Tissue-Infiltrating Lymphocytes as a Predictive Factor for Recurrence in Patients with Curatively Resected Colon Cancer: A Propensity Score Matching Analysis

Lin Fung Chan^a Sotaro Sadahiro^a Toshiyuki Suzuki^a Kazutake Okada^a
Hiroshi Miyakita^a Seiichiro Yamamoto^a Hiroshi Kajiwara^b

^aDepartment of Surgery, School of Medicine, Tokai University, Isehara, Japan; ^bDepartment of Pathology, School of Medicine, Tokai University, Isehara, Japan

Keywords

Tissue-infiltrating lymphocytes · Colon cancer · Prognostic factor

Abstract

Background: In patients with colorectal cancer, the rate of recurrence increases as the histologic stage progresses. However, the prediction of recurrence in individual patients is difficult. Many studies have reported on the relation between outcomes and tissue-infiltrating lymphocytes (TILs). The aim of our study was to clarify the relation between TILs and oncologic outcomes in patients with colon cancer using propensity score matching analysis. **Methods:** The study group comprised 513 patients with colon cancer who received curative resection. By using propensity score matching for sex, age, tumor location, T stage, N stage, histologic type, and adjuvant therapy as conventional prognostic factors, 61 patients with recurrence and 61 patients with no recurrence were selected. Hematoxylin-eosin staining and immunohistochemical staining using CD3, CD8, CD4, and FoxP3 were performed for lymphocytes in the primary tis-

sue. The results were evaluated separately in the whole tumor, the central part, and the invasive margin. **Results:** The median follow-up period was 53 months. Among the 513 patients, 70 had recurrence and 443 had no recurrence. In the comparison of outcomes between the 61 patients with recurrence and the 61 patients with no recurrence, univariate analysis showed that the disease-free survival rate was significantly higher among the patients with positive TILs in the whole tumor and in the invasive margin ($p = 0.016$ and $p = 0.012$, respectively) and with CD8+ cells in the central part ($p = 0.039$) than among those with negative results. A multivariate analysis showed that TILs in the invasive margin (hazard ratio 1.81; 95% confidence interval, 1.03–3.05; $p = 0.037$) and CD8+ cell density in the central part (hazard ratio 1.76; 95% confidence interval, 1.07–2.93; $p = 0.023$) were prognostic factors that were independent from conventional prognostic factors. **Conclusions:** In patients with curatively resected colon cancer, TILs in the invasive margin and CD8+ cell density in the central part may be prognostic factors suggesting host antitumor immune response.

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Introduction

In the USA, colorectal cancer is the third most common cancer and the third leading cause of cancer-related death [1]. In Japan, colorectal cancer is the third leading cause of cancer-related death and the most common type of cancer [2]. Stage I, II, and III colorectal cancers can be curatively resected in nearly all patients. In one study, the recurrence rate was 5% with stage I disease, 12% with stage II disease, and 33% with stage III disease [3]. As the histologic grade of the tumor progresses, the rate of recurrence thus increases. In patients with stage III and high-risk stage II disease, adjuvant chemotherapy should be indicated.

However, the rate of recurrence in individual patients is difficult to accurately predict. Several studies reported that risk factors for recurrence included T4 invasion, the number of retrieved lymph nodes, tumor perforation, obstruction, and poorly differentiated adenocarcinoma, but these factors remain to be established [4–6]. As for lymphocyte infiltration in the cancer tissue, prognostic factors have been reported to be the density of CD8+ T cells and the ratio of CD8+ cells to FoxP3+ cells in patients with ovarian cancer; the densities of CD8+ and CD20+ cells in patients with non-small cell lung cancer; and the densities of CD8+ and FoxP3+ cells in patients with hepatocellular carcinoma [7–9]. In patients with colorectal cancer, the densities of CD3+, CD4+, CD8+, CD56+, and CD57+ cells have been reported to be related to survival rates [10–12]. However, the clinical significance of the densities of CD3+, CD4+, CD8+, CD56+, and CD57+ cells as prognostic factors has not yet been established.

We therefore performed a propensity score matching analysis using clinicopathological risk factors as covariates to examine the relation between tissue-infiltrating lymphocytes (TILs) and outcomes in patients with recurrence and in patients without recurrence following curative resection.

Subjects and Methods

Patient Selection and Data Extraction

Among patients with pathologic stage I, II, and III colon cancers who underwent curative surgery in Tokai University Hospital between January 2005 and December 2015, we studied 513 patients with colon cancer after excluding patients with cancer of the splenic flexure, a boundary between the right side and the left side of the colon. Data extracted from the patient records included baseline

characteristics (sex and age) and primary tumor characteristics (tumor location, pathologic T stage, pathologic N stage, pathologic TNM stage, and histologic type) at initial surgery, as well as postoperative adjuvant chemotherapy.

Postoperative Surveillance

Patients with colon cancer who received curative resection entered our surveillance program and were followed up on an outpatient basis. All patients underwent follow-up examinations every 3–4 months for the first 2 years, every 6–12 months from 3 to 5 years, and annually thereafter. For most patients, follow-up evaluations at each visit included medical history-taking, physical examinations, measurement of serum carcinoembryonic antigen levels, chest radiography, and abdominal ultrasonography. Chest and abdominal CT scans were performed every 6 months for up to 5 years and annually thereafter. Barium enema or colonoscopy was performed during the first, third, and fifth years. Abnormal physical findings or laboratory results mandated further screening by abdominal and pelvic CT, chest CT, and magnetic resonance imaging, as required [13–15].

Propensity Score Matching

To eliminate differences in conventional clinicopathological prognostic factors between patients with recurrence and those with no recurrence, propensity score matching was performed using sex, age, tumor location (right-sided colon cancer including tumors arising from the cecum, ascending colon, hepatic flexure, and transverse colon, or left-sided colon cancer including tumors arising from the descending colon, sigmoid colon, and rectosigmoid), pathologic Tumor-Node-Metastasis (TNM) stage (TNM classification of the Union for International Cancer Control and the American Joint Committee on Cancer) [16, 17], histologic type, and the presence or absence of adjuvant chemotherapy as covariates. Propensity score matching analysis was performed using a caliper width of 0.2 and nearest neighbor matching at a 1:1 ratio.

Histologic Measurements of TILs

After propensity score matching, we evaluated TILs and lymphocyte infiltration in the primary tissue of 122 patients. The TIL counts were quantitatively evaluated using hematoxylin-eosin (H-E)-stained tumor tissue specimens. The subtypes of TILs were evaluated by immunohistochemical staining with the surface markers of lymphocytes (CD3, CD4, CD8, and FoxP3) as follows. Formalin-fixed paraffin-embedded sections were used. Antibodies for the pan-T-cell marker CD3 (ready to use, clone 2GV6; Roche, Basel, Switzerland), helper T-cell marker CD4 (diluted 1:100, clone 4B12; DAKO, Glostrup, Denmark), cytolytic T-cell marker CD8 (diluted 1:40, C8/144B; DAKO), and regulatory T-cell marker FoxP3 (diluted 1:100, clone SP97; Thermo Fisher Scientific, Waltham, MA, USA) were used as the primary monoclonal antibodies.

An avidin-biotin-peroxidase complex method with diaminobenzidine as a chromogen was used according to the manufacturer's instructions. For CD3 and CD8 immunostaining, the VENTANA Discovery ULTRA Slide Staining System (Roche Diagnostics, Risch-Rotkreuz, Switzerland) was used. For CD4 and FoxP3 immunostaining, the Leica BOND-MAX Fully Automated Immunohistochemistry System (Leica Biosystems, Nussloch, Germany) was used. VENTANA Cell Conditioning Solution (CCI) was used

Fig. 1. **a** Absence or a few CD8+ cells infiltrating the central part (grade 0). **b** Mild infiltration of CD8+ cells in the central part (grade 1). **c** Moderate infiltration of CD8+ cells in the central part (grade 2). **d** Strong infiltration of CD8+ cells in the central part (grade 3).

for 60 min for CD3 and CD8 staining. BOND Epitope Retrieval Solution 2 (AR9640) was used for 20 min for CD4 and FoxP3 staining.

The results were evaluated in three parts consisting of the whole tumor, the central part of the tumor, and the invasive margin. The degree of infiltration of TILs (cell density) was graded according to a 4-grade scale: grade 0, no infiltration; grade 1, a small amount of infiltration; grade 2, a moderate amount of infiltration; and grade 3, a large amount of infiltration. Grade 0 or 1 was regarded as negative, and grade 2 or 3 was regarded as positive (Fig. 1).

On staining, FoxP3+ cells were found to be widely scattered. Therefore, to examine the density of FoxP3+ cells, the number of stained lymphocytes was counted in 10 views at a magnification of $\times 400$. Using receiver operating characteristic curves, 60 FoxP3+ cells per 10 views were decided to be the cutoff level; ≥ 60 FoxP3+ cells were regarded as positive, and < 60 FoxP3+ cells were regarded as negative.

Statistical Analysis

All statistical analyses were performed using JMP version 13 software (SAS Institute Japan, Tokyo, Japan). Categorical variables were analyzed using Fisher's exact test, and continuous variables were analyzed using Student's *t* test. The best cutoff points were obtained by receiver operating characteristic curve analysis.

The disease-free survival (DFS) rate was calculated by the Kaplan-Meier method. Multivariate analysis was performed using a Cox proportional-hazards model. *p* values of < 0.05 were considered to indicate statistical significance.

Results

The median follow-up period was 53 months (range 7–125). Among the 513 patients, 70 had recurrence and 443 had no recurrence. After propensity score matching, 61 pa-

Table 1. Relationship between recurrence and baseline patient characteristics before and after propensity score (PS) matching

	Before PS matching (n = 513)			Recurrence after PS matching (n = 122)		
	recurrence (n = 70)	no recurrence (n = 443)	p	recurrence (n = 61)	no recurrence (n = 61)	p
Sex, n (%)						
Male	33 (7)	243 (47)	0.25	28 (23)	33 (27)	0.47
Female	37 (7)	200 (39)		33 (27)	28 (23)	
Age, years	69±9.1	68±11.2		70±8.6	71±11.1	
Location of the tumor ¹						
Right side of colon	34 (7)	229 (45)	0.7	30 (25)	34 (28)	0.59
Left side of colon	36 (7)	214 (41)		31 (25)	27 (22)	
Pathological T stage						
T1	3 (1)	115 (22)	<0.01	3 (2)	3 (2)	0.96
T2	7 (1)	69 (13)		7 (6)	7 (6)	
T3	33 (7)	188 (37)		31 (25)	28 (23)	
T4	27 (5)	71 (14)		20 (17)	23 (19)	
Pathological N stage						
N0	33 (7)	327 (64)	<0.01	30 (25)	32 (26)	0.97
N1	21 (4)	95 (18)		19 (16)	17 (13)	
N2	16 (3)	21 (4)		12 (10)	12 (10)	
Pathological TNM stage						
I	8 (2)	158 (31)	<0.01	8 (7)	6 (5)	0.69
II	24 (5)	168 (33)		21 (17)	25 (20)	
III	38 (7)	117 (22)		32 (26)	30 (25)	
Histologic type						
Well	26 (5)	213 (42)	0.18	25 (20)	23 (19)	0.71
Moderate	39 (8)	212 (41)		34 (28)	33 (27)	
Poor	2 (0.4)	6 (1)		1 (1)	1 (1)	
Mucinous	3 (0.6)	12 (2)		1 (1)	4 (3)	
Postoperative adjuvant chemotherapy						
Positive	25 (5)	135 (26)	0.41	35 (29)	28 (23)	0.28
Negative	45 (9)	308 (60)		26 (21)	33 (27)	

Values denote n (%) or mean ± SD. ¹ Right-sided colon cancers included tumors arising from the cecum, ascending colon, hepatic flexure, and transverse colon, while left-sided colon cancers included tumors arising from the descending colon and sigmoid colon.

tients in each group were analyzed. No significant differences were observed with regard to baseline characteristics (sex, age, tumor location, pathologic T stage, pathologic N stage, pathologic TNM stage, histologic type, and adjuvant chemotherapy) between the groups (Table 1).

TILs on H-E Staining and Density of Lymphocytes on Immunohistochemical Staining

The positivity rates in the whole tumor were 77% for TILs, 96% for CD3+ cell density, 78% for CD4+ cell density, 93% for CD8+ cell density, and 56% for FoxP3+ cell density. The positivity rates in the central part on H-E staining were 49% for TILs, 67% for CD3+ cells, 54% for

CD4+ cells, and 49% for CD8+ cells. The positivity rates in the invasive margin on H-E staining were 77% for TILs, 96% for CD3+ cells, 74% for CD4+ cells, and 90% for CD8+ cells (Fig. 2).

Univariate Analysis of Clinicopathological Variables in Relation to DFS

Table 2 shows the results of univariate analysis of the relation of DFS to clinicopathological factors and TIL-associated factors that have been reported to be related to outcomes. There was no significant relation of DFS to sex, age, tumor location, histologic type, or the presence or absence of adjuvant therapy. With regard to the

Fig. 2. Examples of stained sections showing different patterns of T-cell infiltration in the microenvironment of colon tumors. **a** Minor T-cell infiltration in the invasive margin. **b** Strong infiltration of T cells in the invasive margin. **c** A few CD8+ cells infiltrating the central part. **d** Strong infiltration of CD8+ cells in the central part.

whole tumor, on H-E staining, the 5-year DFS rate was significantly higher among patients who were positive for TILs (48%) than among those who were negative for TILs (27%) ($p = 0.016$). There was no relation between DFS and the densities of CD3+, CD4+, CD8+, or FoxP3+ cells. Regarding the central part, the 5-year DFS rate was significantly higher among patients with a positive density of CD8+ cells (51%) than among those with a negative density (34%) ($p = 0.039$). There was no relation of DFS to TILs and the densities of CD3+ or CD4+ cells.

As regards the invasive margin, the 5-year DFS rate was significantly higher among patients who were positive for TILs (48%) than among those who were negative for TILs (26%) ($p = 0.012$). There were no differences in

the densities of CD3+, CD4+, or CD8+ cells between patients with positive results and those with negative results.

Multivariate Analysis of Clinicopathological Variables in Relation to DFS

Using clinicopathological risk factors and factors found to be related to DFS on univariate analysis (TILs in the invasive margin on H-E staining and CD8+ cell density in the central part), multivariate analysis was performed using a Cox proportional-hazards model (Table 3). TILs in the invasive margin were associated with a hazard ratio of 1.81 (95% confidence interval, 1.03–3.05; $p = 0.037$). CD8+ cell density in the central part was associated with a hazard ratio of 1.76 (95% confidence in-

Table 2. Univariate analysis of the parameters associated with DFS

	N (%)	5-year DFS	p value		N (%)	5-year DFS	p value
Sex				Central part of tumor			
Male	61 (50)	47%	0.942	TIL (H-E stained)			
Female	61 (50)	39%		Positive	60 (49)	44%	0.27
				Negative	62 (51)	42%	
Age				CD3+			
≤70 years	56 (46)	42%	0.412	Positive	82 (67)	43%	0.46
>70 years	66 (54)	45%		Negative	40 (33)	44%	
Location of the tumor¹				CD4+			
Right side of the colon	57 (47)	44%	0.787	Positive	65 (54)	40%	0.54
Left side of the colon	65 (53)	41%		Negative	57 (46)	47%	
Histologic type				CD8+			
Well	48 (39)	43%	0.811	Positive	60 (49)	51%	0.039
Moderate	67 (55)	40%		Negative	62 (51)	34%	
Poor	2 (2)	50%					
Mucinous	5 (4)	75%		Invasive margin of tumor			
Adjuvant chemotherapy				TIL (H-E stained)			
Yes	63 (52)	42%	0.507	Positive	95 (78)	48%	0.012
No	59 (48)	45%		Negative	27 (22)	26%	
Whole tumor				CD3+			
TIL (H-E stained) ¹				Positive	118 (97)	43%	0.95
Positive	96 (79)	48%	0.016	Negative	4 (3)	50%	
Negative	26 (21)	27%		CD4+			
CD3+				Positive	91 (75)	40%	0.39
Positive	118 (97)	43%	0.95	Negative	31 (25)	51%	
Negative	4 (3)	50%		CD8+			
CD4+				Positive	110 (90)	42%	0.66
Positive	97 (80)	39%	0.18	Negative	12 (10)	55%	
Negative	25 (20)	56%					
CD8+							
Positive	113 (93)	43%	0.97				
Negative	9 (7)	51%					
FoxP3							
Positive	68 (56)	42%	0.58				
Negative	54 (44)	44%					

DFS, disease-free survival; TIL, tumor-infiltrating lymphocyte.
¹ Right-sided colon cancers included tumors arising from the cecum, ascending colon, hepatic flexure, and transverse colon, while left-sided colon cancers included tumors arising from the descending colon and sigmoid colon.

terval, 1.07–2.93; $p = 0.023$). TILs in the invasive margin and CD8+ cell density in the central part were independent prognostic factors.

Discussion

In clinical practice, the outcomes of patients with curatively resected colorectal cancer can be predicted on the basis of the TNM classification. However, patients with stage IIB or IIC disease have poorer outcomes than those with stage IIIA disease. Therefore, factors other than the previously used T stage and N stage are consid-

ered to determine outcomes [18]. Stage II disease was diagnosed in about 30–40% of patients who underwent surgery for colon cancer [19]. Some studies reported that adjuvant therapy can improve outcomes in patients with stage II disease and risk factors for recurrence. However, risk factors for recurrence have yet to be established [20, 21].

Host immune response has been recognized to be an important determinant of the outcomes of human tumors [22]. In patients with colorectal cancer, infiltration of inflammatory cells is related to survival rates, independently of histologic tumor grades. In addition to tumor-associated macrophages, dendritic cells, and neutrophils,

Table 3. Multivariate analysis of parameters associated with disease-free survival

	N (%)	5-year DFS	TIL (H&E stained)			CD8+ cell density		
			HR	95% CI	<i>p</i> value	HR	95% CI	<i>p</i> value
<i>Sex</i>								
Male	61 (50)	47%	1.0	Reference		1.0	Reference	
Female	61 (50)	39%	1.03	0.63–1.68	0.88	0.99	0.61–1.61	0.98
<i>Age</i>								
≤70 years	56 (46)	42%	1.0	Reference		1.0	Reference	
>70 years	66 (54)	45%	1.15	0.72–1.86	0.54	1.06	0.66–1.72	0.78
<i>Location of the tumor¹</i>								
Right side of the colon	57 (47)	44%	1.0	Reference		1.0	Reference	
Left side of the colon	65 (53)	41%	1.33	0.83–2.16	0.22	1.26	0.79–2.02	0.32
<i>Histologic type</i>								
Well	48 (39)	43%	1.0	Reference		1.0	Reference	
Moderate	67 (55)	40%	1.01	0.61–1.68	0.96	1.03	0.63–1.17	0.89
Poor	2 (2)	50%	1.02	0.05–5.01	0.97	1.21	0.06–6.11	0.85
Mucinous	5 (4)	75%	1.32	0.38–3.51	0.62	1.66	0.46–4.68	0.39
<i>Adjuvant chemotherapy</i>								
Yes	63 (52)	42%	1.0	Reference		1.0	Reference	
No	59 (48)	45%	0.93	0.58–1.48	0.77	0.95	0.60–1.52	0.85
<i>Invasive margin of tumor</i>								
TIL (H-E stained)								
Positive	95 (78)	48%	1.0	Reference				
Negative	27 (22)	27%	1.81	1.03–3.05	0.037			
<i>Central part of tumor</i>								
CD8+								
Positive	60 (49)	51%				1.0	Reference	
Negative	62 (51)	34%				1.76	1.07–2.93	0.023

TIL, tumor-infiltrating lymphocyte. ¹ Right-sided colon cancers included tumors arising from the cecum, ascending colon, hepatic flexure, and transverse colon, while left-sided colon cancers included tumors arising from the descending colon and sigmoid colon.

the presence of TILs may be an effective indicator of immune response [23]. As for subtypes of T cells, some studies reported a relation of CD3+ [24, 25], CD8+ [10, 24, 26, 27], CD45RO+ cells [23, 28], and FoxP3+ cells [29, 30] to outcomes.

To clarify the relation between TILs and outcomes, we performed propensity score matching in the present study, using previously reported clinicopathological risk factors for recurrence, and compared these factors between the two groups, with no significant differences. Consequently, we found that TILs on H-E staining and the density of CD8+ cells in the central part were determinant prognostic factors. Our results differ from those of previous studies. Many studies reported that CD8+ cells in the central part and the invasive margin are related to prognosis [10, 24, 26]. Other studies reported that

CD8+ cells only in the central part are related to prognosis. The results remain controversial [27]. Our results reconfirm that host immune response is an important determinant prognostic factor of human cancer. However, further studies are required to determine appropriate methods for evaluating immune response and the part of the tumor to be assessed.

Conclusions

We performed propensity score matching using conventional clinicopathological factors in patients with curatively resected colon cancer to examine the relation between TILs and outcomes and found that TILs in the invasive margin and the density of CD8+ cells in the central

part may be independent prognostic factors. These results suggest a close relation between the host's immune response and prognosis.

Statement of Ethics

This study was approved by the Institutional Review Board of Tokai University (16R-267), and all patients provided written informed consent.

Disclosure Statement

The authors have no potential conflicts of interest to declare.

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Author Contributions

Conception and design: L.F. Chan and S. Sadahiro; provision of study materials or patients: L.F. Chan, S. Sadahiro, T. Suzuki, K. Okada, and H. Miyakita; collection and assembly of data: L.F. Chan, K. Okada, and T. Suzuki; data analysis and interpretation: L.F. Chan, S. Sadahiro, H. Kajiwar, manuscript writing: L.F. Chan and S. Sadahiro; final approval of the manuscript: L.F. Chan, S. Sadahiro, T. Suzuki, K. Okada, H. Miyakita, S. Yamamoto, and H. Kajiwar.

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