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ORIGINAL ARTICLE

Systemic Analysis of Predictive Biomarkers for Recurrence in Colorectal Cancer Patients Treated with Curative Surgery

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Abstract

Background Preoperative serum systemic inflammatory response (SIR) in patients with colorectal cancer (CRC) has been reported to be a predictive biomarker of early recurrence. The molecular status of CRC, including microsatellite instability (MSI), *BRAF* and *KRAS* mutations, and tumor-infiltrating lymphocytes (TILs), has also been associated with recurrence in CRC patients treated with curative surgery.

Aim We investigated the impacts of SIR status, TILs, and MSI on recurrence in curative CRC patients.

Methods In this retrospective study, we enrolled 157 patients with stage I–III CRC undergoing curative surgery, for whom preoperative neutrophil/lymphocyte ratio (NLR), platelet/lymphocyte ratio (PLR), and C-reactive protein (CRP) data were available as indicators of SIR status. Molecular status was evaluated by counting TILs as the numbers of intratumoral Foxp3- and CD8-positive T cells by immunohistochemistry. MSI status was determined using five mononucleotide repeat microsatellite markers.

Results Kaplan–Meier analysis of SIR indicators revealed that higher CRP, NLR, and PLR were associated with significantly poorer disease-free survival (DFS). Low levels of infiltrating CD8-positive T cells in CRC tissue was a significant predictor of poor DFS. Multivariate analysis showed that few infiltrating CD8-positive T cells and high serum CRP levels were independent predictive factors for recurrence. Furthermore, the combination of high CRP and few infiltrating CD8-positive T cells increased the predictive accuracy in these patients.

Conclusions The results of this study suggest that both CRP levels in preoperative serum and CD8 T cells in CRC tissue are useful biomarkers for predicting early relapse in CRC patients treated with curative surgery.

Keywords Recurrence · Colorectal cancer · Systemic inflammatory response · Tumor-infiltrating lymphocyte · Microsatellite instability

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Introduction

Colorectal cancer (CRC) is the third most common cancer and fourth leading cause of cancer-related deaths worldwide [1]. Despite improvements in surgical techniques, fatal disease recurrence occurs in 20–25 % of curatively treated patients [2]. Patients' prognosis depends on the stage or anatomic extent of disease, based on the Union for International Cancer Control (UICC) tumor-node-metastasis (TNM) classification. The function of TNM staging has expanded from predicting prognosis to informing treatment choices [3, 4]. Several large randomized trials have suggested that all node-positive patients should receive adjuvant chemotherapy, while the value of adjuvant therapy for node-negative cases is controversial [3, 5]. The predictive value of TNM staging for identifying node-negative patients at risk of early recurrence in CRC is therefore limited. Accordingly, extensive research has been devoted to studying clinicopathological features and/or predictive molecular factors that may supplement TNM classification for predicting prognosis and recurrence in patients with CRC undergoing curative surgery.

The systemic inflammatory response (SIR) status is thought to be secondary to hypoxia or tumor necrosis and is associated with anti-apoptotic characteristics of cancer cells [6]. Preoperative serum SIR has been reported as a predictive biomarker of early recurrence in CRC patients treated with curative surgery [7], while neutrophil/lymphocyte ratio (NLR), platelet/lymphocyte ratio (PLR), and C-reactive protein (CRP) are considered to be indicators of SIR status.

The molecular status of CRC, including microsatellite instability (MSI) and tumor-infiltrating lymphocytes (TILs), is also associated with high risk of recurrence in CRC patients treated with curative surgery. TILs can act as an indicator of the host's tumoral immune response and have been associated with recurrence and improved clinical outcome in CRC, as well as providing an attractive target for immunotherapy [8–12]. MSI represents an alternative pathway of colorectal carcinogenesis, in which tumors arise as a result of mutation or hypermethylation in the DNA mismatch repair system. MSI status has been reported to be an independent prognostic predictor of improved survival and time to recurrence [13].Therefore, both SIR-related host and local tumoral factors should be investigated to determine the factors predicting early recurrence in CRC patients treated with curative surgery.

This study therefore aimed to elucidate the clinical significance of both preoperative serum SIR and local tumor TILs and MSI status to identify biomarkers of recurrence risk in CRC patients treated with curative surgery.

Methods

Patients and Specimens

A total of 157 patients with stage I, II, or III primary CRC who underwent surgical resection at the Department of Gastrointestinal and Pediatric Surgery, Mie University, Mie, Japan, from 2007 to 2011 were analyzed. Patients with the following criteria were excluded: preoperative radiotherapy or chemotherapy, diagnosis of multiple CRCs, history of cancer in another organ, familial CRC, and inflammatory bowel disease. All patients were classified postoperatively based on the UICC TNM classification [14], using 10 % formalin-fixed, paraffin-embedded (FFPE) specimens. The histomorphology of the primary tumors and lymph nodes was confirmed by the Department of Pathology, Mie University, Mie, Japan. Written informed consent was obtained from all patients according to the guidelines approved by our institutional research board.

Laboratory Measurements of Neutrophils, Lymphocytes, CRP, and Carcinoembryonic Antigen

Neutrophils, lymphocytes, CRP, and carcinoembryonic antigen (CEA) were measured in routine blood samples obtained within 1 week before operation. Patients were divided into two groups using an NLR cutoff value of 3, based on a previous report [15]. Patients were also categorized according to PLR $\leq$150 or >150, as reported previously [16]. The cutoff value for CRP was 0.5 mg/dl ($\leq$0.5 and >0.5 mg/dl) and for CEA was 5 ng/μl ($\leq$5 and >5 ng/μl), according to the normal range at our institute.

Immunohistochemistry

FFPE specimens were sliced into 5-μm sections and subjected to immunohistochemical analysis to detect Foxp3 and CD8 expression. After deparaffinization and dehydration, the sections were placed in 10 mM sodium citrate buffer (pH 6.0) and autoclaved at 121 °C for 10 min for antigen retrieval. The sections were incubated in 3 % hydrogen peroxide for 10 min, blocked, and incubated in normal goat serum (Vector Laboratories Inc, Burlingame, CA, USA) overnight at 4 °C for Foxp3 and for 1 h at room temperature for CD8. The primary antibodies used were monoclonal mouse anti-human Foxp3 antibody (clone: 236A/E, Abcam, Cambridge, UK; dilution 1:100) for regulatory T cells and monoclonal rabbit anti-human CD8 (clone: EP1150, GeneTex, San Antonio, TX, USA; dilution 1:1000) for cytotoxic T cells. Antibody binding was visualized using Envision reagents (Dako REAL EnVision Detection System; peroxidase/DAB+, Dako Cytomation,

Glostrup, Denmark). All the sections were counterstained with hematoxylin–eosin prior to dehydration and mounting.

Scoring Foxp3- and CD8-Positive T Cells

Foxp3- and CD8-positive T cells were counted using a scanner system under a Biorevo BZ-9000 microscope (Keyence, Osaka, Japan). Each slide was scanned microscopically, and intratumoral Foxp3- and CD8-positive T cells were photographed at a magnification of 400× in three representative high-power fields. Foxp3- and CD8-positive T cells with any detectable staining above background levels were considered positive.

KRAS/BRAF Mutation and MSI Analysis

FFPE sections (10 μm thick) from 157 surgical CRC patients were evaluated for mutations. Hematoxylin–eosin-stained FFPE sections were microdissected to extract DNA from the tumor cells. Genomic DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. DNA quantity and quality were assessed using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Houston, TX, USA). *KRAS* (exons 2 and 3) and *BRAF* (V600E) mutations were analyzed by pyrosequencing using the primers listed in Table 1. Reactions were run on a PyroMark Q96 ID system (Qiagen). MSI status was determined by polymerase chain reaction analyses of five mononucleotide repeat microsatellite markers (BAT-25, BAT-26, NR-21, NR-24, and NR-27), as recommended previously [17]. The primer sequences have been described previously [17]. Tumors with instability at more than three of these markers were classified as showing MSI, and those with instability at fewer than two markers as showing microsatellite stability (MSS).

Statistical Analysis

The associations between SIR status, TILs, MSI status, and clinicopathological features were analyzed using Chi-square tests. Disease-free survival (DFS) curves were analyzed using the Kaplan–Meier method, and differences were examined using log-rank tests. Cox's proportional hazards regression tests were used to estimate univariate and multivariate hazard ratios for recurrence. Multivariate survival analyses were performed using factors identified as significant in univariate analyses. All P values were two-sided, and $P < 0.05$ was considered statistically significant. All statistical analyses were carried out using JMP version 10 (SAS Institute, Cary, NC, USA).

Table 1 Primer sequences for *KRAS* and *BRAF* mutation analysis

BRAF	
Forward	GAA GAC CTC ACA GTA AAA ATA G
Reverse	Bio-ATA GCC TCA ATT CTT ACC ATC C
Sequencing	AGG TGA TTT TGG TCT AGC TAC AG
KRAS	
Forward	GGC CTG CTG AAA ATG ACT GA
Reverse	Bio-TAG CTG TAT CGT CAA GGC ACT CT
Sequencing	TTG TGG TAG TTG GAG CT

Results

Patient Characteristics

The study included a total of 90 men and 67 women with an average age of 66.9 years (range 35–89 years). The median follow-up time was 20.5 months (range 0.2–62.4 months). All patients were classified postoperatively based on the histopathological analysis using the UICC TNM classification criteria: stage I ($n = 48$), stage II ($n = 55$), and stage III ($n = 54$). Twenty-nine patients (18.4 %) developed recurrence.

Immunohistochemical Analysis of Tumor-Infiltrating Foxp3- and CD8-Positive T Cells

Intratumoral Foxp3- and CD8-positive T cells were detected (Fig. 1), with median numbers of 11 (range 0–99) and 15 (range 1–144), respectively. Patients were classified into high- and low-expressing groups for each antigen using the median values as cutoff points.

KRAS/BRAF Mutation and Tumor MSI Analysis

KRAS and *BRAF* mutations were analyzed in CRCs from 152 patients. Fifty patients (32.9 %) had *KRAS* codon mutations, and five (0.03 %) harbored *BRAF* mutations. A total of 98 patients (64.5 %) had no mutations in either the *BRAF* or *KRAS* gene (wild type). MSI was analyzed in 151 patients, of whom 142 showed MSS and nine showed MSI.

Associations Between SIR Status as Host Factor and Clinicopathological Features

We analyzed the association between preoperative SIR status and clinicopathological features (Table 2). High NLR was significantly associated with lymphatic invasion ($P = 0.0371$) and recurrence ($P = 0.0062$), while high PLR was also associated with recurrence ($P = 0.006$). In addition, high CRP level was significantly associated with

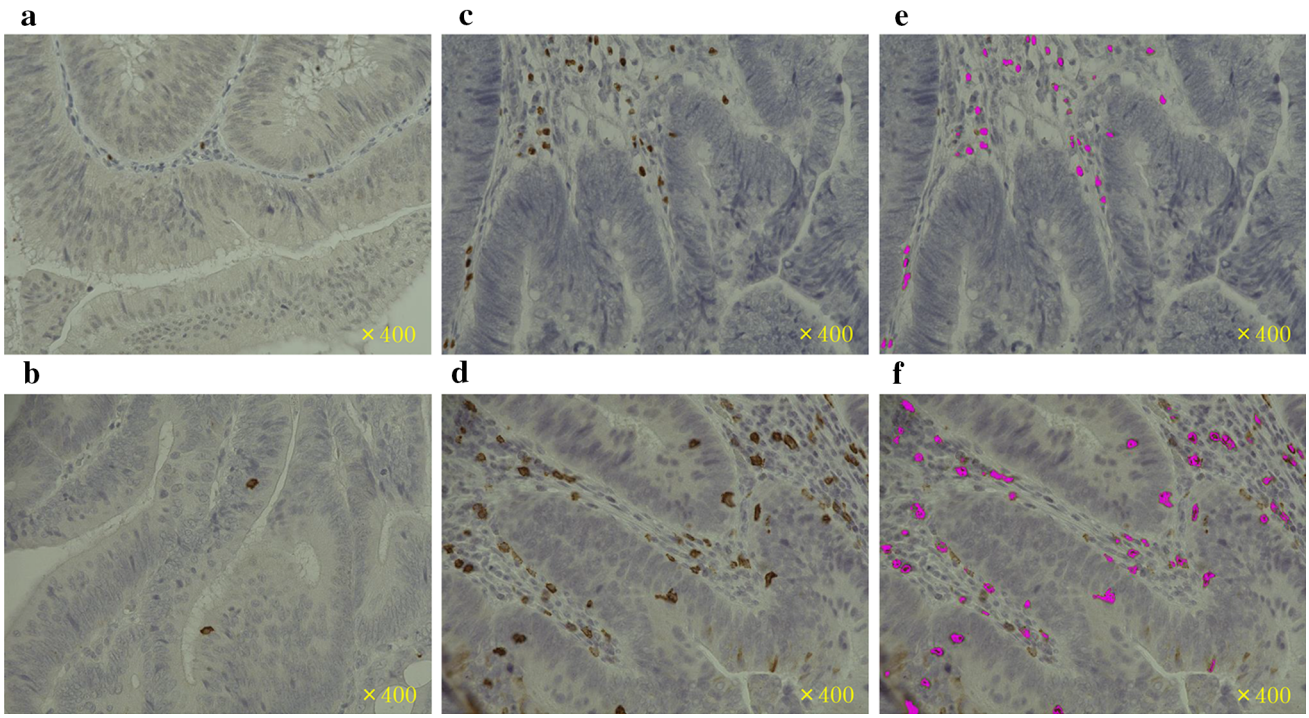


Fig. 1 Immunohistochemical staining of Foxp3- and CD8-positive TILs. **a** Foxp3-positive TILs/low density (original magnification ×400). **b** CD8-positive TILs/low density (original magnification ×400). **c** Foxp3-positive TILs/high density (original magnification ×400). **d** CD8-positive TILs/high density (original magnification ×400). **e**, **f** Intratumoral densities of positively stained cells were measured using an automatic image analysis system

T classification ($P = 0.0004$), recurrence ($P = 0.0016$), and TNM stage ($P = 0.0091$).

Associations Between TILs, MSI, and CEA as Tumor Factors and Clinicopathological Features

We analyzed the correlations between TILs, MSI, and CEA as tumor factors and clinicopathological features (Table 3). A low CD8-positive T cell count was significantly associated with venous invasion ($P = 0.0185$) and recurrence ($P = 0.0084$). However, Foxp3-positive T cell was not associated with clinicopathological features. MSI was significantly associated with undifferentiated histology ($P = 0.022$). High CEA levels were significantly associated with age ($P = 0.0488$), T classification ($P < 0.0001$), lymphatic invasion ($P = 0.0133$), venous invasion ($P = 0.0234$), recurrence ($P = 0.0015$), and TNM stage ($P = 0.0006$).

Associations Between MSI and TILs

We also investigated the associations between MSI and TILs. There was a trend toward more infiltrating Foxp3-positive T cells in patients with MSI, though the association was not significant (103 cells in MSI vs. 74 cells in MSS; $P = 0.0585$) (Fig. 2a), and significantly more infiltrating CD8-positive T cells were detected in patients with MSI (130 cells in MSI vs. 73 cells in MSS; $P = 0.0001$) (Fig. 2b).

Few Infiltrating CD8-Positive T Cells and High CRP Levels Are Independent Predictors of Recurrence in CRC Patients Treated with Curative Surgery

We analyzed DFS according to SIR status. Kaplan–Meier analysis showed that patients with high NLR, PLR, and CRP as host factors had significantly poorer DFS than patients with lower levels ($P = 0.0232$, $P = 0.0233$, $P = 0.0017$, respectively) (Fig. 3a–c). We also investigated DFS according to tumor status, including TILs, MSI, and preoperative CEA. Patients with fewer infiltrating Foxp3-positive T cells had poorer DFS than those with more infiltration, though the difference was not significant ($P = 0.2568$) (Fig. 3d), while patients with fewer infiltrating CD8-positive T cells had significantly poorer DFS than those with more infiltration ($P = 0.0028$) (Fig. 3e). In contrast, there was no association between MSI and DFS (Fig. 3f). High preoperative CEA levels were significantly associated with early recurrence ($P = 0.0010$) (Fig. 3g).

Table 2 Correlations between SIR status and clinicopathological features in CRC patients treated with curative surgery

Variables	*n*	NLR		*P*	*n*	PLR		*P*	*n*	CRP (mg/dl)		*P*
		High ($n = 53$)	Low ($n = 99$)			High ($n = 84$)	Low ($n = 68$)			High ($n = 33$)	Low ($n = 119$)	
Age (year)												
≤67	81	26	55	0.4441	81	42	39	0.3663	77	14	63	0.285
>67	71	27	44		71	42	29		75	19	56	
Gender												
Male	87	30	57	0.9081	87	43	44	0.094	88	23	65	0.1207
Female	65	23	42		65	41	24		64	10	54	
T classification												
T1, T2	62	17	45	0.1097	62	35	27	0.8068	64	5	59	0.0004
T3, T4	90	36	54		90	49	41		88	28	60	
Lymph node metastasis												
Present	53	21	32	0.3682	53	32	21	0.3535	53	14	39	0.3033
Absent	99	32	67		99	52	47		99	19	80	
Histology												
Undifferentiated	8	5	3	0.1055	8	7	1	0.0656	6	3	3	0.096
Differentiated	140	48	92		140	76	64		142	30	112	
Lymphatic invasion												
Present	101	41	60	0.0371	101	59	42	0.2713	100	25	75	0.1725
Absent	51	12	39		51	25	26		52	8	44	
Venous invasion												
Present	38	16	22	0.2797	38	20	18	0.7064	38	9	29	0.7333
Absent	114	37	77		114	64	50		114	24	90	
Recurrence												
Present	28	16	12	0.0062	28	22	6	0.006	27	12	15	0.0016
Absent	124	37	87		124	62	62		125	21	104	
Stage												
I	45	11	34	0.176	45	23	22	0.3414	47	3	44	0.0091
II	55	20	35		55	28	27		53	15	38	
III	52	22	30		52	33	19		52	15	37	

The median age was 67 years

We also performed univariate analysis to detect important factors associated with DFS. T3/4 classification ($P < 0.0001$), lymphatic node metastasis ($P = 0.0028$), poor differentiation/mucinous ($P = 0.0120$), lymphatic invasion ($P = 0.0307$), venous invasion ($P = 0.0002$), high CEA levels ($P = 0.0010$), high CRP levels ($P = 0.0044$), high NLR ($P = 0.0266$), high PLR ($P = 0.0182$), and few infiltrating CD8-positive T cells ($P = 0.0026$) were all significant predictive factors for poor DFS (Table 4). Multivariate analysis of these factors identified few infiltrating CD8-positive T cells (hazard ratio (HR) = 4.3, $P = 0.0059$) as a tumor factor and high CRP (HR = 3.07, $P = 0.0145$) as a host factor, as independent predictive markers for recurrence in CRC patients treated with curative surgery (Table 4).

Combination of Few Infiltrating CD8-Positive T Cells and High CRP Levels Increase the Predictive Accuracy for Early Recurrence in CRC Patients Treated with Curative Surgery

We assessed the effect of combining independent predictive factors (preoperative CRP and intratumoral CD8 T cells) on the predictive accuracy for early recurrence in CRC patients treated with curative surgery. We divided the patients into three groups based on the following scores: high CRP, +1; low CRP, 0; few infiltrating CD8-positive T cells, +1; more infiltrating CD8-positive T cells, 0. Sixteen patients had a score of 2, 77 had a score of 1, and 59 had a score of 0. Kaplan–Meier analysis showed that patients with a score of 2 had significantly poorer DFS than the

Table 3 Correlations between tumor factors including TILs, MSI, and CEA and clinicopathological features in CRC patients treated with curative surgery

Variables	*n*	FOXP3+ T cell		*P*	*n*	CD8+ T cell		*P*	*n*	MSI		*P*	*n*	CEA (ng/μl)		*P*
		High ($n = 77$)	Low ($n = 80$)			High ($n = 78$)	Low ($n = 79$)			High ($n = 9$)	Low ($n = 142$)			High ($n = 72$)	Low ($n = 85$)	
Age (year)																
≤67	81	44	37	0.1722	81	44	37	0.23	77	4	73	0.6853	81	31	50	0.0488
>67	76	33	43		76	34	42		74	5	69		76	41	35	
Gender																
Male	90	43	47	0.7129	90	47	43	0.4605	87	3	84	0.1285	90	43	47	0.5762
Female	67	34	33		67	31	36		64	6	58		67	29	38	
T classification																
T1, T2	65	30	35	0.5425	65	37	28	0.1272	61	3	58	0.6561	65	17	48	<0.0001
T3, T4	92	47	45		92	41	51		90	6	84		92	55	37	
Lymph node metastasis																
Present	55	30	25	0.3113	55	22	33	0.0748	53	2	51	0.4039	55	30	25	0.1088
Absent	102	47	55		102	56	46		98	7	91		102	42	60	
Histology																
Undifferentiated	8	3	5	0.4561	8	5	3	0.4793	8	2	6	0.022	8	6	2	0.0881
Differentiated	145	74	71		145	72	73		139	7	132		145	64	81	
Lymphatic invasion																
Present	104	51	53	0.9983	104	48	56	0.2156	99	5	94	0.5147	104	55	49	0.0133
Absent	53	26	27		53	30	23		52	4	48		53	17	36	
Venous invasion																
Present	39	17	22	0.4319	39	13	26	0.0185	38	1	37	0.3164	39	24	15	0.0234
Absent	118	60	58		118	65	53		113	8	105		118	48	70	
Recurrence																
Present	29	11	18	0.1849	29	8	21	0.0084	28	2	26	0.7696	29	21	8	0.0015
Absent	128	66	62		128	70	58		123	7	116		128	51	77	
Stage																
I	48	22	26	0.692	48	29	19	0.0925	46	2	44	0.4129	48	11	37	0.0006
II	55	26	29		55	28	27		53	5	48		55	30	25	
III	54	29	25		51	21	33		52	2	50		54	31	23	

The median age was 67 years

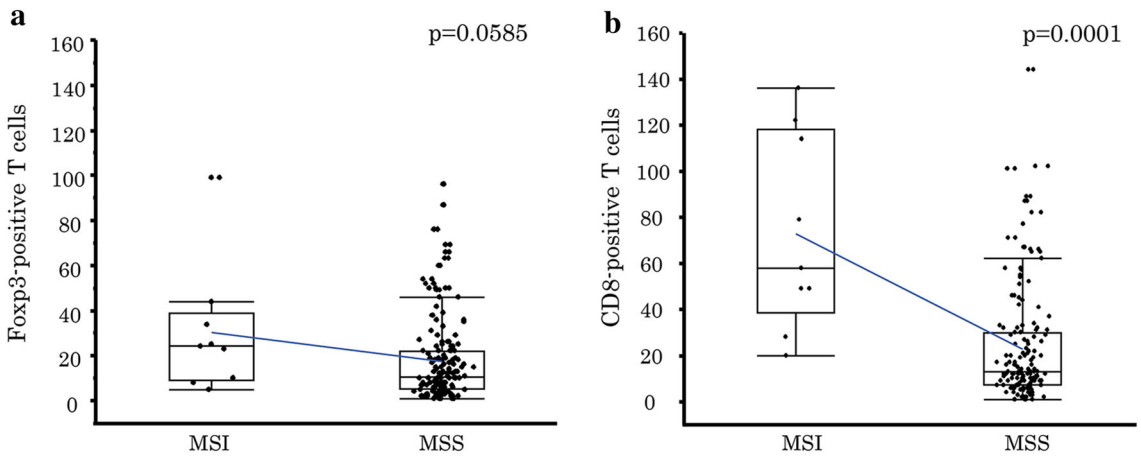


Fig. 2 Numbers of TILs in MSI and MSS tumors. **a** Numbers of Foxp3-positive T cells in MSI and MSS tumors. **b** Numbers of CD8-positive T cells in MSI and MSS tumors

other two groups ($P < 0.0001$) (Fig. 3h) and that the risk of recurrence was increased when both independent factors were combined (HR = 5.26).

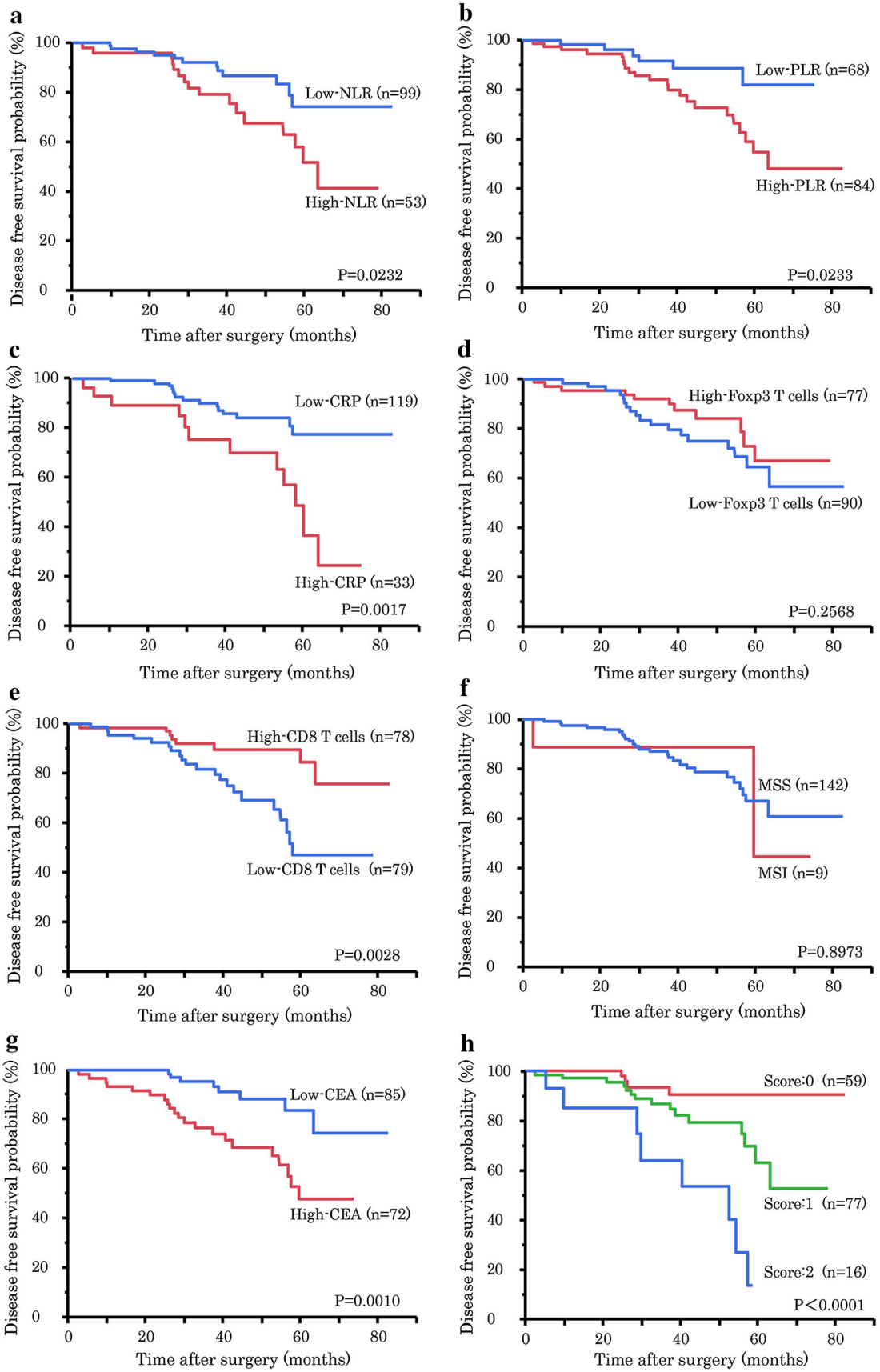
Discussion

The UICC staging system provides the most reliable indication of prognosis and is useful for discriminating between patients with early-stage disease and those with advanced disease. However, its ability to predict prognosis in patients with intermediate levels of tumor invasion (stage II or stage III) is less accurate. Sensitive biomarkers are therefore needed to allow the targeting of postoperative adjuvant chemotherapy to those patients at highest risk of early relapse, with a resultant improvement in survival. This study provides the first comprehensive analysis for identifying predictive biomarkers based on both the host immune response and the local tumor molecular status. We showed that high SIR status, including NLR, PLR, and CRP levels as host factors, and few intratumoral CD8-positive T cells and high CEA levels as tumor factors, was significantly associated with early recurrence in patients with surgically resected CRC. In addition, multivariate analysis revealed that high CRP levels and few intratumoral CD8-positive T cells were independent predictors of recurrence. Furthermore, the combination of both host and tumor factors increased the predictive accuracy for determining recurrence risk in CRC patients treated with curative surgery. This new predictive test, which is based on the widely available results of CRP assays, combined with CD8 immunohistochemistry that is feasible in most laboratories, represents a step forward for accurately identifying curatively treated CRC patients at risk of recurrence in the clinical setting.

Cancer progression depends on complex interactions between the tumor, its microenvironment, and the host immune response. Inflammation has been implicated in the pathogenesis of many adult malignancies and is now recognized as a hallmark of tumorigenesis [18]. Park et al. [19] showed that the host inflammatory response to CRC influenced disease recurrence and survival via the SIR. However, the tumor may also encourage the inflow of inflammatory lymphocytes, resulting in cell destruction within the surrounding tissue, generating a more widespread, nonspecific inflammatory response [20]. Several studies have shown an association between the local inflammatory response, indicated by increased T cell tumor infiltration, and improved prognosis and recurrence in CRC [8, 21, 22]. It is therefore important to carry out a comprehensive analysis of biomarkers of early recurrence based on both systemic and local inflammatory factors in patients with CRC treated with curative intent.

SIR status, reflected by CRP, NLR, and PLR, is thought to be a surrogate indicator of the host immune response to tumors and has been shown to act as a biomarker of outcome in a variety of malignancies [23–25].

CRP is an essential acute-phase reactant that acts as a surveillance molecule for activation of the adaptive immune system. It is synthesized in hepatocytes and is upregulated by cytokines such as interleukin-6 and tumor necrosis factor-α [26]. Several studies have demonstrated associations between high CRP levels and increased risk of early recurrence and poor outcome in CRC patients treated with curative surgery [27–29]. However, although CRP represents an excellent biomarker for oncological outcome, we should be aware of its limitations; CRP levels cannot discriminate between patients with several cancers, including CRC, and those with inflammatory conditions such as inflammatory bowel disease, collagen disease, rheumatic disease, and cardiovascular disease [30].


a
Disease free survival probability (%)
Low-NLR (n=99)
High-NLR (n=53)
P=0.0232
Time after surgery (months)
b
Low-PLR (n=68)
High-PLR (n=84)
P=0.0233
c
Low-CRP (n=119)
High-CRP (n=33)
P=0.0017
d
High-Foxp3 T cells (n=77)
Low-Foxp3 T cells (n=90)
P=0.2568
e
High-CD8 T cells (n=78)
Low-CD8 T cells (n=79)
P=0.0028
f
MSS (n=142)
MSI (n=9)
P=0.8973
g
Low-CEA (n=85)
High-CEA (n=72)
P=0.0010
h
Score:0 (n=59)
Score:1 (n=77)
Score:2 (n=16)
P<0.0001

◀**Fig. 3** Kaplan–Meier curves for DFS classified according to SIR status, TILs, MSI status, and CEA levels in CRC patients treated with curative surgery. **a** DFS according to NLR. **b** DFS according to PLR. **c** DFS according to CRP levels. **d** DFS according to density of Foxp3-positive T cells. **e** DFS according to density of CD8-positive T cells. **f** DFS according to MSI status. **g** DFS according to CEA levels. **h** DFS according to combined score of few infiltrating CD8-positive T cells and high CRP levels

Patients with high NLRs also have the balance tipped in favor of pro-tumor inflammatory status, meaning that a high NLR is a predictor of recurrence in CRC patients treated with curative surgery [31, 32]. Both thrombocytosis and lymphocytopenia have also been shown to correlate with the degree of host systemic inflammation, and the ratio of these factors (PLR) might reflect a novel inflammatory factor incorporating these two individual hematologic factors [33]. Szkandera et al. [34] found that PLR was a predictive marker of recurrence in CRC patients treated with curative surgery. Collectively, SIR status thus comprises potential biomarkers for predicting early recurrence in CRC, though no previous studies have compared the predictive values of CRP level, NLR, and PLR in terms of CRC recurrence. However, the current multivariate analysis demonstrated that high CRP level was the best component of SIR for predicting recurrence in CRC patients treated with curative surgery.

CRCs are generally immunogenic and often infiltrated by T cells [35], suggesting that clinical outcome and recurrence may depend on the status of the local adaptive immune response. Upon antigenic stimulation, CD8-positive T cells differentiate into effector cells that kill tumor cells by releasing toxic granules such as granzyme B and perforins [36, 37]. A high density of TILs in CRC tissue is associated with increased tumor cell apoptosis [38]. However, regulatory T cells expressing the Foxp3 transcription factor may block the adaptive immune response [39]. Foxp3-positive T cells can suppress host-mediated antitumor immunity and tumor-specific cytotoxicity, suggesting regulatory T cell deletion as a potential therapeutic strategy [40]. Recent evidence has also indicated that signaling by the T cell chemoattractant CCL5 can recruit Foxp3-positive T cells to tumors and enhance their ability to kill CD8-positive T cells, thereby providing a mechanism of immune escape [41]. A high density of infiltrating Foxp3-positive T cells has thus been shown to be associated with an adverse prognosis in some tumor types [39, 42]. However, several reports have indicated favorable impact of infiltrating Foxp-3 T cells in other tumors, including CRC [43–47], and the role of Foxp3-positive T cells thus remains controversial. The results of the present study revealed that, as components of the local inflammatory response, infiltrating CD8-negative T lymphocytes, but not Foxp3-positive T lymphocytes, were significantly associated with poor DFS and were an independent predictive factor for early recurrence in CRC patients treated with curative surgery.

The majority of hereditary nonpolyposis CRC tumors are characterized by MSI, while only 10–15 % of sporadic CRC cases display MSI, predominantly caused by epigenetic hypermethylation of the MLH1 mismatch repair gene.

Table 4 Univariate and multivariate analyses of DFS in CRC patients treated with curative surgery

Variables	Univariate analysis		*P*	Multivariate analysis		*P*
	HR	95 % CI		HR	95 % CI	
Age (>67 vs. ≤67 years)	1.4	0.67–2.92	0.3692	–	–	–
Gender (female vs. male)	1.5	0.72–3.17	0.2782	–	–	–
T classification (T3, 4 vs. T1, 2)	9.56	2.86–59.27	<0.0001	4.25	1.10–28.18	0.0349
Lymphatic node metastasis (positive vs. absent)	3.08	1.47–6.75	0.0028	2.43	1.01–6.16	0.0478
Pathology (poor or muc vs. mod/well differentiated)	4.31	1.44–10.56	0.012	–	–	–
Lymphatic invasion (present vs. absent)	3.13	1.1–13.16	0.0307	–	–	–
Venous invasion (present vs. absent)	4.07	1.96–8.63	0.0002	–	–	–
CEA (>5 vs. ≤5 ng/μl)	3.61	1.66–8.69	0.001	–	–	–
CRP (>0.5 vs. ≤0.5 mg/dl)	3.18	1.46–6.79	0.0044	3.07	1.25–7.55	0.0145
NLR (>3 vs. ≤3)	2.33	1.10–5.04	0.0266	–	–	–
PLR (>150 vs. ≤150)	2.73	1.18–7.41	0.0182	–	–	–
FOXP3+ T cell (low vs. high)	1.54	0.74–3.37	0.254	–	–	–
CD8+ T cell (low vs. high)	3.27	1.50–7.93	0.0026	4.3	1.50–14.14	0.0059

Median age was 67 years

Median numbers of Foxp3- and CD8-positive T cells were 11 and 15

HR hazard ratio, *CI* confidence interval

A previous systematic review of the prognostic and predictive values of MSI status found that MSI was associated with better overall survival and DFS [48]. Closer analysis of the clinical data suggested no benefit from 5-fluorouracil treatment in patients with MSI CRC, supported by the fact that patients with MSI tumors have a better prognosis than those with MSS [49]. The better outcome in patients with MSI may be partly attributable to the generation of neoantigens as a result of mutational frameshifts within the coding regions of specific genes, as a consequence of inactivation of DNA mismatch repair in CRC epithelial cells. This attracts specific immune cells that help to contain the tumor and limit metastasis [50, 51]. Several previous studies in CRC patients have demonstrated an association between MSI and increased intraepithelial CD8-positive T cells compared with patients with MSS [51–53]. In addition, the density of intratumoral Foxp3-positive T cells is significantly higher in MSI compared with MSS CRC, paralleling the enhanced number of CD8-positive cells [54]. Our current study found no significant relationship between MSI status and recurrence, possibly because of the small sample size; however, we did demonstrate a significant association between TILs and MSI status in CRC.

In conclusion, this study demonstrates that recurrence in CRC patients treated with curative surgery is affected by both systemic and local inflammatory statuses. Host inflammatory status, represented by preoperative serum CRP levels, and local tumor inflammatory status, represented by CD8-positive infiltrating T cells, were independent predictive factors for recurrence in curatively resected CRC patients. The combination of CRP level and intratumoral CD8-positive T cells could thus be used to identify CRC patients who require adjuvant chemotherapy after curative surgery.

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