Histological Inflammatory Cell Infiltration Is Associated with the Number of Lymph Nodes Retrieved in Colorectal Cancer

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Abstract. *Background: Antitumor immune response is suggested to be a factor affecting the number of nodes retrieved after colorectal cancer surgery. The purpose of this study was to evaluate the correlation of antitumor immune response with the number of retrieved nodes. Patients and Methods: Patients with colorectal cancer (n=63, TNM stage II and III) were enrolled. Inflammatory cell infiltration (ICI) was assessed on hematoxylin and eosin staining and T-cell markers (CD3, CD8, CD45RO) were evaluated using immunohistochemical methods. Results: On univariate analysis, high ICI, CD3 and CD8 expression were associated with a greater number of nodes being retrieved. On multivariate analysis, tumors of the right colon (p=0.01) and high ICI (p=0.04) were independent predictors of a greater retrieval of nodes. TNM stage III tumor with low ICI was associated with reduced cancer-specific survival (p=0.02). Conclusion: ICI influences the number of nodes retrieved and affects survival of patients with stage III disease. Antitumor immune response may be an underlying factor determining the number of nodes retrieved after surgery for colorectal cancer.*

The number of nodes retrieved after colorectal surgery is influenced by the extent of regional lymphadenectomy and the effort of the pathological examination. The number of lymph nodes is also influenced by tumor characteristics such as anatomic location and histological grade, as well as patient factors such as age, gender, and obesity. Well-differentiated histology, older age, male sex, and obesity were factors associated with fewer nodes being retrieved (1-5).

In addition, antitumor immune response to the primary tumor is suggested to be a factor that affects the number of nodes. Since lymph nodes are secondary lymphoid organs, the primary tumor may potentially influence the immune environment around regional lymph nodes and induce reactive changes in the nodes themselves. Increases in node size due to reactive changes can be helpful to pathologists, making nodes easier to identify and thereby increasing the number of nodes obtained from surgery. It has been shown that colorectal carcinomas are commonly infiltrated by cytotoxic and memory T-lymphocytes (6). Prominent lymphocytic infiltration of the primary tumor is associated with a higher number of nodes retrieved after colorectal cancer surgery (7).

To date, as far as we are aware of, there is no study that has investigated the relationship between the number of nodes retrieved and the antitumor immune response to the primary tumor using immunohistochemistry (IHC). Thus, we evaluated the correlation of the number of retrieved lymph nodes with inflammatory cell infiltration and IHC expression of T-cell markers in patients with colorectal cancer.

Patients and Methods

Patients. A total of 63 patients who underwent major colorectal surgery with curative intent and adjuvant chemotherapy or chemoradiation therapy for stage II or III colorectal cancer between June 2005 and November 2005 were enrolled in this study. Patients were excluded if they had neoadjuvant chemoradiation therapy or inflammatory bowel disease. All enrolled patients were registered in a dedicated database and underwent close follow-up. Out of the 63 patients, 37 had stage II disease and the remaining 26 patients had stage III colorectal cancer. Patient follow-up lasted until either death or the cut-off date of December 31, 2012. Nine patients (14.3%) were lost to follow-up. The median follow-up interval was 77 months (range=7-91 months). This study was approved by the Institutional Review Board at our institution (no. 4-2010-0151).

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Key Words: Colorectal cancer, antitumor immune response, inflammatory cell infiltration, CD3, CD8, lymph node retrieval.

Surgery and adjuvant therapy. All surgeries were performed by an experienced colorectal surgeon (NKK). Details of the operative procedure and adjuvant treatment were described elsewhere (8).

Pathological lymph node examination. A senior gastrointestinal pathologist (MYC) reviewed all gross and microscopic pathological findings. Lymph nodes were identified *via* gross examination and manual palpation. The method of node retrieval was standardized and did not change significantly during the study period (9). All lymph nodes were stained with hematoxylin and eosin (H&E), and were examined for the presence of tumor metastasis under light microscopy. A fat clearing method was not performed in order to maximize the number of present nodes. Microsatellite instability (MSI) analysis was performed in all cases and details of the MSI analysis are described elsewhere (10).

IHC staining. IHC staining was conducted for markers of T-cell-mediated immunity (CD3, CD8 and CD45RO) in the primary tumor tissue.

Tumor samples were formalin-fixed and embedded in paraffin. Prepared paraffin-embedded tissue blocks were sectioned at 4 μm, treated with xylene, and rehydrated with alcohol. Antigens were retrieved *via* three, five minute incubations with antigen-retrieval buffer (LabVision, Fremont, CA, USA) at 99˚C. The sections were then cooled to 65˚C for 20 min. Endogenous peroxidase activity was blocked by treating sections with Tris-buffered saline Triton X-100 (TBST buffer) and 0.3% hydrogen peroxide for 10 min.

The following primary antibodies were then applied for two hours at room temperature: rabbit polyclonal antibody to CD3 (Cell Marque, Rocklin, CA, USA), mouse monoclonal antibody to CD8 (Cell Marque), mouse monoclonal antibody to CD45RO (Cell Marque). The sections were then washed in TBST. Secondary antibodies were subsequently applied at room temperature for 30 minutes, followed by a wash in TBST. 3-Amino-9-ethyl carbazole (AEC) was applied for 5-10 min for the peroxidase reaction and the sections were counterstained with hematoxylin. A tissue microarray technique was not used.

Assessments of inflammatory cell infiltration and IHC. The inflammatory cells in the tumor sections consisted mainly of lymphocytes and plasma cells (in most cases), occasionally being mixed with neutrophils, eosinophils and macrophages (few cases). Therefore, distinction between the types of inflammatory cells was not made. The degree of inflammatory cell infiltration was separately assessed in the center of the tumor (CT) and invasive margin of the tumor (IM) as reported previously (11), with some modification as follows (Figures 1 and 2). Under a microscope (×40), the whole tumor area (CT and IM) was first evaluated for the presence and multifocality of lymphoid follicle formation, abscess formation and large inflammatory cell aggregates. We then examined the level of infiltration in the respective tumor areas under a ×20 objective lens (×200). A four-point grading system was used as follows: few and focal infiltrations without formation of aggregates, score 1; multifocal infiltration without lymphoid follicles, score 2; up to three lymphoid follicles or aggregates, score 3; and more than four lymphoid follicles or aggregates, score 4. The same 4-point grading method was used for estimating the infiltration of inflammatory cells expressing CD3, CD8 (Figure 3), and CD45RO in the CT and IM compartments.

Statistical analysis. All statistical analyses were performed using IBM SPSS Statistics for Windows, version 20.0 (IBM, Armonk, NY, USA). For statistical analyses, the inflammatory cell infiltration, CD3, CD8, and CD45RO score were calculated as the sum of the scores for the CT (1 to 4) and the IM (1 to 4) compartments. Based on the sum of these scores, the range was classified into two groups of low (1 to 4) and high (5 to 8) scores for further analysis. A *p*-value of less than 0.05 was considered statistically significant.

Table I. *Clinicopathological characteristics associated with the number of nodes retrieved at surgery for colorectal cancer.*

	Patients (n=63) N (%)	Number of nodes retrieved	
		Mean (SD)	*p*-Value
Age (years)			
<70	48 (76.2)	27 (11)	0.51
≥70	15 (23.8)	25 (14)	
Gender			
Male	34 (54.0)	28 (13)	0.41
Female	29 (46.0)	25 (11)	
BMI (kg/m^2)			
<25	47 (74.6)	26 (13)	0.91
≥25	16 (25.4)	26 (10)	
Location			
Right colon	14 (22.2)	35 (14)	0.004
Left colon	28 (44.4)	25 (10)	
Rectum	21 (33.3)	22 (10)	
TNM stage			
II	37 (58.7)	26 (13)	0.53
III	26 (41.3)	28 (11)	
Pathological T classification			
T1 and 2	3 (4.8)	15 (8)	0.11
T3	60 (95.2)	27 (12)	
Histologic grade			
WD and MD	59 (93.7)	26 (12)	0.15
PD and Mucinous	4 (6.3)	35 (18)	
Tumor diameter (cm)			
<4	22 (34.9)	22 (11)	0.03
≥4	41 (65.1)	29 (12)	
MSI phenotype			
MSS, MSI-low	57 (90.5)	25 (11)	0.001
MSI-high	6 (9.5)	42 (12)	

SD, Standard deviation; BMI, body-mass index; TNM, tumor node metastasis; WD, well-differentiated; MD, moderately-differentiated; PD, poorly-differentiated; MSI, microsatellite instability; MSS, microsatellite stability.

Results

Clinicopathological characteristics associated with the number of nodes retrieved. There were no differences in the mean number of nodes retrieved with respect to age, gender, body-mass index, TNM stage, pathological T classification, or histological grade.

Right-sided colon cancer (p=0.004), larger tumor diameter (p=0.03), and MSI-high tumors (p=0.001) were associated with a greater number of retrieved nodes (Table I).

Table II. *Correlation of the number of nodes retrieved at surgery with inflammatory cell infiltration and immunohistochemical expression.*

	Score[a]	Patients (n=63) N (%)	Number of nodes retrieved	
			Mean (SD)	*p*-Value
Inflammatory cell infiltration	Low	22 (34.9)	20 (9)	0.003
	High	41 (65.1)	30 (13)	
CD3	Low	34 (54.0)	23 (10)	0.02
	High	29 (46.0)	30 (14)	
CD8	Low	41 (65.1)	23 (10)	<0.001
	High	22 (34.9)	34 (13)	
CD45RO	Low	46 (73.0)	25 (11)	0.2
	High	17 (27.0)	30 (14)	

[a]Low: 1-4; high: 5-8. MSI, Microsatellite instability; SD, standard deviation; CD, cluster of differentiation.

Correlation of inflammatory cell infiltration and T-cell markers with the number of nodes retrieved. High inflammatory cell infiltration (p=0.003), and high CD3 (p=0.02) and CD8 expression (p<0.001) were associated with a greater retrieval of nodes, although CD45RO expression was not (Table II).

Multiple regression analysis of factors associated with the number of nodes retrieved. Multivariate analysis showed that right-sided colon cancer (p=0.01) and high inflammatory cell infiltration (p=0.04) are predictive of the retrieval of a greater number of nodes (Table III).

Prognostic factors associated with cancer-specific survival. Univariate analysis using Kaplan-Meier curves showed that older age (>70 years) (p=0.03) and TNM III stage (p=0.049) were associated with inferior cancer-specific survival. The degree of inflammatory cell infiltration alone did not affect survival (p=0.67). In combination, however, TNM stage III and low inflammatory cell infiltration led to an unfavorable 5-year survival rate (42.9%) when compared to TNM stage II (89.2%) and TNM stage III with high inflammatory cell infiltration (73.7%) (p=0.02).

Multivariate analysis using Cox proportional hazards model showed that TNM stage III with low inflammatory cell infiltration was an independent prognostic factor for significantly decreased cancer-specific survival (hazard ratio=4.7, p=0.02) (Table IV).

Discussion

The major finding of this study was that a histologically-determined antitumor immune response, as shown by inflammatory cell infiltration, influences the number of retrieved lymph nodes. Inflammatory cell infiltration also affects cancer-specific survival of patients with stage III colorectal cancer.

We postulated that an enhanced immune response to the primary tumor by the host is associated with a greater number of lymph nodes retrieved. To our knowledge, this is the first study to investigate the association between inflammatory cell infiltration and T-cell marker expression, and the number of nodes retrieved in colorectal cancer surgery. In the present study, potential confounding factors such as operative technique and pathological examination were homogeneous during the 6-month study period (8, 9).

We evaluated the association between clinicopathological characteristics and the number of retrieved nodes. The correlation between right-sided colon cancer and a greater number of nodes may be the result of larger surgical specimens that are typically obtained during right *versus* left colectomy which may contain a large amount of mesenteric fat (12). In addition, larger tumors may tend to undergo necrosis due to inadequate blood supply, which induces reactive changes in the regional nodes and eventually results in lymph node enlargement (2). The correlation of MSI-high tumor phenotype with a greater number of nodes retrieved is likely because MSI-high tumors are characterized by the presence of tumor-infiltrating lymphocytes and proximal colonic tumor location (13-15).

We assessed the antitumor immune response using inflammatory cell infiltration and immunohistochemical T-cell marker expression. To date, there is no consensus on objective criteria for histological determination of tumor immune response however, previous investigations have found that histological examination of lymphocytic infiltration and Crohn;s like lymphoid infiltraion are reliable and reproducible methods when assessing the tumor-induced immune response (16-18). Thus, we evaluated inflammatory cell infiltration at both the center of the tumor and the invasive margin, and used a four-tiered scoring system (11, 19). The previous methods for evaluating the grade of inflammatory cell infiltration had either only used a two-tiered (presence or absence), or four-tiered scoring method,

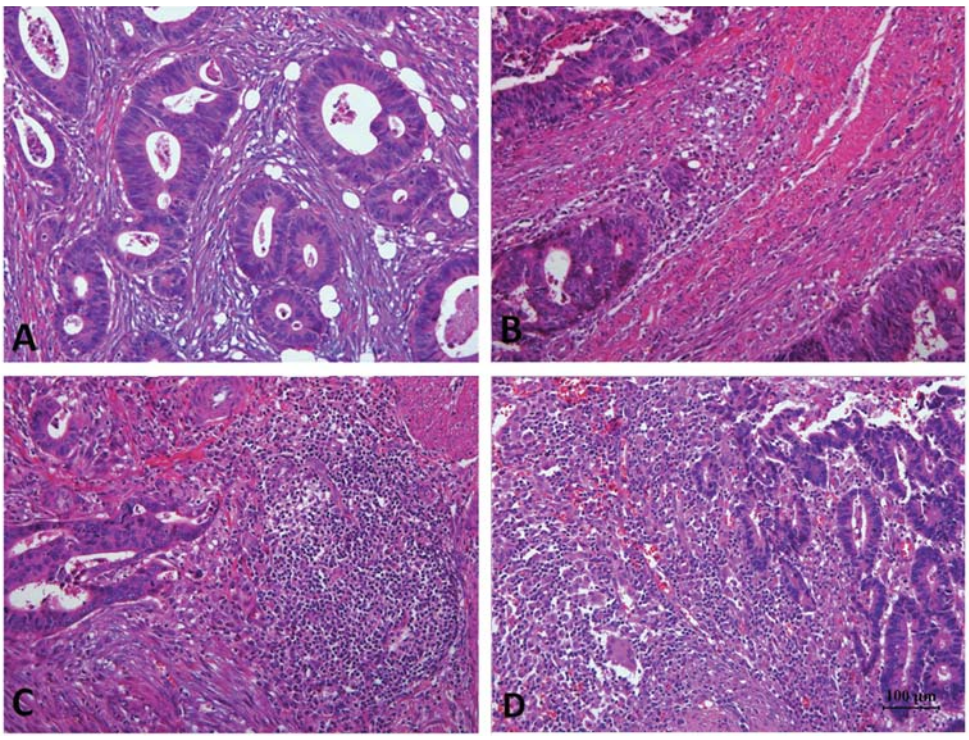

Figure 1. *Inflammatory cell infiltration in the center of tumors. The infiltration of inflammatory cells was graded as few and focal infiltration without aggregates (A); multifocal infiltration without lymphoid follicles (B); the presence of moderate aggregates (C); and abscess formation (D). Hematoxylin and eosin staining, ×200.*

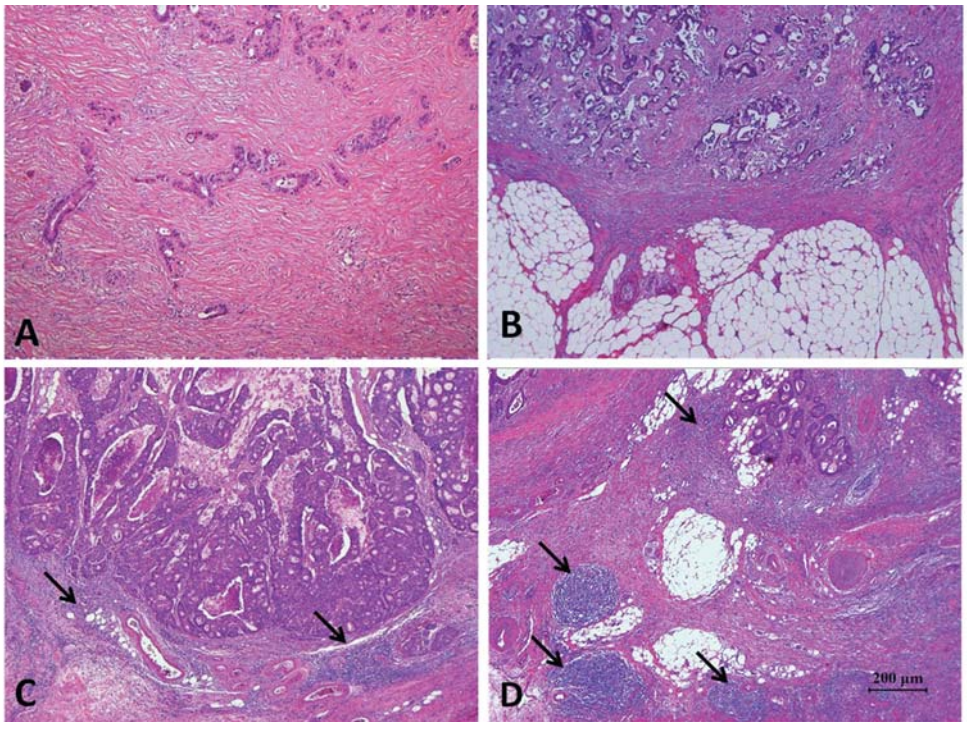

Figure 2. *Inflammatory cell infiltration at the invasive margin of tumors. The inflammatory cell infiltration is sparse and rare (A); multifocal infiltration without the presence of lymphoid follicles (B); up to three lymphoid follicles or aggregates (arrows) (C); and the presence of more than four lymphoid follicles or peritumoral abscess formation (arrows) (D). Hematoxylin and eosin staining, ×40.*

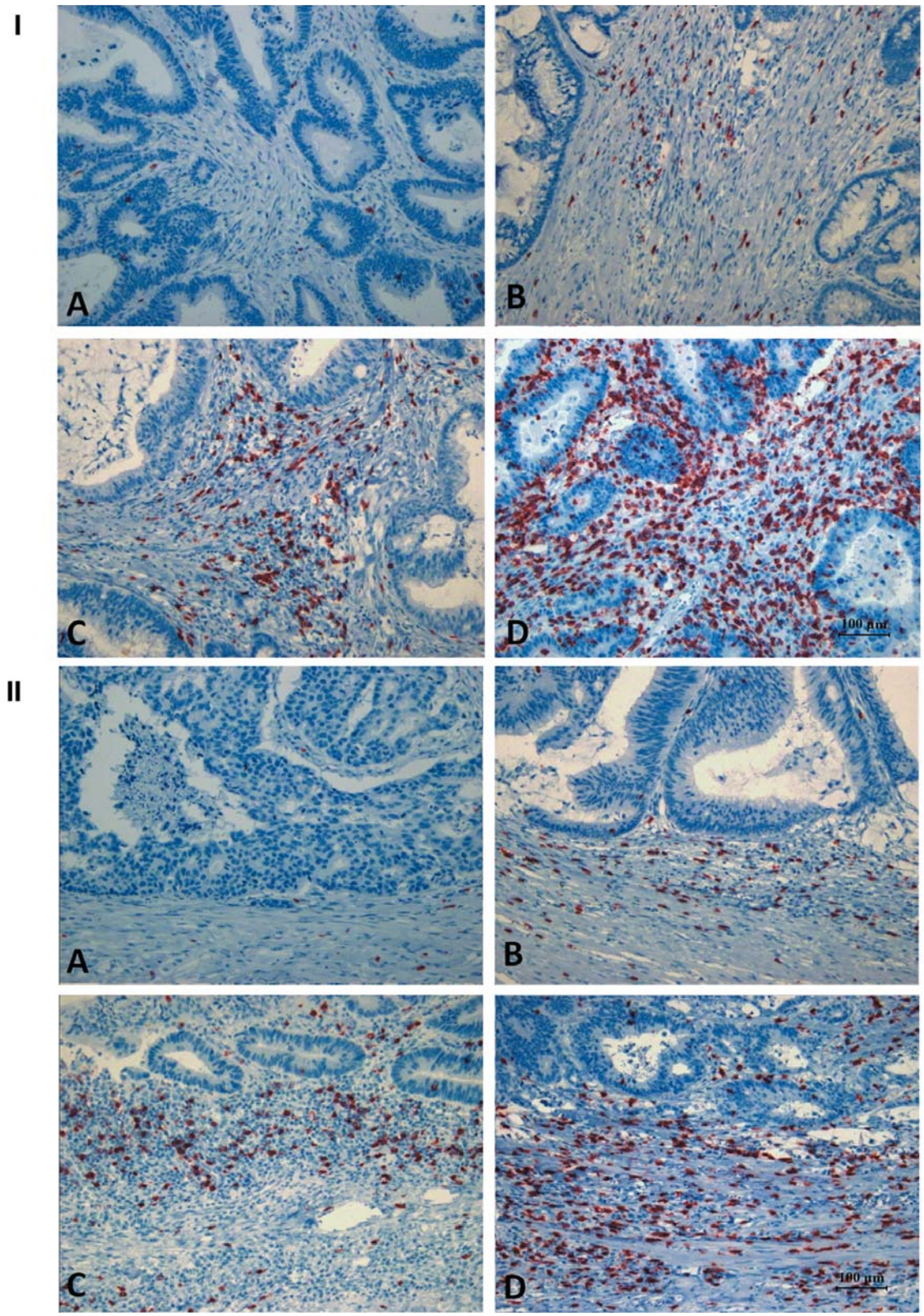

Figure 3. *Immunohistochemical expression of CD8 in the center (I) and invasive margin (II) of tumors. The infiltration of CD8-positive cells was graded as few and focal infiltration without aggregates (A); multifocal minimal infiltration without lymphoid follicles (B); the presence of moderate aggregates (C); and severe aggregates (D). CD8 staining, ×200.*

Table III. *Multiple regression analysis of factors associated with the number of nodes retrieved at surgery for colorectal cancer.*

	Regression coefficient	95% Confidence interval	*p*-Value
Tumor location Right colon *versus* left colon and rectum	−4.2	−7.5 to −0.9	0.01
Tumor diameter (cm) <4 *versus* ≥4	4.2	−1.4 to 9.8	0.13
MSI phenotype MSS, MSI-low *versus* MSI-high	8.1	−1.3 to 17.4	0.09
Inflammatory cell infiltration scorea Low *versus* high	1.5	0.1 to 2.8	0.04
CD3 score[a] Low *versus* high	2.8	−2.6 to 8.2	0.3
CD8 score[a] Low *versus* high	4.0	−2.0 to 10.0	0.19

[a]Low: 1-4; high: 5-8. MSI, Microsatellite instability; MSS, microsatellite stability; CD, cluster of differentiation

Table IV. *Univariate and multivariate analyses for prognostic factors associated with cancer-specific survival.*

		Kaplan-Meier analysis with log-rank test		Cox proportional hazards model	
		5-year CSS (%)	*p*-Value	HR (95% CI)	*p*-Value
Age (years)	≤70	85.4	0.03	1	0.07
	>70	60.0		2.6 (0.9 to7.3)	
Tumor location	Right colon	78.6	0.87	NA	
	Left colon	78.6			
	Rectum	81.0			
Number of nodes retrieved	≤15	75.0	0.92	1	0.93
	>15	80.4		1.1 (0.3 to 4.3)	
MSI phenotype	MSS, MSI-low	78.9	0.57	1	0.62
	MSI-high	83.3		0.6 (0.1 to 4.9)	
TNM stage	II	89.2	0.049	NA	
	III	65.4			
Inflammatory cell infiltration score	Low	77.3	0.67	NA	
	High	80.5			
TNM stage with inflammatory cell infiltration score	Stage II	89.2	0.02	1	0.07
	Stage III, with high score	73.7		1.9 (0.6 to 6.2)	0.27
	Stage III, with low score	42.9		4.7 (1.3 to 17.3)	0.02
CD3 score	Low	79.4	0.75	NA	
	High	79.3			
CD8 score	Low	80.5	0.43	NA	
	High	77.3			

CSS, Cancer-specific survival; HR, hazard ratio; CI, confidence interval; NA, not applied; MSI, microsatellite instability; MSS, microsatellite stability; TNM, tumor node metastasis; CD, cluster of differentiation; low: 1-4; high: 5-8.

similar to ours, but only evaluating focal areas of the tumor under medium-power microscopy (×100). First of all, to our experience tumors always have inflammatory reaction, in contrast to the scoring criteria of a previous study (20). Secondly, any given tumor might have areas that if seen under high-power microscopy may contain a considerable number of inflammatory cells, which may lead to false high positivity with any scoring method. Interestingly, a scoring method based on counting the number of inflammatory cells per high power microscopy field (×400) has recently been used (20). Importantly, examination of the multifocality of inflammatory cell infiltration and the proper evaluation of

the pattern of inflammatory cell infiltration at the invasive margins are feasible only by evaluating the tumor area at first under low power (×40) and if multifocality and pattern are included in the scoring criteria.

We also evaluated the relationship between T-cell marker expression and the number of nodes retrieved. High expressions of CD3 and CD8 were associated with a greater number of nodes retrieved in univariate analysis. Taken together, right-sided colon cancer and high inflammatory cell infiltration were independent predictors of greater number of node retrieval on multivariate analysis. This indicates that inflammatory cell infiltration was a more reliable predictor for node retrieval than were T-cell markers. Although the T-cell-mediated immune reaction plays a great role between the tumor and the host, the antitumor immune reaction is linked to a wide range of immune cells, such as T- and B-lymphocytes, natural killer cells, dendritic cells, macrophages, neutrophils, eosinophils, and mast cells (21). Accordingly, the T-cell-mediated immune reaction is a part of the immune response occurring in the primary tumor. Unfortunately, we did not evaluate other types of immune cells using IHC.

Survival analyses showed that patients with low inflammatory cell infiltration and stage III disease had an unfavorable cancer-specific survival. Low inflammatory cell infiltration was predictive of poor survival, but not independent of tumor stage. In addition, the number of nodes retrieved (≥15 *versus* <15) alone did not influence survival. Previous studies demonstrated that patients with prominent lymphocytic infiltration in the primary tumor had a favorable prognosis compared to those without infiltration (22, 23).

This study has limitations in that it is small single-center study and only T-cell markers were evaluated using IHC.

In summary, histological antitumor immune response as determined by inflammatory cell infiltration in the primary tumor influences the number of nodes retrieved and also affects cancer-specific survival in patients with stage III colorectal cancer. This suggests that inflammatory cell infiltraton may be an underlying factor in determining the number of nodes retrieved at surgery. The association between a greater number of lymph nodes and longer survival found in previous studies (5, 6, 9, 24) may also be explained by the fact that a more robust immune reaction, in patients with a greater retrieval of nodes, leads to more favorable survival.

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