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The Correlation of Microsatellite Instability and Tumor-infiltrating Lymphocytes in Hereditary Non-polyposis Colorectal Cancer (HNPCC) and Sporadic Colorectal Cancers: the Significance of Different Types of Lymphocyte Infiltration

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Background: Tumor-infiltrating lymphocytes (TIL) are strictly divided into two categories: those lymphocytes in stroma and those in between cancer cells. However, there has been no fully adequate comparison of these two categories, especially analysis in relation to microsatellite instability (MSI).

Methods: The materials were derived from patients with colorectal cancer who underwent surgery in Jichi Medical School and Omiya Medical Center. There were 19 hereditary non-polyposis colorectal cancer (HNPCC) patients who were compatible with Japanese criteria A and 106 patients with sporadic colorectal cancer (sCRC) in either Dukes B or C stage. As microsatellite markers, the global standard five markers were selected. Immunohistochemical analysis was performed using the anti-CD3, -CD4, -CD8 and -S-100 antibodies and the results were evaluated according to the degree of infiltration, which was classified into three grades.

Results: As for stroma-infiltrating lymphocytes (SIL) in sCRCs, severe infiltration was observed in 20% of high microsatellite instability (MSI-H) patients and 12.8% of low microsatellite instability (MSI-L)/stable microsatellite (MSS) patients without a statistically significant difference. In contrast, severe infiltration of intra-tumor cell-infiltrating lymphocytes (ITCIL) was observed in 41.7% of MSI-H sCRC patients and 4.3% of MSI-L/MSS patients. Thus, there was a close correlation between ITCIL severity and increased microsatellite instability ($P < 0.001$). In examination of ITCIL, patients with severe infiltration tended to show a better survival rate than those with moderate or mild infiltration.

Conclusions: The present study suggests that different factors are involved in the infiltration of SIL and ITCIL. Although there were no statistically significant differences, the cumulative survival rates tended to be higher in severe ITCIL cases than in those with moderate and poor ITCIL ($P < 0.09$). We suggest that there might be a possibility of ITCIL having a role for a better prognosis after colorectal cancer surgery, which is closely related to MSI.

Key words: microsatellite instability (MSI) – tumor infiltrating lymphocytes (TIL) – hereditary non-polyposis colorectal cancer (HNPCC) – sporadic colorectal cancer – survival rate

INTRODUCTION

Recent advances in molecular biology have demonstrated that some cancers are genetic diseases caused by accumulation of genetic abnormalities such as mutations and deletions. Such genetic changes lead to activation of oncogenes and/or inactivation of cancer suppressor genes, which may result in transformation from normal to cancer cells.

In the case of colorectal cancer, various causative genes have been cloned one after another from the research on hereditary colorectal cancers such as familial adenomatous polyposis or hereditary non-polyposis colorectal cancer (HNPCC). The APC gene (1,2) was cloned as one of the important genes involved in the genetic changes in adenoma carcinoma sequence proposed by Vogelstein (3). Various mismatch repair genes (hMSH2, hMLH1, hPMS1, hPMS2, hMSH6), which are

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causative genes for HNPCC, were also identified (4–7). The PTEN gene for Cowden disease (8) and the SKT11 gene for Peutz–Jegher's syndrome (9) have recently been discovered. While we expect more colorectal cancer-related genes to be discovered in the future, it is clear that there are at least two main carcinogenic mechanisms: loss of heterozygosity (LOH) and mismatch repair (MMR) deficiency (10).

HNPCC is a type of MMR deficiency colorectal cancer that often affects young people and has peculiar characteristics as follows. It occurs in the right-half of the colon, is poorly differentiated, is accompanied by mucinous carcinoma, tends to have associated development of other organ malignancies such as the endometrium, ovary, stomach and small intestine, is accompanied by severe lymphocyte infiltration, and has a favorable prognosis (11). Infiltrating lymphocytes are often referred to as tumor-infiltrating lymphocytes (TIL), and are believed to be directly related to the antitumor immune response. Several studies have found that infiltration by TILs is often severe in sporadic microsatellite instability-high (MSI-H) tumors, and numerous reports have suggested a correlation between MSI and TIL (12–15). According the site of lymphocyte infiltration, TIL can be roughly divided into two types: stromal infiltrating lymphocytes (SIL) and intra-tumor-cell infiltrating lymphocytes (ITCIL). However, in some previous reports (16–19) the distinction between SIL and ITCIL was not clear. When dividing TIL into SIL and ITCIL, it is clinically important to determine which type is more closely related to MSI and which is more useful as a survival and recurrence indicator.

In an attempt to clarify these points, we investigated tumor site, lymphocyte surface markers, degree of infiltration by various lymphocytes and survival rates based on clinico-pathological background factors among patients with HNPCC and sporadic colorectal cancer (sCRC).

PATIENTS AND METHODS

PATIENTS

The sizes of colorectal tumors were expressed in maximum diameter (mm), and all other factors were assessed according to the General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus (20). Of patients with primary colorectal cancer who underwent either an endoscopic resection or a surgical resection at the Department of Surgery, Jichi Medical School (JMS) and Omiya Medical Center of Jichi Medical School, between 1983 and 1999, 19 patients with HNPCC satisfied Japanese clinical criteria A (colorectal cancer patients with two or more first-degree relatives with colorectal cancers in two generations) (21). As to sCRC, of patients with colorectal cancer who underwent surgical procedure at the JMS between 1996 and 1997, 106 patients with primary colorectal cancers (Dukes B or C, and stages II or III), who underwent curative surgery and were followed until at least December 2001, served as subjects. There were 47 cases of stage II, 39 cases of stage IIIa and 20 cases of stage IIIb. With regard to Dukes Analysis, there were 47 cases of Dukes B and 59 cases of Dukes C.

METHODS

Resected tissue samples were collected from the 19 patients with HNPCC and the 106 patients with sCRC, and paraffin sections were prepared. MSI was assessed by first extracting DNA from paraffin sections using micro-dissection, performing PCR with at least five markers, including the standard internal markers, and assessing the results using either ABI Prism 310 or 3100 Genetic Analyzer (Applied Biosystems, Tokyo, Japan). Furthermore, the frequency and severity of SIL and ITCIL were determined by subjecting resected tissue samples to histological staining using hematoxylin and eosin (HE) as well as immunological staining using anti-CD3, -CD4, -CD8 and -S-100 antibodies.

TISSUE SAMPLING AND DNA EXTRACTION

Tissue samples were fixed in 20% formaldehyde, embedded in paraffin and sliced into thin sections. DNA was extracted by the micro-dissection method, which has been employed in our previously reported studies (22–24). In each case, a HE staining block having normal and cancer areas was selected under a microscope and was then sliced into 10 μm paraffin sections. Next, micro-dissection was performed under a microscope using a 24G needle within ~2–3 mm^2 of cancer and normal areas. After deparaffinization using 95% ethanol, the DNA samples were incubated overnight at 55°C in 100 μl of digestion buffer (5.0 μl of 1 M Tris–HCl pH 8.0, 0.5 μl of 0.2 M ethylenediamine tetraacetic acid, 0.5 μl of 0.4% Tween 20 and 3.75 μl of 20 mg/ml proteinase K). DNA samples were then heated for 10 min at 100°C in order to inactivate proteinase K and were stored in a freezer at –20°C.

ANALYSIS OF MSI

MSI was analyzed using at least six markers, including the five standard international markers (BAT25, BAT26, D5S346, D2S123, D17S250). A fluorescent primer was used for all markers, and their color allocations were as follows: FAM, D2S123, D17S250; HEX, BAT26, D5S346; and NED: BAT25. PCR was performed under the following conditions: D2S123-FAM: 5′-AAACAGGATGCCTGCCTTTA, D2S123-R: 5′-GGACTTTCCACCTATGGGAC, 94°C for 2 min (94°C for 30 s, 55°C for 30 s and 72°C for 30 s) 30 cycles, 72°C for 7 min; D17S250-FAM: 5′-GGAAGAATCAAATAGACAAT, D17S250-R: 5′-GCTGGCCATATATATATTTAAACC, 94°C for 2 min (94°C for 30 s, 50°C for 30 s, 72°C for 30 s) 30 cycles, 72°C for 7 min; BAT26-HEX: 5′-TGACTACTTTTGACTTCAGCC, BAT26-R: 5′-AACCATTCAACATTTTTAACCC, 94°C for 2 min (94°C for 30 s, 49°C for 30 s, 72°C for 30 s) 30 cycles, 72°C for 7 min; D5S346-HEX: 5′-ACTCACTCTAGTGATAAATCGGG, D5S346-R: 5′-AGCAGATAAGACAGTATTACTAGTT, 94°C for 2 min (94°C for 30 s, 53°C for 30 s, 72°C for 30 s) 30 cycles, 72°C for 7 min; and BAT25-NED: 5′-

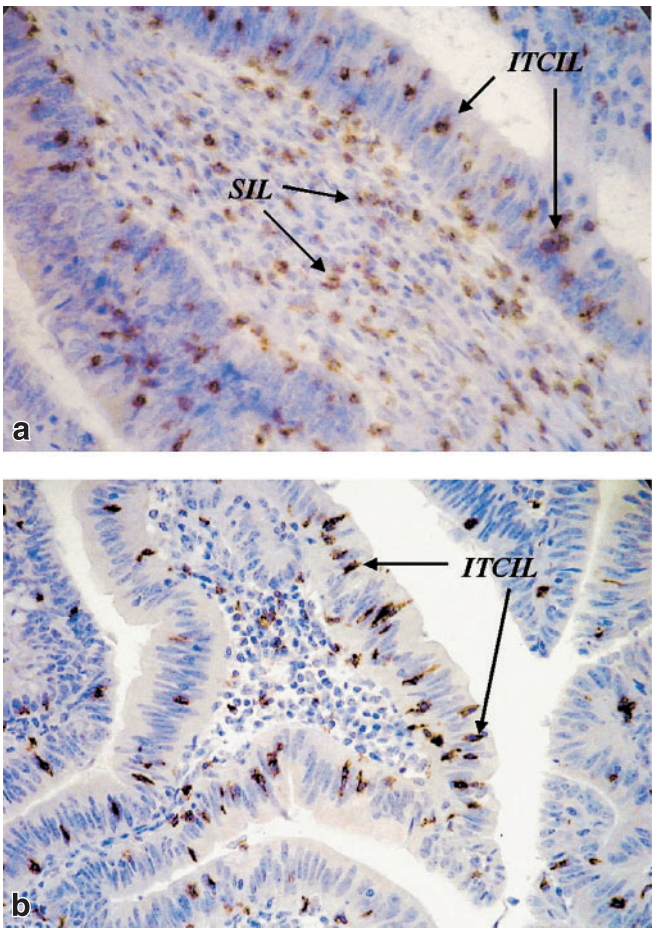

Figure 1. Immunohistochemistry for CD8 antibody. (a) This case shows that the degrees of SIL and ITCIL are severe (×400). (b) This case shows that the degree of ITCIL is severe and that of SIL is moderate (×400).

TCGCCTCCAAGAATGTAAGT, BAT25-R: 5′-TCTGCATTTTAACTATGGCTC, 94°C for 2 min (94°C for 30 s, 51°C for 30 s, 72°C for 30 s) 30 cycles, 72°C for 7 min.

PCR products were analyzed by Gene Scan using Prism 3100, and MSI was classified into three grades as follows: high MSI (MSI-H) ≥40% instability, low MSI (MSI-L) <40% instability and stable (MSS).

Immunohistochemical analysis

In all patients, 4-μm thick paraffin sections were made, and immunologically stained by the labeled streptavidin biotin method. Briefly, paraffin sections were subjected to 0.5% periodate oxidation for 15 min to block endogenous peroxidase, placed in a pressure cooker for 2 min to activate antigens (25), and reacted with phosphate buffered saline containing 10% normal rabbit or goat serum for 30 min. The following primary antibodies were used: anti-CD3 (mouse, X100, DAKO), anti-CD4 (mouse, X20, DAKO), anti-CD8 (mouse, X20, DAKO) and anti-S-100 (rabbit, X1000, DAKO). While the anti-CD4 antibody was allowed to react for 3 days, the other antibodies were allowed to react overnight at 4°C in a moist chamber. After washing the resulting sections with phosphate buffered saline twice, secondary antibodies were added, and color development was conducted by allowing the sections to react with a DAB H_2O_2 solution for 40 s (20 mg of 3,3′-diaminobenzidine tetrahydrochloride 4HCl (Wako Jyunyaku, Tokyo, Japan), 180 ml of 1 M Tris–HCl buffered solution, and 5 μl of 30% H_2O_2). The resulting sections were washed using water, stained using hematoxylin and examined under a light microscope. One of the sections was stained using HE for morphological examination.

Assessment of infiltrating lymphocytes

Infiltrating lymphocytes were divided into two types: ITCIL that infiltrated among the tumor cells and beyond the basal membrane of the tumor cells, which were made up cancerous glands, and SIL that infiltrated the stroma around the nests of cancerous glands (Fig. 1). When assessing ITCIL, a region of interest was established at an area with cancerous glands.

Lymphocytes that reacted positively to the various antibodies were quantitatively assessed as follows: under a light microscope at ×400 magnification, five visual fields were randomly selected and the number of positive cells was counted to determine the number of lymphocytes per 250 μm^2. For anti-CD8 antibody-positive cells, the degree of infiltration was arbitrarily classified into three grades based on the number of infiltrating cells per unit area: poor <40; moderate ≥40 but <80; and severe ≥80.

Statistical analysis

For statistical analysis, Fisher exact test was performed to make comparisons among two groups, and chi-square tests were used. The Kruskal–Wallis test was used for multiple comparison procedures. A P value of <0.05 was considered to be statistically significant. Stat-Mate software (Microsoft Excel, statistical soft, Atms, Co. Ltd, Tokyo) for Windows was used.

Analysis of cumulative survival rates

Multivariate analyses on various colorectal cancer-related factors, MSI status and survival rate were carried out. Patients were diagnosed as having had recurrence of colorectal cancer when postoperative clinical examination or diagnostic imaging confirmed findings of recurrence. A Kaplan–Meier test (log rank) was used to calculate cumulative survival rates (Stat-Mate for Windows). Among the 106 patients with sCRC, three multivariate analyses were conducted: (i) MSI: histological type, SIL, ITCIL, lymph node metastasis and tumor site; (ii) M: MSI, SIL, ITCIL, histological type and tumor site; (iii) survival at 4 years after surgery: MSI, histological type, SIL, ITCIL, lymph node metastasis and tumor site. A dummy variable was introduced to both explanatory and target variables in order to analyze binary data. Dr. SPSS (8.0.1.J) for Windows was used for multivariate analyses.

Table 1. Characterestics of patients with colorectal cancers and sCRCs according to MSI status

	Colorectal cancers		*P* value	sCRCs		*P* value
	HNPCC (*n* = 19)	sCRCs (*n* = 106)		MSI-H (*n* = 12)	MSI-L/MSS (*n* = 94)	
Class of age, no (%)						
<60	11 (57.9)	33 (31.1)	<0.05	4 (33.3)	29 (30.9)	0.876
≥60	8 (42.1)	73 (68.9)		8 (66.7)	65 (69.1)	
Sex, no (%)						
Males	8 (42.1)	61 (57.5)	0.213	5 (41.7)	56 (59.6)	0.237
Females	11 (57.9)	45 (42.5)		7 (58.3)	38 (40.4)	
Status, no (%)						
Proximal	11 (57.9)	46 (43.4)	0.243	10 (77.8)	36 (38.3)	<0.01
Distal	8 (42.1)	60 (56.6)		2 (21.2)	58 (61.7)	
Pathological stage, no (%)						
II	7 (36.8)	47 (44.3)	0.366	10 (83.3)	37 (39.4)	<0.01
III	4 (21.1)	59 (55.7)		2 (16.7)	57 (60.7)	
IIIa	4 (21.1)	39 (36.8)		2 (16.7)	37 (39.4)	
IIIb	0 (0)	20 (18.9)		0 (0)	20 (21.3)	
Histological type, no (%)						
Well	15 (78.9)	68 (64.2)	0.423	7 (58.3)	61 (64.9)	<0.001
Moderate	3 (15.8)	27 (25.5)		0 (0)	27 (28.7)	
Poor	1 (5.3)	5 (4.7)		4 (33.3)	1 (1.1)	
Mucinous	0 (0)	6 (5.7)		1 (8.3)	5 (5.3)	

sCRCs: sporadic colorectal cancers.

RESULTS

The various clinicopathological findings, SIL, ITCIL and survival rates were compared by dividing the patients into three groups (HNPCC, sporadic MSI-H and sporadic MSI-L/MSS) and by subdividing patients with sporadic colorectal cancers into two groups with respect to tumor site (proximal: cecum, ascending colon or transverse colon; distal: descending colon, sigmoid colon or rectum).

Clinicopathological Findings

Of the 19 HNPCC patients, nine were men and 10 were women, with an average age of 58.2 ± 21 years (range, 41–81 years). Of the patients with sporadic colorectal cancer, 45 were men and 35 were women, with an average age of 62.4 ± 43 years (range, 44–83 years). There were no significant gender differences among the HNPCC, sporadic MSI-H and sporadic MSI-L/MSS groups. As to tumor site, while 83.3% were proximal for the sporadic MSI-H group, 16.7% were proximal for the sporadic MSI-L/MSS group ($P < 0.01$). As to age, more than half of the HNPCC patients were younger than 60 years, six patients (31.6%) were in their 40s, and there was a significant difference between HNPCCs and sCRCs ($P < 0.05$). Regarding histological stage, sporadic stage II accounted for 83.3% of the MSI-H tumors, but for only 39.4% of the MSI-L/MSS tumors. The incidence of lymph node metastasis for the sporadic MSI-H group was significantly lower than sporadic MSI-L/MSS tumors ($P < 0.01$). The most common histological type in sporadic cases was well differentiated, followed by moderately differentiated and poorly differentiated, and the incidence of poorly differentiated carcinoma was significantly higher in MSH-H cases ($P < 0.05$). HNPCC patients also showed similar distribution of histological types as in MSI-H sporadic colorectal cancers (Table 1).

When the patients with sCRC were divided into two groups with respect to tumor site (proximal and distal), there were no significant differences in age, gender or histological type. While proximal MSI-H cancer accounted for 21.7% of proximal colorectal cancer, distal MSI-H cancer only accounted for 3.3% of distal colorectal cancer ($P < 0.01$).

Stromal-infiltrating Lymphocytes (SIL)

In the tumor stroma, all four types of lymphocytes were observed. The number of positive cells tended to be highest for the sporadic MSI-H group, followed by the HNPCC group and then the MSI-L/MSS group, and the breakdown of the different

Table 2. Breakdown of SIL for four colorectal cancer groups (/250 μm^2)

	CD8	CD3	CD4	S-100
HNPCC	45.2	143.3	82.5	16.2
HNPCC MSI-H	51.5	165.4	91.2	22.3*,‡
Sporadic MSI-H	47.6	153.2	84.7	20.1*,†
Sporadic MSI-L/MSS	35.8	112.4	55.5	12.4†,‡

*$P = 0.982$; †$P = 0.501$; ‡$P = 0.482$.

Table 3. The comparison between SIL and ITCIL in cancer tissue and clinicopathologic features in HNPCC and sCRC

	Histological type			*P* value	Lymph node metastasis		*P* value	Lymphatic invasion				*P* value	Venous invasion				*P* value
	well	mod	poor		n(–)	n(+)		ly0	ly1	ly2	ly3		v0	v1	v2	v3	
SIL																	
HNPCC																	
Severe	4	0	0		3	1		0	3	1	0		0	3	1	0	
Moderate	3	2	1	0.102	3	3	0.518	2	2	1	1	0.676	1	3	2	0	0.979
Poor	8	1	0		7	2		0	5	4	0		2	3	4	0	
sCRC																	
Severe	6	2	3		6	5		4	3	4	0		7	2	1	1	
Moderate	26	6	2	0.082	18	16	0.832	6	17	10	1	<0.05	11	15	8	0	<0.05
Poor	36	19	0		26	29		3	23	25	4		10	23	16	6	
ITCIL																	
HNPCC																	
Severe	6	2	0		4	4		2	4	2	0		2	5	1	0	
Moderate	1	1	0	0.562	1	1	0.208	0	1	0	1	0.298	0	0	2	0	0.089
Poor	8	0	1		8	1		0	5	4	0		1	4	4	0	
sCRC																	
Severe	6	1	2		6	3		6	1	2	0		7	1	1	0	
Moderate	10	1	1	0.544	6	6	0.576	1	6	5	0	<0.05	3	8	1	0	<0.01
Poor	52	25	2		38	41		7	33	34	5		19	29	24	7	

lymphocytes was similar between the sporadic MSI-H group and the HNPCC group; the pattern of lymphocyte infiltration specific to HNPCC was not recognized. Although the numbers of positive cells for the sporadic MSI-L/MSS group were lower when compared to the other two groups, there were no statistically significant differences ($P = 0.904$). In all groups, the most common lymphocytes were anti-CD3 antibody-positive lymphocytes, followed in order by anti-CD4, anti-CD8 and anti-S-100 (all antibody-positive lymphocytes) (Table 2).

The comparison between SIL and ITCIL in cancer tissue and clinicopathologic features in HNPCC and sCRCs is shown in Table 3. Histological type and the status of lymph node metastasis did not show significant differences among different grades of SIL and ITCL infiltration in HNPCC and sCRCs. On the other hand, lymphatic and venous invasion were significantly lower in severe SIL and severe ITCIL cases of sCRCs.

The ratio of anti-CD8 antibody-positive SIL was high for the HNPCC MSI-H group and the sporadic MSI-H group at 20.0% and 25.0%, respectively, but low for the sporadic MSI-L/MSS group at 12.8%. However, no statistically significant differences were found (Table 4).

Furthermore, while the cumulative survival rate for severe SIL was higher, there were no statistically significant differences between different grades of SIL severity ($P = 0.314$). In this analysis, there were no tendencies of better survival in severe SIL cases, because the moderate SIL cases showed higher survival rates than the other two groups (Fig. 2).

Intra-tumor-cell Infiltrating Lymphocytes (ITCIL)

In all groups, almost all ITCIL were anti-CD8 antibody-positive lymphocytes and as a result the other types of lymphocytes were hardly seen. Comparison was made between HNCPP and sCRC cases on the ITCIL in cancer tissue and clinicopathologic features. There was no significant difference between HNPCC and sCRC in pathological types or lymph node metas-

Table 4. Relationship between MSI and anti-CD8 antibody-positive SIL

	Poor	Moderate	Severe	Total
HNPCC	9 (47.4)	6 (31.6)	4 (21.1)	19
HNPCC MSI-H	6 (40.0)	6 (40.0)	3 (20)	15*,‡
Sporadic MSI-H	4 (33.3)	5 (41.7)	3 (25.0)	12*,†
Sporadic MSI-L/MSS	55 (58.5)	27 (28.7)	12 (12.8)	94†,‡

*$P = 0.695$; †$P = 0.089$; ‡$P = 0.183$.

Table 5. Relationship between MSI and ITCIL severity

	Poor	Moderate	Severe	Total
HNPCC	9 (47.4)	2 (10.5)	8 (42.1)	19
HNPCC MSI-H	6 (40.0)	2 (13.3)	7 (46.7)	15*,‡
Sporadic MSI-H	3 (25.0)	4 (33.3)	5 (41.7)	12*,†
Sporadic MSI-L/MSS	79 (84.0)	11 (11.7)	4 (4.3)	94†,‡

*$P = 0.813$; †$P < 0.001$; ‡$P < 0.001$.

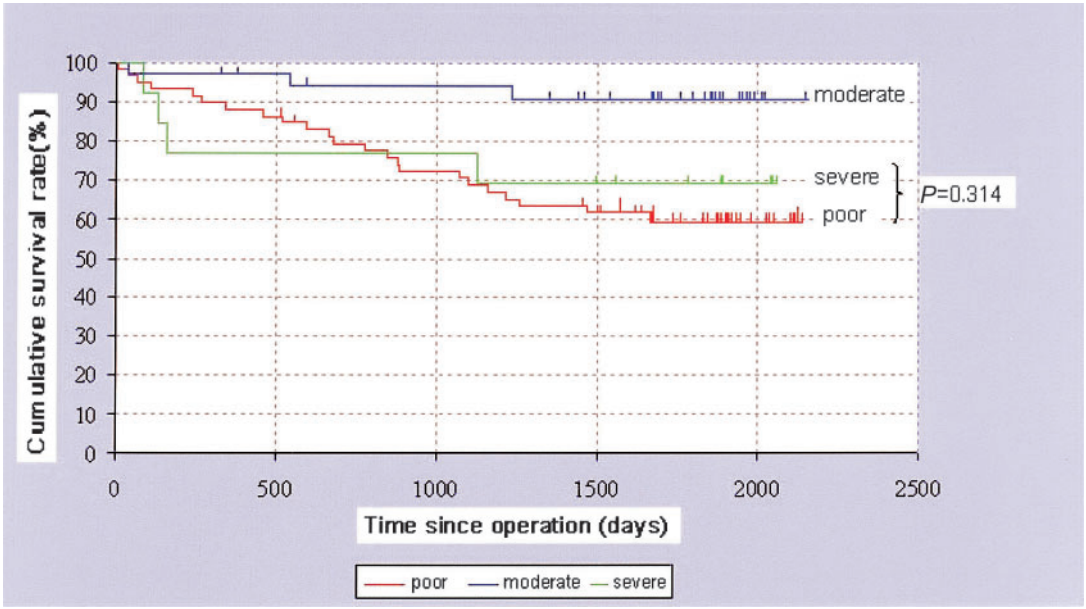

Figure 2. Comparison of postoperative cumulative survival rate with degree of SIL.

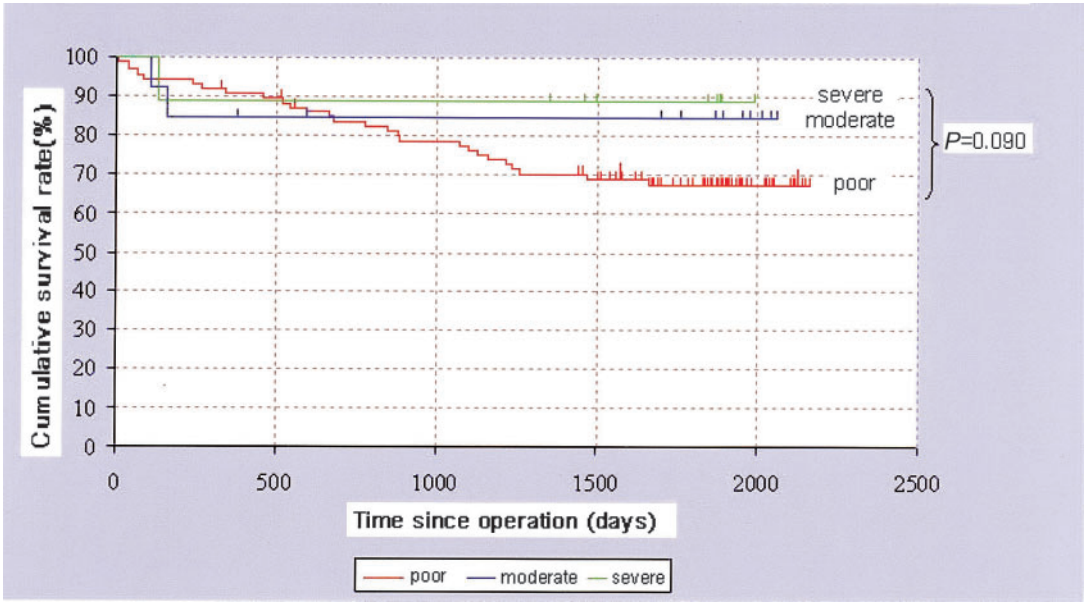

Figure 3. Comparison of postoperative cumulative survival rate with degree of ITCIL.

tasis. However, lymphoid and venous infiltrations were less significant in severe ITCIL than in sCRC (Table 3).

Severe ITCIL cases accounted for only 4.3% of sporadic MSI-L/MSS cases. On the other hand, they accounted for 41.7% and 46.7% of sporadic MSI-H and HNPCC MSI-H cases, respectively, confirming a close correlation between ITCIL severity and increased MIS ($P < 0.001$) (Table 5). Similar to SIL, the pattern of lymphocyte infiltration specific to HNPCC was not recognized. While the cumulative survival rate for severe ITCIL cases was higher, there was no statistically significant difference between different grades of ITCIL severity ($P = 0.090$) (Fig. 3). As for the relationship in the infiltration grade between SIL and ITCIL in sCRCs, although statistical analysis was not possible, we did not observe any relationship between them (Table 6).

Multivariate Analyses on MSI, Histological Type, SIL, ITCIL, Lymph Node Metastasis ('n'-factor), Tumor Site and Survival Rates at 4 Years after Surgery

In an analysis taking MSI as a dependent variable, there was a significant correlation between ITCIL and tumor site ($P = 0.0044$ and 0.0332, respectively) (Table 7). As shown by the Kruskal–Wallis test, there was no significant correlation with

Table 6. The relationship between anti-CD8 antibody-positive SIL and ITCIL grade in sCRCs

SIL	ITCIL			
	Severe	Moderate	Poor	Total
Severe	4	2	9	15
Moderate	3	10	19	32
Poor	2	3	54	59
Total	9	15	82	106

Table 7. Analysis handling MSI as a dependent variable

Factors	Category	*P* value	Odds ratio
SIL	Severe vs moderate vs poor	0.2819	1.8841
ITCIL	Severe vs moderate vs poor	0.0044	5.2461
n	(+) vs (–)	0.0511	0.142
Location	Proximal vs distal	0.0322	9.0887
Histological type	Well vs moderate vs poor vs mucinous	0.4335	1.4175

Table 8. Analysis handling n as a dependent variable

Factors	Category	*P* value	Odds ratio
SIL	severe vs moderate vs poor	0.4368	1.2665
ITCIL	severe vs moderate vs poor	0.4555	1.3678
Location	proximal vs distal	0.3861	0.6902
Histological type	well vs moderate vs poor vs mucinous	0.2081	1.4055
MSI	MSS/MSI-L vs MSI-H	0.0262	0.1116

Table 9. Analysis handling survival at 4 years after surgery as a dependent variable

Factors	Category	*P* value	Odds ratio
SIL	Severe vs moderate vs poor	0.3301	1.4575
ITCIL	Severe vs moderate vs poor	0.5681	1.3777
n	(+) vs (–)	0.0022	0.1998
Location	Proximal vs distal	0.6205	0.7807
Histological type	Well vs moderate vs poor vs mucinous	0.245	0.7188
MSI	MSS/MSI-L vs MSI-H	0.5824	1.956

SIL. In an analysis handling 'n'-factor as a dependent variable, there was a significant correlation with MSI (P = 0.0262) (Table 8). In analyses taking survival rates at 4 years after surgery as dependent variables, there was a significant correlation with lymph node metastasis (n) (P = 0.0022 and 0.0454, respectively), thus reaffirming that lymph node metastasis is an important factor for survival. Furthermore, as shown by the Kaplan–Meier test, there was no significant correlation with SIL or ITCIL (P = 0.3301 and 0.5681, respectively) (Table 9).

DISCUSSION

Numerous factors influence the prognosis and recurrence of colorectal cancer, TIL is one among them. Because TIL is believed to play an important role in the immune response for cancer, studies have been conducted to investigate surface differentiation antigens on the cell membrane of TIL to ascertain cytotoxicity of TIL (26) and also to analyze TIL by activating lymphocytes (27,28) in an attempt to clarify the clinical significance of TIL. However, it is not widely known that TIL can be roughly divided into two groups with respect to the extent of infiltration (SIL and ITCIL), and that the composition and characteristics of lymphocytes differ between SIL and ITCIL.

SIL are present in the tumor stroma and consist mostly of pan T cells, but contain other types of lymphocytes, such as anti-CD4, -CD3 and -S-100 antibody-positive lymphocytes. On the other hand, ITCIL exist among tumor cells and consist mostly of anti-CD8-antibody-positive cells, thus exhibiting a clear difference from SIL. In the process known as the tumor immune response system (29), antigen-presenting cells (APCs) first present cancer specific antigen peptides, then helper T cells are activated, and the activated helper T cells release cytokines (IL-2, IL-4, GM-CSF and IFN-γ), which in turn activate various immuno-competent cells (including anti-CD8 antibody-positive cells). As our results showed the presence of different types of lymphocytes in SIL and ITCIL, we suggest that ITCIL have a specific role in the immune response system mentioned above.

Several studies have reported the involvement of MSI in TIL (12–15). Therefore, we performed a Kruskal–Wallis test and multivariate analysis to investigate whether MSI was closely related to SIL or ITCIL. The results of both tests showed that only ITCIL correlated well with MSI, which strongly suggested that separate mechanisms are involved for SIL and ITCIL, and that ITCIL may be induced by MSI. Among the HNPCC, sporadic MSI-H and sporadic MSI-L/MSS groups. there was a significant difference in ITCIL, while there was none in SIL. This strongly suggests the involvement of MSI in the presence of ITCIL. In addition, the HNPCC group and the sporadic MSI-H group exhibited similar lymphocyte infiltration findings, indicating that MSI is involved in the development of ITCIL.

Reduced expression of HLA-class I antigens has been proposed as one of the mechanisms for tumor escape. Gutierrez (30), Allen (31) and Toyoda (32) documented a correlation between TIL and HLA antigen expression. It is possible that TIL are attracted to cancer cells by recognizing tumor antigens and HLA antigens on their surface, and signal the cytokines produced by TIL and microenvironmental changes surrounding cancer cells. They subsequently induce or enhance the HLA antigen expression on the cancer cells. Furthermore, MSI could: (i) support the expression of auxiliary signal molecules that are necessary for the activation of T lymphocytes; (ii) induce unknown molecules that are antagonistic to immuno-

suppressive agents produced by cancer cells; and (iii) release antibody masking (when non-cytotoxic antibodies bind specifically with tumor antigens, the antigens are masked, thus evading attacks from cytotoxic T lymphocytes). We are planning to investigate these possibilities in the future.

The fact that there was a strong correlation between ITCIL and MSI suggests the prospect of using ITCIL for MSI-H colorectal cancer screening. MSI assessment is clinically advantageous in identifying HNPCC patients, predicting prognosis and planning treatment, but it is cost- and time-intensive and requires very complicated procedures, such as DNA extraction from healthy and cancer regions, PCR using microsatellite markers, gel electrophoresis and gene scanning. Although routine ITCIL assessment during a pathological test requires a certain amount of training, ITCIL could be used as a convenient MSI screening tool (14).

Numerous reports have confirmed the relationship between the prognosis of colorectal cancer and TIL severity (16,17, 33–35), but some recent studies did not (36), and thus the evidence is inconclusive. Our study showed that there was no statistically significant difference in the cumulative survival rates among the different grades of both SIL and ITCIL. However, so far as ITCIL is concerned, there was a tendency of having a better survival rate in cases with severe ITCIL in comparison to the moderate or poor ITCIL cases. On the other hand, SIL did not show any specific tendency regarding the survival rate. Furthermore, the results of multivariate analyses on survival also showed no significant correlation. TIL are known to induce apoptosis of target cells through: (i) perforin and granzyme (37); (ii) Fas-L (38); and (iii) tumor necrosis factor (TNF)-α or TNF-β (39). However, our data strongly suggest a limit to the antitumor effects of TIL and the existence of factors, in addition to TIL, that are more closely related to prognosis. In our study, lymph node metastasis was related to MSI-H, and MSI-H was in turn related to severe ITCIL in multivariate analysis. Although there was no statistically significant relationship between ITCIL and survival rate, there might be a possibility of MSI-H having a role in bringing a better prognosis by inducing severe grade ITCIL.

The results of the present study show that SILs consisted of a variety of lymphocytes (anti-CD4, -CD8 and -S-100-positive cells), while ITCILs primarily consisted of anti-CD8 antibody-positive cells. The present study strongly suggests that different factors are involved in the infiltration of SIL and ITCIL, and that intra-tumor cell-infiltration of lymphocytes is caused by MSI. Although there were no statistically significant differences, the cumulative survival rates tended to be higher in severe ITCIL cases than in those with moderate and poor ITCIL cases (P = 0.09). Considering the relatively small number of cases in this study, we consider that there may be a possibility of ITCIL having a role, closely related to MSI, in bringing about better prognosis after colorectal cancer surgery.

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