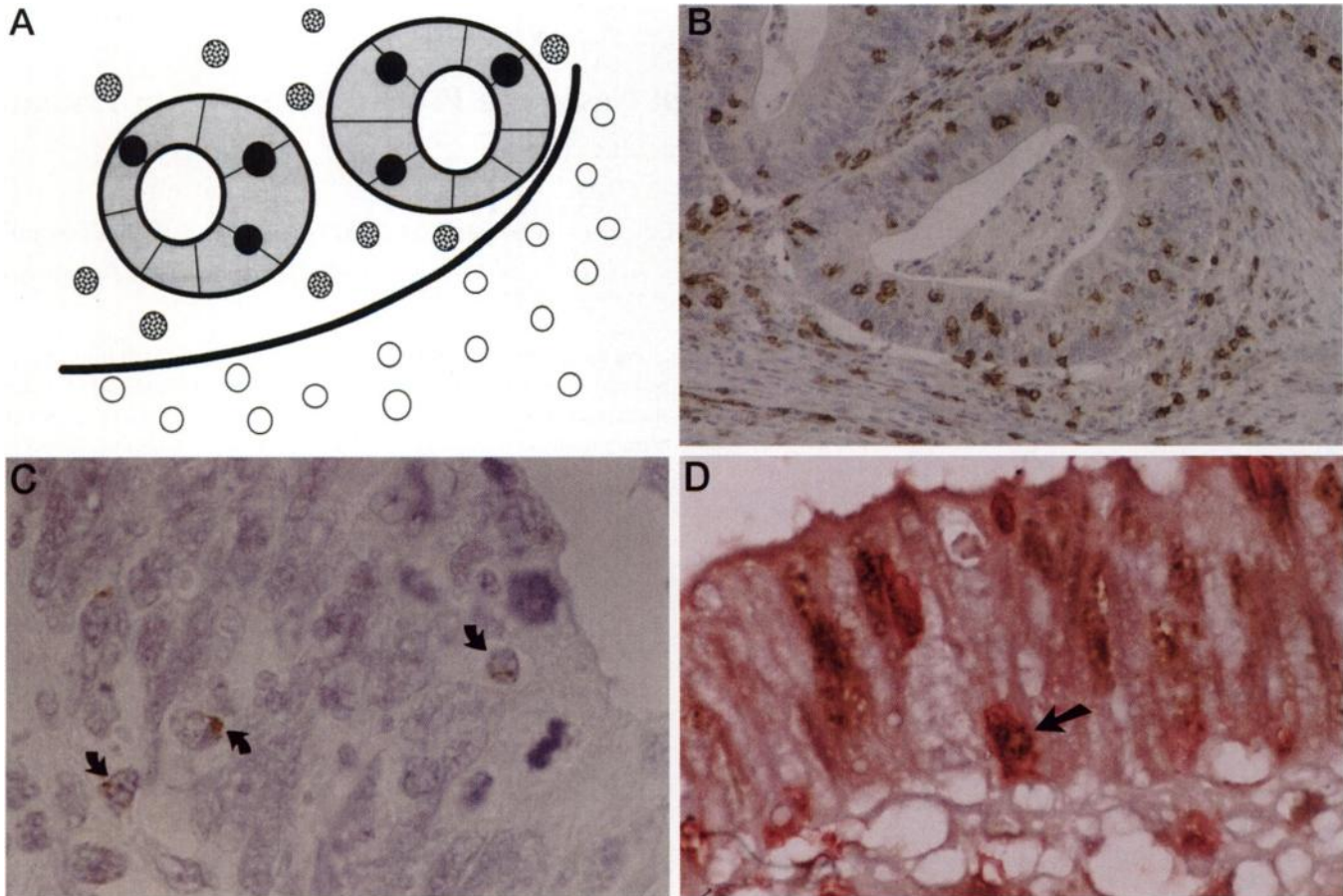


Fig. 1. A, schematic representation of CD8<sup>+</sup> T cells at three different localization patterns: ○, along the invasive margin; ⊙, in cancer stroma; and ●, within cancer cell nests. B, immunohistochemistry for CD8. CD8<sup>+</sup> T cells are present within cancer cell nests (among cancer cells) and in the stroma. ×80. C, immunohistochemistry for granzyme B. Arrows, positive cells. Note sparsely granular immunoreactive pattern. ×330. D, double immunohistochemistry for CD8 (red) and Ki-67 (brown). Arrow, double-positive cells. ×330.

20–49, and over 50 were scored as 0, I, II, and III, respectively. For semi-quantification of CD8<sup>+</sup> T cells in cancer stroma, 17 cases were not included because it was difficult to distinguish CD8<sup>+</sup> T cells in cancer stroma from those along invasive margin in those 17 cases.

**Statistical Analysis.** We quantified or semi-quantified each variable as described above and then made correlation with the patients' survival by Kaplan-Meier method for each variable (monivariate analysis) using computer software Stata (Stata Corp., College Station, TX). We judged the difference as significant when both log-rank and generalized Wilcoxon tests were significant. For multivariate analysis, we adopted proportional hazards model (Cox) and logistic model (Stata).

**Correlation between CD8<sup>+</sup> T Cells within Cancer Cell Nests and Dukes' Staging.** All cases were scored into 0, I, II, or III by the degree of CD8<sup>+</sup> T cells within cancer cell nests as described above and into Dukes' A, B, C, or D. The correlation between the two was tested by Spearman's test (Stata).

**Double Immunohistochemistry (Performed in Representative 15 Cases).** Double immunohistochemical analysis for CD8 and Ki-67 was performed as described previously with a modification (9, 15). After autoclave pretreatment, anti-CD8 monoclonal antibody was applied for 60 min. Histofine kit for alkaline phosphatase was used with a chromogen new fuchsin (red). To denature the antibody used, specimens were soaked in boiled water for 10 min. Mouse monoclonal antibody for proliferating cells (clone Ki-67; DAKO; 1:20) was applied overnight and then reacted with Envision (K1490; DAKO). The chromogen was 3,3'-diaminobenzidine tetrahydrochloride (brown). The median of the labeling index for Ki-67 of CD8<sup>+</sup> T cells within cancer cell nests was compared with that of CD8<sup>+</sup> T cells along the invasive margin by Mann-Whitney's *U* test (Stata).

## Results

**Immunohistochemistry.** CD8<sup>+</sup> T cells were distributed mainly along the invasive margin and in the stroma. This distribution pattern was similar to that of lymphocytes by conventional H&E stain. CD8<sup>+</sup> T cells were also detected among cancer cells in 75 of 131 cases, which were scored as I, II, and III, as described in "Materials and Methods" (Fig. 1B). These cells were designated as CD8<sup>+</sup> T cells within cancer cell nests.

Granzyme B<sup>+</sup> lymphocytes were detected within cancer cell nests (Fig. 1C) and also in the stroma (data not shown). The average ratio of lymphocytes positive for granzyme B among CD8<sup>+</sup> T cells within cancer cell nests was 28% in 23 cases classified as score III. This suggests that a part of CD8<sup>+</sup> T cells within cancer cell nests shows activated, cytotoxic phenotype. We analyzed the pathophysiological

Table 1 Statistical analysis among CD8<sup>+</sup> T cells at different localizations

Note that only CD8<sup>+</sup> T cells within cancer cell nests has a significant impact on the patients' survival by multivariate analysis. All variables were scored into four groups (see "Materials and Methods" for details).

	Kaplan-Meier <i>P</i> <sup>a</sup>	Proportional hazards model (Cox)	
		Hazard ratio	<i>P</i>
CD8 <sup>+</sup> T cells along invasive margin	0.21	0.91	0.68
CD8 <sup>+</sup> T cells in cancer stroma	0.014	0.81	0.56
CD8 <sup>+</sup> T cells within cancer cell nests	0.0003	0.52	0.016

<sup>a</sup> Log-rank test.

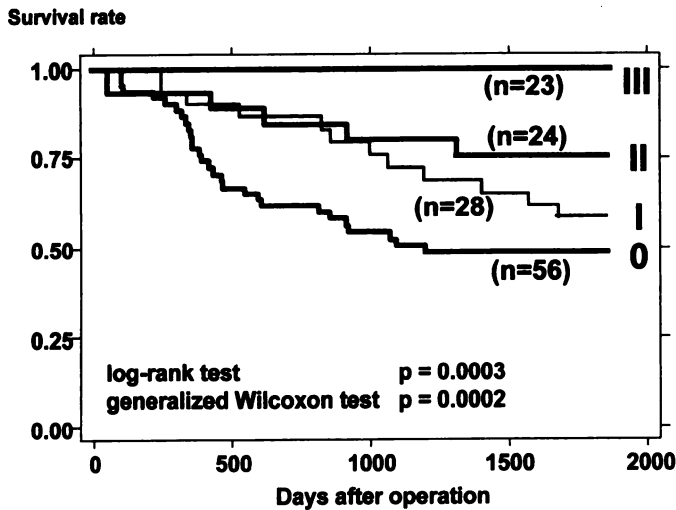


Fig. 2. Survival comparisons of patients with colorectal cancer by scores of CD8<sup>+</sup> T cells within cancer cell nests (Kaplan-Meier method). Note the correlation of patients' survival and score of T cells. For details of scoring, see "Materials and Methods" (score 0 corresponds to nil).

significance of these CD8<sup>+</sup> T cells as shown below together with other prognostic factors.

**Statistical Analysis among CD8<sup>+</sup> T Cells at Different Localization.** Monovariate analysis revealed that CD8<sup>+</sup> T cells within cancer cell nests and those in the stroma had significant impact on the patients' survival; those along the invasive margin bore no impact (Table 1). By multivariate analysis, only CD8<sup>+</sup> T cells within cancer cell nests were judged to be significant (Table 1). As shown in Fig. 2, the patients showed better survival as the score of CD8<sup>+</sup> T cells within cancer cell nests increased.

**Multivariate Analysis with Other Prognostic Factors.** To further analyze the pathophysiological significance of CD8<sup>+</sup> T cells within cancer cell nests, we performed multivariate analysis with other prognostic factors (Table 2). The significant risk factor was Dukes' staging with the hazard ratio and odds ratio over 1.0, indicating that this factor is a risk factor. CD8<sup>+</sup> T cells within cancer cell nests are also significant. Note that the hazard ratio and odds ratio are below 1.0, because this factor is favorable for survival. CD8<sup>+</sup> T cells within cancer cell nests possessed similar impact on survival as Dukes' stages did, because the reciprocal number of hazard ratio or odds ratio of CD8<sup>+</sup> T cells within cancer cell nests was similar to those of Dukes' staging (Table 2). These data indicate that infiltration of CD8<sup>+</sup> T cells within cancer cell nests can be a reliable prognostic factor. Although significant by monovariate analysis, patterns of invasion (either expanding or infiltrating) and histological type were not significant by multivariate analysis.

Table 2 Statistical analysis on CD8<sup>+</sup> T cells within cancer cell nests and other prognostic factors

CD8<sup>+</sup> T cells within cancer cell nests and Dukes' staging are significant by multivariate analysis. CD8<sup>+</sup> T cells were classified into 0–III; Dukes' staging into A–D; inflammatory cells along invasive margin into 0–III (including both mononuclear and polymorphonuclear leukocytes); pattern of invasion into expanding and infiltrating; and histological type into well, moderately and poorly differentiated adenocarcinomas. Except CD8<sup>+</sup> T cells, other variables were judged by H&E staining.

	Kaplan-Meier <i>P</i> <sup>a</sup>	Proportional hazards model (Cox)			Logistic model		
		Hazard ratio	95% CI <sup>b</sup>	<i>P</i>	Odds ratio	95% CI <sup>b</sup>	<i>P</i>
CD8 <sup>+</sup> T cells within cancer cell nests <sup>c</sup>	0.0003	0.605	0.41–0.89	0.011	0.54	0.33–0.88	0.013
Dukes' staging	0.0001	2.35	1.40–4.05	0.002	2.29	1.20–4.40	0.012
Inflammatory cells along invasive margin <sup>c</sup>	0.023	0.95	0.57–1.60	0.86	0.91	0.47–1.78	0.78
Pattern of invasion	0.0002	1.38	0.64–2.96	0.42	1.78	0.67–4.70	0.25
Histological type	0.01	1.26	0.79–2.01	0.32	1.46	0.76–2.80	0.26

<sup>a</sup> Log-rank test.

<sup>b</sup> CI, confidence interval.

<sup>c</sup> Variable (factor) related with a favorable survival with the hazard ratio or odds ratio below 1.0.

**Correlation between CD8<sup>+</sup> T Cells within Cancer Cell Nests and Dukes' Staging.** There was a statistically significant inverse correlation between CD8<sup>+</sup> T-cells within cancer cell nests and stages (Table 3). Higher scores (II and III) of infiltration of cancer cell nests by CD8<sup>+</sup> T cells were more frequent in cases of Dukes' A and B. Because both variables had statistically significant impact on the survival by multivariate analysis, Dukes' staging and CD8<sup>+</sup> T cells within cancer cell nests influence the patients' survival cooperatively with each other.

**Proliferative Activity of CD8<sup>+</sup> T Cells.** To estimate the proliferative activity of CD8<sup>+</sup> T cells, we performed double immunohistochemistry for CD8 and Ki-67 (9, 15) (Fig. 1D). This double staining, in a way, corresponds to *in vitro* mixed lymphocyte reaction, which tests the antigen-specific T-cell responses. CD8<sup>+</sup> T cells within cancer cell nests had significantly higher labeling index (6.3%) than those along invasive margin (2.8%) (*P* < 0.05).

## Discussion

In the present report, we analyzed TILs among different localization patterns to demonstrate, for the first time, that CD8<sup>+</sup> T cells infiltrated within cancer cell nests can be a prognostic factor. The impact of this factor is similar to that of Dukes' staging. The effects by CD8<sup>+</sup> T cells within cancer cell nests could be theoretically related to the effector function of activated killer T cells. The occurrence of granzyme B<sup>+</sup> cells suggests that part of CD8<sup>+</sup> T cells within cancer cell nests are activated CTLs (16). Stimulation of naive T cells is required to induce their proliferation and differentiation into activated T cells. The second signal by the costimulatory molecules is required for this process (17). For the demonstration of this activation mechanism, we have already reported that costimulatory molecules B7-1 and B7-2 are expressed on macrophages distributed along the invasive margin of colon cancer, where T cells are colocalized (15). Close localization of cancer cells and B7<sup>+</sup> macrophages suggests an occurrence of antigen presentation in which certain tumor antigens may be included (15). After being activated with the costimulatory function, T cells may migrate into cancer cell nests, exhibiting a higher proliferation activity.

Table 3 Correlation between CD8 T cells within cancer cell nests and Dukes' staging

Figures in the table represent case number. Correlation coefficient,  $-0.38$ ; *P* < 0.001.

Dukes' stage	Score of CD8 <sup>+</sup> T cells within cancer cell nests				total
	0	I	II	III	
A	1	6	4	10	21
B	20	11	10	7	48
C	28	10	10	6	54
D	7	1	0	0	8
Total	56	28	24	23	131

MHC class I molecule is required to be expressed by cancer cells for the recognition of cancer cells by T cells. In colorectal cancer, cancer cells usually express MHC class I molecules (18). In cervical neoplasia, no clear correlation was reported between the expression of MHC class I molecules by neoplastic cells and infiltration of CD8<sup>+</sup> T cells into the neoplastic tissue (19).

Occurrence of metachronous metastasis in the liver or in the lung via a hematogenous route is one of the major causes of death in patients with colorectal cancer. Considering this, our data on CD8<sup>+</sup> T cells suggest that these T cells may function not only locally but systemically in the liver and lung as well to suppress micrometastasis after being activated in the cancer tissue. On the possible local effects, degeneration of tumor cells was reported to be associated with invasion of cancer tissue by CD4<sup>+</sup> T cells and CD11c<sup>+</sup> macrophages (18). The mechanism for this finding may be different from the effect of CD8<sup>+</sup> T cells observed in this study. We did not observe apparent findings of cancer cell degeneration or cell death in carcinoma nests infiltrated by CD8<sup>+</sup> T cells.

There was significant correlation between the degree of CD8<sup>+</sup> T-cell infiltration within cancer cell nest and Dukes' staging, with both factors bearing significant impact on the patients' survival. This not only suggests a cooperative function by both factors but also indicates that immune reactions, if present, could confine cancer stages to earlier ones by diminishing the aggressiveness of cancer cells.

We dealt with CD8<sup>+</sup> T cells in the present study. As discussed above, the interactions are expected between T cells and macrophages. We dealt with all immune/inflammatory cells as a whole in the present study for comparison to CD8<sup>+</sup> T cells. We need to further analyze the detailed pathophysiological impacts of other immune/inflammatory cells separately using specific immunohistochemical markers.

Our method is easy to perform and more specific compared with previous assessment methods of TILs, indicating that infiltration of cancer cell nests by CD8<sup>+</sup> T cells can be a reliable marker to predict a longer survival of patients with colorectal cancer.

#### Acknowledgments

We are grateful to Dr. Masaaki Miyazawa for valuable discussion and to Dr. Ikuro Sato and Soichiro Shizawa for advice on the statistical analysis.

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