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## Article

# Expression of Immune-Related and Inflammatory Markers and Their Prognostic Impact in Colorectal Cancer Patients

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**Abstract:** The tumor microenvironment of colorectal cancer (CRC) is heterogenous; thus, it is likely that multiple immune-related and inflammatory markers are simultaneously expressed in the tumor. The aim of this study was to identify immune-related and inflammatory markers expressed in freshly frozen CRC tissues and to investigate whether they are related to the clinicopathological features and prognosis of CRC. Seventy patients with CRC who underwent curative surgical resection between December 2014 and January 2017 were included in this study. Tissue samples were obtained from tumor and non-tumor areas in the patients' colons. The concentrations of immune-related markers (APRIL/TNFSF13, BAFF, LAG-3, PD-1, PD-L1, and CTLA-4) and inflammatory markers (CHIT, MMP-3, osteocalcin, pentraxin-3, sTNF-R1, and sTNF-R2) in the samples were measured using the Bio-plex Multiplex Immunoassay system. The concentrations of APRIL/TNFSF13, BAFF, and MMP-3 in the samples were significantly high; thus, we conducted analyses based on the cutoff values for these three markers. The high APRIL/TNFSH13 expression group showed a significantly higher rate of metastatic lesions than the low expression group, whereas the high MMP-3 expression group had higher CEA levels, more lymph node metastases, and more advanced disease stages than the low expression group. The five-year disease-free survival of the high MMP-3 expression group was significantly shorter than that of the low expression group (65.1% vs. 90.2%,  $p=0.033$ ). This study provides evidence that the APRIL/TNFSF13, BAFF, and MMP-3 pathway is overexpressed CRC tissues and is associated with unfavorable clinicopathological features and poor prognosis in CRC patients. These markers could serve as diagnostic or prognostic biomarkers for CRC.

**Keywords:** colorectal neoplasm; biomarkers; Bioplex; immune checkpoint proteins

## 1. Introduction

In recent years, immunotherapy using immune checkpoint inhibitors (ICIs) has been receiving increasing attention as an effective therapeutic option for the treatment of advanced, metastatic, and recurrent colorectal cancer (CRC) [1,2]. Immunotherapy using ICIs can reverse tumor immune escape by suppressing immune checkpoint pathways. Representative immune checkpoint target molecules include programmed cell death protein 1 (PD-1), programmed death ligand 1 (PD-L1), and cytotoxic

T-lymphocyte-associated protein 4 (CTLA-4). The efficacy of ICI therapy for the treatment of various malignant tumors, such as melanoma, lung cancer, and prostate cancer, has been demonstrated [3–6]. For the treatment of CRC, ICI therapy has been partially proven to be effective and is used in clinical settings. Currently, ICIs are used on a limited basis for the treatment of patients with stage IV metastatic CRC and have been reported to have some effect in patients with the DNA mismatch repair-deficient (dMMR)/microsatellite instability-high (MSI-H) genetic phenotype. However, some patients do not respond to immunotherapy; therefore, its effect has not been clearly identified [7,8]. Additionally, ICIs are ineffective for the treatment of tumors with specific genetic phenotypes, such as the mismatch-repair-proficient (pMMR) and microsatellite-stable (MSS) phenotypes, or tumors with low levels of microsatellite instability (MSI-L) [9]. As tumors with the MSI-H phenotype account for only 5–15% of all CRC cases, development of immunotherapy for pMMR/MSS tumors with few genetic variants is urgently needed [10]. To do so, it is essential to discover not only MSI status but also novel immune-related markers that can predict the efficacy of immunotherapy for cancer treatment. Overexpression of various immune-related markers in tumors has been reported in several studies. However, to the best of our knowledge, there has been no clear conclusion that these markers are related to clinicopathological features and long-term outcomes [11–13].

Given the heterogeneous nature of the tumor microenvironment (TME), it is likely that multiple immune-related and inflammatory markers are simultaneously expressed in tumors, contributing to the complexity of tumor-immune interactions. Understanding the expression patterns and effects of these markers in the TME is critical for developing effective cancer therapies that harness the power of the immune system to fight cancer [14].

Therefore, the aim of this study was to identify immune-related and inflammatory markers expressed in CRC tissue that had been stored as freshly frozen samples and to investigate whether they are related to clinicopathological features and prognosis. In particular, rather than use a traditional enzyme-linked immunosorbent assay (ELISA), we attempted to efficiently identify these markers using the Bio-plex Multiplex immunoassay, which has not been used in previous studies.

2. Results

2.1. Patient Characteristics

Seventy patients were included in this study. The mean age of the patients was 69.6 years and 38 (54.3%) of them were male. Nineteen (27.1%) and 27 (25.7%) patients had right- and left-sided colon cancer, respectively, whereas 24 (34.3%) had rectal cancer. Fifty-five (78.6%) patients underwent minimally invasive surgery, including laparoscopic or robotic surgery. Thirty-one (44.3%) patients had stage III disease, which was the most common disease stage, and the average number of metastatic lymph nodes recorded was 2.2. Sixty-three (91%) patients had MSS (91%), four had MSI-H (5%), and three (4%) had unverifiable MSI information. Forty-six (65.7%) patients received postoperative chemotherapy, whereas only one (1.4%) patient received radiation treatment. During the follow-up period, 17 (24.3%) patients showed cancer recurrence, and five (7.1%) patients died. The patient characteristics are summarized in Table 1.

Table 1. Baseline characteristics.

	Number of patients (%) (n=70)
Age, mean ± SD	69.6 ± 10.8
Gender	
Male	38 (54.3)
Female	32 (45.7)
Body mass index, mean ± SD	23.4 ± 3.5
ASA score	
II	35 (50.0)
III	35 (50.0)

<b>Medical history</b>	
None	19 (27.1)
One	18 (25.7)
Two or more	33 (47.1)
<b>Tumor location</b>	
Right	19 (27.1)
Left	27 (38.6)
Rectum	24 (34.3)
<b>CEA</b>	
<5	45 (64.3)
≥5	25 (35.7)
<b>Operation method</b>	
Open	15 (21.4)
MIS	55 (78.6)
<b>T stage</b>	
Tis	1 (1.4)
3	53 (75.7)
4	16 (22.9)
<b>N stage</b>	
0	28 (40.0)
1	28 (40.0)
2	14 (20.0)
<b>M stage</b>	
0	57 (81.4)
1	13 (18.6)
<b>TNM stage</b>	
0	1 (1.4)
2	25 (35.7)
3	31 (44.3)
4	13 (18.6)
<b>Metastatic lymph node, mean ± SD</b>	2.2 ± 3.6
<b>Harvested lymph node, mean ± SD</b>	24.8 ± 11.1
<b>Tumor differentiation</b>	
Well differentiation	13 (18.8)
Moderate differentiation	53 (76.8)
Poorly differentiation	1 (1.4)
Mucinous adenocarcinoma	2 (2.9)
<b>Tumor size (cm), mean ± SD</b>	5.0 ± 2.0
<b>Lymphatic invasion</b>	
Negative	38 (54.3)
Positive	32 (45.7)
<b>Venous invasion</b>	
Negative	63 (90.0)
Positive	7 (10.0)
<b>Perineural invasion</b>	
Negative	50 (71.4)
Positive	20 (28.6)
<b>EGFR</b>	
Negative	5 (7.6)
Positive	61 (92.4)
<b>MSI</b>	
MSS	63 (94.0)

MSI-H	4 (6.0)
<b>KRAS</b>	
Wild	39 (58.2)
Mutant	28 (41.8)
<b>NRAS</b>	
Wild	47 (92.2)
Mutant	2 (3.9)
<b>BRAF</b>	
Wild	62 (95.4)
Mutant	3 (4.6)
<b>Laboratory markers, mean ± SD</b>	
WBC (10 <sup>3</sup> /μL)	7.6 ± 3.1
Hb (g/dL)	11.9 ± 2.4
PLT (10 <sup>3</sup> /μL)	288.0 ± 100.3
Neutrophil count (10 <sup>3</sup> /μL)	5.4 ± 3.0
Lymphocyte count (10 <sup>3</sup> /μL)	1.5 ± 0.6
NLR	4.5 ± 4.9
C-reactive protein (mg/dL)	1.7 ± 2.7
Albumin (g/dL)	3.8 ± 0.6
<b>Chemotherapy</b>	
No	24 (34.3)
Yes	46 (65.7)
<b>Radiotherapy</b>	
No	69 (98.6)
Yes	1 (1.4)
<b>Recurrence</b>	
No	42 (60.0)
Yes	17 (24.3)
<b>Death</b>	
No	45 (64.3)
Yes	5 (7.1)

SD, standard deviation; ASA, American Society of Anesthesiologists; CEA, Carcinoembryonic antigen; MIS, minimally invasive surgery; EGFR, Epidermal growth factor receptor; MSI, Microsatellite instability; WBC, white blood cell; Hb, hemoglobin; PLT, platelet; NLR, neutrophil-lymphocyte ratio.

2.2. Immune-Related and Inflammatory Markers in Tumor Tissues

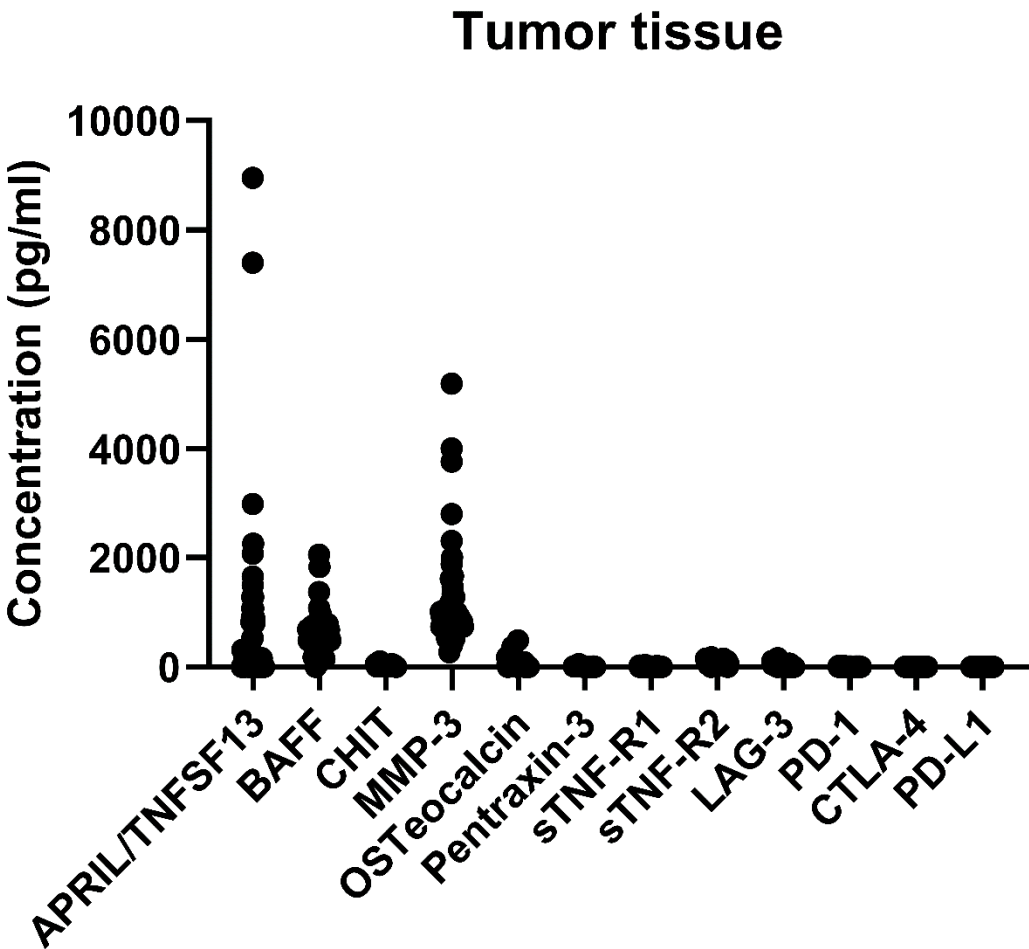
Table 2 shows the results of the multiplex immunoassay for immune-related and inflammatory markers in tumor tissues. Among the various markers identified in the tumor tissues, a proliferation-inducing ligand (APRIL/TNFSF13), B-cell activating factor (BAFF), and matrix metalloproteinase-3 (MMP-3) showed significantly high concentrations. Scatter plots that show the distribution of the immune marker levels are outlined in Figure 1.

Table 2. Immune-related and inflammatory markers in tumor tissues.

	Median [IQR]	Range
APRIL/TNFSF13	166.02 [0, 806.4]	0~8954.58
BAFF	485.6 [355.3, 664.0]	0~2053.2
CHIT	21.3 [10.24, 31.24]	0~101.19
MMP-3	905.1 [736.2, 1106.9]	270.5~5198.8
Osteocalcin	16.33 [2.37, 41.34]	0~487.48
Pentraxin-3	8.98 [7.41, 12.19]	3.23~57.43
sTNF-R1	6.67 [5.43, 7.87]	2.28~36.20

sTNF-R2	60.99 [35.78, 106.93]	0~177.87
LAG-3	0 [0, 11.46]	0~164.96
PD-1	5.3 [5.30, 10.84]	0~21.77
PD-L1	0 [0, 0.43]	0~4.49
CTLA-4	0 [0, 0]	0~3.1

IQR, interquartile range; APRIL/TNFSF13, A proliferation-inducing ligand/ Tumor necrosis factor lsuperfamily member 13; BAFF, B lymphocyte activating factor; CHIT, Chitinase 1; MMP-3, matrix metallopeptidase 3; sTNF-R, Soluble Tumor Necrosis Factor Receptor Type; LAG-3, Lymphocyte activation gene-3; PD-1, Programmed cell death protein 1; PD-L1, Programmed death-ligand 1; CTLA-4, Cytotoxic T-Lymphocyte Associated Protein 4



**Figure 1.** Levels of immune-related and inflammatory markers in tumor tissues. Scatter plots showing the distribution of levels of twelve markers. APRIL/TNFSF13, A proliferation-inducing ligand/ Tumor necrosis factor lsuperfamily member 13; BAFF, B lymphocyte activating factor; CHIT, Chitinase 1; MMP-3, matrix metallopeptidase 3; sTNF-R, Soluble Tumor Necrosis Factor Receptor Type; LAG-3, Lymphocyte activation gene-3; PD-1, Programmed cell death protein 1; PD-L1, Programmed death-ligand 1; CTLA-4, Cytotoxic T-Lymphocyte Associated Protein 4.

*2.3. Relationships between Immune-Related and Inflammatory Markers and Clinicopathologic Features*

We set cut-off values for each of the three above-mentioned immune-related and inflammatory markers significantly high in tumor tissues and analyzed the differences in clinicopathological features between the low and high expression groups. APRIL/TNFSH13 and BAFF were grouped based on quartile 3 values of 806.4 and 664.0, respectively, whereas MMP-3 was grouped based on the quartile 1 value of 736.2. There were no differences in basic patient characteristics, such as age, body mass index, American Society of Anesthesiologists score, and medical history, between the subgroups. The high APRIL/TNFSH13 expression group showed a significantly higher rate of



metastatic lesions (ML) than the low APRIL/TNFSF13 expression group (11.8% vs. 36.8%,  $P=0.03$ ); therefore, the proportion of patients with stage IV disease was high. Additionally, neutrophil-lymphocyte ratio, one of the serologic markers, was significantly higher in the APRIL/TNFSF13 high expression group than the low APRIL/TNFSF13 expression group (4.1 vs. 2.7, respectively,  $p=0.04$ ). The high BAFF expression group had a significantly higher five-year recurrence rate than the low BAFF expression group (12 [23.1%] vs. 5 [27.8%],  $p=0.03$ ). Compared to the low expression group, the MMP-3 high expression group had more patients with a CEA level  $\geq 5$  (44.7% vs. 17.4%,  $p=0.04$ ). In addition, the high MMP-3 expression group had a higher proportion of patients with T4 disease than the low MMP-3 expression group (15 [31.9%] vs. 1 [4.3%],  $p=0.01$ ). Furthermore, the mean number of metastatic lymph nodes in the high MMP-3 expression group was 2.7, which was significantly higher than that of the low MMP-3 expression group, 1.1 ( $p=0.02$ ), and there were many patients in advanced stages disease, such as stage 3 and 4 disease ( $p=0.04$ ) (Table 3).

**Table 3.** Correlation of immunologic markers with clinicopathologic features.

	APRIL/TNFSF13 (806.4)			BAFF (664.0)			MMP-3 (736.2)		
	Low (N=51)	High (N=19)	$p$	Low (N=52)	High (N=18)	$p$	Low (N=23)	High (N=47)	$p$
<b>Age, mean <math>\pm</math> SD</b>	69.8 $\pm$ 10.7	69.2 $\pm$ 11.0	0.82	69.4 $\pm$ 11.1	70.3 $\pm$ 10.1	0.74	70.7 $\pm$ 11.4	69.1 $\pm$ 10.5	0.59
<b>Gender</b>									
Male	27 (52.9)	11 (57.9)	0.92	25 (48.1)	13 (72.2)	0.13	15 (65.2)	23 (48.9)	0.30
Female	24 (47.1)	8 (42.1)		27 (51.9)	5 (27.8)		8 (34.8)	24 (51.1)	
<b>BMI</b>	23.5 $\pm$ 3.7	23.1 $\pm$ 3.1	0.70	23.4 $\pm$ 3.7	23.4 $\pm$ 3.0	0.96	23.3 $\pm$ 3.6	23.4 $\pm$ 3.5	0.90
<b>ASA score</b>									
II	28 (54.9)	7 (36.8)	0.28	29 (55.8)	6 (33.3)	0.17	13 (56.5)	22 (46.8)	0.61
III	23 (45.1)	12 (63.2)		23 (44.2)	12 (66.7)		10 (43.5)	25 (53.2)	
<b>Medical history</b>									
None	14 (27.5)	5 (26.3)	0.95*	12 (23.1)	7 (38.9)	0.22*	6 (26.1)	13 (27.7)	0.81
One	14 (27.5)	4 (21.1)		16 (30.8)	2 (11.1)		5 (21.7)	13 (27.7)	
T or more	23 (45.1)	10 (52.6)		24 (46.2)	9 (50.0)		12 (52.2)	21 (44.7)	
<b>Tumor location</b>									
Right	15 (29.4)	4 (21.1)	0.65	17 (32.7)	2 (11.1)	0.13*	8 (34.8)	11 (23.4)	0.51
Left	20 (39.2)	7 (36.8)		20 (38.5)	7 (38.9)		7 (30.4)	20 (42.6)	
Rectum	16 (31.4)	8 (42.1)		15 (28.8)	9 (50.0)		8 (34.8)	16 (34.0)	
<b>CEA</b>									
<5	34 (66.7)	11 (57.9)	0.68	34 (65.4)	11 (61.1)	0.96	19 (82.6)	26 (55.3)	0.04
$\geq 5$	17 (33.3)	8 (42.1)		18 (34.6)	7 (38.9)		4 (17.4)	21 (44.7)	
<b>Operation method</b>									
Open	10 (19.6)	5 (26.3)	0.53*	11 (21.2)	4 (22.2)	1*	6 (26.1)	9 (19.1)	0.54*
MIS	41 (80.4)	14 (73.7)		41 (78.8)	14 (77.8)		17 (73.9)	38 (80.9)	
<b>T stage</b>									
Tis	0 (0.0)	1 (5.3)	0.29*	0 (0.0)	1 (5.6)	0.09*	0 (0.0)	1 (2.1)	0.01*
3	40 (78.4)	13 (68.4)		42 (80.8)	11 (61.1)		22 (95.7)	31 (66.0)	
4	11 (21.6)	5 (26.3)		10 (19.2)	6 (33.3)		1 (4.3)	15 (31.9)	
<b>N stage</b>									
0	22 (43.1)	6 (31.6)	0.60*	20 (38.5)	8 (44.4)	0.81*	13 (56.5)	15 (31.9)	0.15*
1	20 (39.2)	8 (42.1)		22 (42.3)	6 (33.3)		6 (26.1)	22 (46.8)	
2	9 (17.6)	5 (26.3)		10 (19.2)	4 (22.2)		4 (17.4)	10 (21.3)	

<b>M stage</b>									
0	45 (88.2)	12 (63.2)	0.03*	45 (86.5)	12 (66.7)	0.08*	21 (91.3)	36 (76.6)	0.19*
1	6 (11.8)	7 (36.8)		7 (13.5)	6 (33.3)		2 (8.7)	11 (23.4)	
<b>TNM stage</b>									
0	0 (0.0)	1 (5.3)	0.02*	0 (0.0)	1 (5.6)	0.06*	0 (0.0)	1 (2.1)	0.04*
2	21 (41.2)	4 (21.1)		19 (36.5)	6 (33.3)		13 (56.5)	12 (25.5)	
3	24 (47.1)	7 (36.8)		26 (50.0)	5 (27.8)		8 (34.8)	23 (48.9)	
4	6 (11.8)	7 (36.8)		7 (13.5)	6 (33.3)		2 (8.7)	11 (23.4)	
<b>Metastatic lymph node</b>	1.8 ± 3.3	3.2 ± 4.3	0.23	2.0 ± 3.3	2.8 ± 4.4	0.45	1.1 ± 1.7	2.7 ± 4.2	0.02
<b>Harvested lymph node</b>	24.4 ± 9.4	25.9 ± 14.9	0.68	25.3 ± 9.8	23.4 ± 14.4	0.62	27.1 ± 13.1	23.7 ± 9.9	0.27
<b>Tumor differentiation</b>									
WD	10 (19.6)	3 (16.7)	0.79*	9 (17.3)	4 (23.5)	0.63*	4 (17.4)	9 (19.6)	0.91*
MD	39 (76.5)	14 (77.8)		41 (78.8)	12 (70.6)		19 (82.6)	34 (73.9)	
PD	1 (2.0)	0 (0.0)		1 (1.9)	0 (0.0)		0 (0.0)	1 (2.2)	
Mucinous	1 (2.0)	1 (5.6)		1 (1.9)	1 (5.9)		0 (0.0)	2 (4.3)	
<b>Tumor size (cm), mean ± SD</b>	4.9 ± 2.2	5.2 ± 1.3	0.50	4.9 ± 2.2	5.1 ± 1.5	0.64	4.8 ± 2.0	5.0 ± 2.1	0.74
<b>Lymphatic invasion</b>									
Negative	28 (54.9)	10 (52.6)	1	28 (53.8)	10 (55.6)	1	11 (47.8)	27 (57.4)	0.61
Positive	23 (45.1)	9 (47.4)		24 (46.2)	8 (44.4)		12 (52.2)	20 (42.6)	
<b>Venous invasion</b>									
Negative	47 (92.2)	16 (84.2)	0.37*	47 (90.4)	16 (88.9)	1*	21 (91.3)	42 (89.4)	1*
Positive	4 (7.8)	3 (15.8)		5 (9.6)	2 (11.1)		2 (8.7)	5 (10.6)	
<b>Perineural invasion</b>									
Negative	40 (78.4)	10 (52.6)	0.06	40 (76.9)	10 (55.6)	0.15	19 (82.6)	31 (66.0)	0.24
Positive	11 (21.6)	9 (47.4)		12 (23.1)	8 (44.4)		4 (17.4)	16 (34.0)	
<b>EGFR</b>									
Negative	1 (2.1)	4 (22.2)	0.01*	1 (2.0)	4 (25.0)	0.01*	0 (0.0)	5 (11.6)	0.15*
Positive	47 (97.9)	14 (77.8)		49 (98.0)	12 (75.0)		23 (100.0)	38 (88.4)	
<b>MSI</b>									
MSS	46 (92.0)	17 (100.0)	0.56*	47 (92.2)	16 (100.0)	0.56*	21 (91.3)	42 (95.5)	0.60*
MSI-H	4 (8.0)	0 (0.0)		4 (7.8)	0 (0.0)		2 (8.7)	2 (4.5)	
<b>KRAS</b>									
Wild	27 (56.2)	12 (63.2)	0.80	29 (59.2)	10 (55.6)	1	12 (57.1)	27 (58.7)	1
Mutant	21 (43.8)	7 (36.8)		20 (40.8)	8 (44.4)		9 (42.9)	19 (41.3)	
<b>NRAS</b>									
Wild	33 (97.1)	14 (93.3)	0.52*	35 (97.2)	12 (92.3)	0.46*	16 (100.0)	31 (93.9)	1*
Mutant	1 (2.9)	1 (6.7)		1 (2.8)	1 (7.7)		0 (0.0)	2 (6.1)	
<b>BRAF</b>									
Wild	45 (95.7)	17 (94.4)	1*	45 (93.8)	17 (100.0)	0.56*	19 (95.0)	43 (95.6)	1*
Mutant	2 (4.3)	1 (5.6)		3 (6.2)	0 (0.0)		1 (5.0)	2 (4.4)	

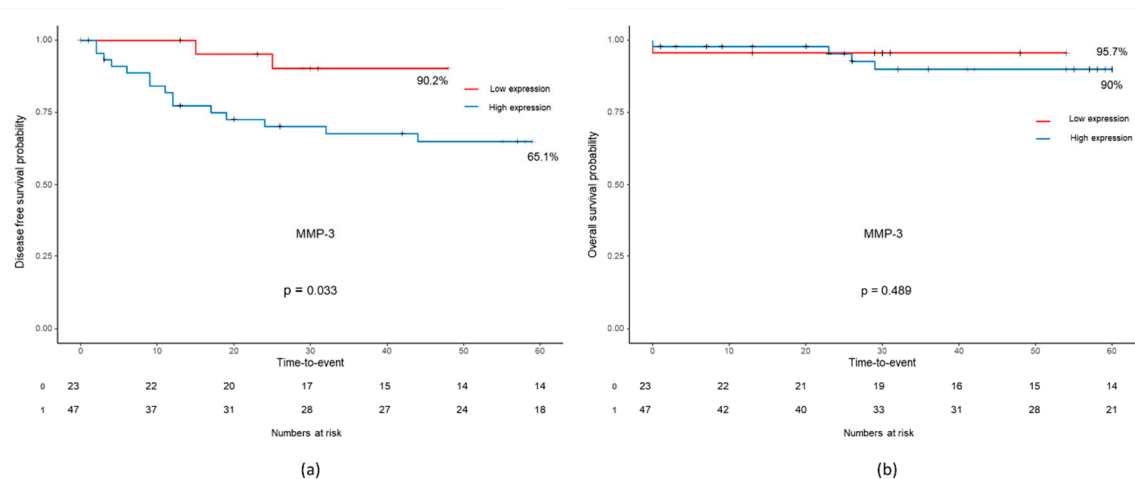


<b>Laboratory markers, median [IQR]</b>									
WBC (10 <sup>3</sup> /μL)	6.6 [5.4, 9.2]	7.1 [6.5, 8.8]	0.53	7.2 [5.5, 9.2]	6.7 [5.9, 8.9]	0.83	6.5 [4.9, 7.6]	7.2 [5.9, 9.4]	0.10
Hb (g/dL)	12.6 [10.4, 13.6]	11.1 [9.7, 12.5]	0.13	12.4 [10.2, 13.4]	12.4 [9.8, 13.8]	0.87	12.4 [10.1, 13.8]	12.3 [10.2, 13.6]	0.58
PLT (10 <sup>3</sup> /μL)	272.0 [209.5, 323.0]	253.0 [231.0, 331.0]	0.92	275.5 [212.2, 333.5]	242.0 [224.5, 294.2]	0.37	260.0 [193.0, 307.0]	259.0 [222.5, 332.5]	0.42
Neutrophil (10 <sup>3</sup> /μL)	4.7 [3.0, 6.4]	5.1 [4.4, 7.1]	0.17	4.7 [3.1, 6.9]	4.9 [4.3, 6.8]	0.38	3.6 [3.0, 5.8]	4.9 [3.7, 7.1]	0.08
Lymphocyte (10 <sup>3</sup> /μL)	1.6 [1.3, 1.9]	1.3 [1.0, 1.8]	0.20	1.5 [1.2, 1.9]	1.3 [1.0, 1.8]	0.49	1.4 [1.2, 1.7]	1.6 [1.1, 1.9]	0.48
NLR	2.7 [2.1, 4.2]	4.1 [2.7, 6.0]	0.04	2.7 [2.2, 4.4]	3.9 [2.7, 5.4]	0.15	2.5 [2.1, 4.1]	3.6 [2.5, 5.2]	0.16
CRP (mg/dL)	0.4 [0.3, 1.6]	1.0 [0.3, 2.3]	0.25	0.5 [0.3, 1.8]	0.7 [0.3, 1.3]	0.97	0.6 [0.3, 1.3]	0.7 [0.3, 1.8]	0.99
Albumin (g/dL)	3.9 [3.6, 4.3]	3.7 [3.2, 4.0]	0.07	3.9 [3.6, 4.2]	3.8 [3.3, 4.2]	0.58	3.8 [3.2, 4.2]	3.9 [3.5, 4.3]	0.31
<b>Chemotherapy</b>									
No	17 (33.3)	7 (36.8)	1	16 (30.8)	8 (44.4)	0.44	9 (39.1)	15 (31.9)	0.74
Yes	34 (66.7)	12 (63.2)		36 (69.2)	10 (55.6)		14 (60.9)	32 (68.1)	
<b>Radiotherapy</b>									
No	50 (98.0)	19 (100.0)	1*	51 (98.1)	18 (100.0)	1*	22 (95.7)	47 (100.0)	0.32*
Yes	1 (2.0)	0 (0.0)		1 (1.9)	0 (0.0)		1 (4.3)	0 (0.0)	
<b>Recurrence</b>									
No	34 (66.7)	8 (42.1)	0.06*	35 (67.3)	7 (38.9)	0.03*	16 (69.6)	26 (55.3)	0.08*
Yes	12 (23.5)	5 (26.3)		12 (23.1)	5 (27.8)		2 (8.7)	15 (31.9)	
<b>Death</b>									
No	36 (70.6)	9 (47.4)	0.11*	37 (71.2)	8 (44.4)	0.08*	16 (69.6)	29 (61.7)	0.78*
Yes	4 (7.8)	1 (5.3)		3 (5.8)	2 (11.1)		1 (4.3)	4 (8.5)	

SD, standard deviation; ASA, American Society of Anesthesiologists; CEA, Carcinoembryonic antigen; MIS, minimally invasive surgery; EGFR, Epidermal growth factor receptor; MSI, Microsatellite instability; ; IQR, Interquartile range; WBC, white blood cell; Hb, hemoglobin; PLT, platelet; NLR, neutrophil-lymphocyte ratio.

#### 2.4. Relationships between Immune-Related and Inflammatory Markers and Long-Term Oncologic Outcomes

Of the three above-mentioned markers, only MMP-3 was associated with five-year disease-free survival (DFS). The five-year DFS of the high MMP-3 expression group was significantly lower than that of the low MMP-3 expression group (65.1% vs. 90.2%,  $p=0.033$ ). In addition, there was no difference in five-year overall survival (OS) between the two groups (90.0% vs. 95.7%,  $p=0.489$ ). For APRIL/TNFSF13 and BAFF, there was no difference between the survival curves of the high and low expression groups (Figure 2).



**Figure 2.** Kaplan–Meier survival curves for disease-free survival (a) and overall survival (b) according to the expression level of MMP-3. MMP-3, matrix metalloproteinase 3.

### 3. Discussion

In this study, we investigated the expression of immune-related and inflammatory markers in CRC tissue samples. The results indicated that APRIL/TNFSF13, BAFF, and MMP-3 are highly expressed in CRCs. Analyses performed according to expression levels of these markers confirmed that high expression of immune-related and inflammatory markers was associated with advanced clinicopathological features. In addition, the results revealed that high expression of MMP-3 was associated with elevated CEA levels, more lymph node metastases, and more advanced disease stages. Furthermore, the results indicated that MMP-3 expression level was associated with long-term prognoses, such as five-year DFS. Moreover, the high APRIL/TNFSH13 expression group showed a higher rate of metastatic lesions than the low APRIL/TNFSH13 expression group.

The MMPs are a family of at least 28 zinc-dependent enzymes. Their main function is catalyzing proteolytic activities and aiding the breakdown of the extracellular matrix. It is upregulated in response to inflammation and has been shown to be involved in the development and progression of several inflammatory and autoimmune diseases, as well as cancer. They induce tumor invasion, neoangiogenesis, and metastasis by degrading the connective tissues between cells and in the lining of blood vessels [15]. Several studies have demonstrated that MMPs are highly expressed in malignant tumors compared with normal tissue [12,16,17]. There are several subtypes of MMPs, and studies on the role of each subtype have been conducted [16,18]. Yu et al. [16] analyzed the mRNA expression levels of all 24 MMPs and their prognostic values in CRC. The authors suggested that the transcriptional levels of MMP1, MMP3, MMP7, MMP9–MMP12, and MMP14 are significantly upregulated in tumors. In addition, their analysis showed that upregulation of MMP11, MMP14, MMP17, and MMP19 is significantly associated with a more advanced tumor stage and worse long-term prognosis. Another study by Islekel et al. [11] demonstrated that the protein expression levels of MMP-3 in tumor tissues are higher than those in normal tissues, and that the expression level of MMP-3 is related to lymph node status. These findings are consistent with the results of the present study.

APRIL/TNFSF13 is a tumor necrosis factor (TNF) protein that plays an important role in the development of B cells, which are involved in immune function [19]. This protein is expressed by immune cells in bone marrow and peripheral tissues under normal physiological conditions. APRIL is produced by various types of tumor cells, including breast, gastric, bladder, and ovarian cancers [20–23]. Several studies have suggested that APRIL is overexpressed in CRC tissues, and that increased APRIL expression is associated with poor prognosis in patients with CRC [24–26]. Similar to the results of the present study, a study by Lascano et al. [24] demonstrated that distant metastasis

is more frequent in patients that show high APRIL expression than in those that show low APRIL expression; however, APRIL expression level is not an independent factor for OS.

BAFF is a member of the TNF superfamily and is mainly produced by myeloid cells. BAFF plays a role in immune function regulating B cell survival, activation, and maturation. Previous studies have demonstrated that BAFF plays a role in neoplasm progression and aggressiveness [27,28]. In addition, both BAFF and APRIL signaling may increase tumor cell proliferation and enhance tumor cell viability in response to chemotherapeutic drugs for hematopoietic malignancies. Interestingly, elevated blood levels of BAFF and APRIL are associated with an advanced disease stage and invasiveness of cancers such as breast cancer, chronic lymphocytic leukemia, and pancreatic cancer [28,29]. However, the relationship between BAFF expression and disease progression is not consistent in all cancers [30]. A study has shown that the expression of BAFF in CRC tissues is higher than that in normal tissues; however, there has been no analysis of the association between BAFF expression and disease severity [13]. In the present study, high BAFF expression was observed; however, its correlations with clinicopathological features and long-term outcomes were not confirmed.

Taken together, the APRIL/TNFSF13, BAFF/TNFSF13B, and MMP-3 pathway plays a critical role in the regulation of the immune system, and dysregulation of this pathway can lead to the development of cancer. Theoretically, these above-mentioned markers that were overexpressed in CRC tissues in the present study can be used as treatment targets. In fact, several studies have been conducted to evaluate the potential of these markers as therapeutic targets. MMP-3 has also been studied as a potential therapeutic target for CRC [31]. The results of an *in vitro* study by Wen et al. suggested that histone deacetylase 11 (HDAC11) inhibits the migration and invasion of CRC cells by downregulating MMP-3 expression. Additionally, the authors found that HDAC11 is downregulated in human CRC tissues, and that decreased HDAC11 level is correlated with advanced clinical stage and lymph node metastasis. Another *in vivo* and *in vitro* study suggested that APRIL knockdown is associated with the modulation of cell proliferation, as well as the reduction of cell migration and invasion *in vitro*. Moreover, APRIL-knockdown cells displayed markedly inhibited tumor growth and decreased liver metastasis in the study. The study also revealed that APRIL could regulate the expression of MMPs, suggesting a link between APRIL and MMPs [32]. Overall, these studies suggest that APRIL/TNFSF13 and MMP-3 may be potential targets for the development of new therapies for CRC. However, further research is required to fully understand the roles of these markers in the development and progression of CRC.

To assess the expression of immune-related and inflammatory markers in the present study, we utilized the Bio-plex Multiplex immunoassay system. This is a highly sensitive assay that is capable of simultaneously measuring multiple analytes in a single sample, making it an ideal tool for studying complex biological systems such as the TME [33]. The multiplex immunoassay is faster than the traditional ELISA and planar microarray, but with comparable and reliable diagnostic accuracy [34–36]. The multiplex immunoassay has been used in a few studies to detect tumor-specific biomarkers in malignant tumors such as melanomas, ovarian cancer, and pancreatic cancer [37,38]. Calu et al. [13] used multiplex immunoassays to identify inflammatory molecules in CRC tissues.

This study has several limitations. First, the number of patients included in this study was relatively small. Second, as this study was conducted using data from a single institution, the characteristics of various types of patients may not have been included. Third, markers other than APRIL/TNFSF13, BAFF, and MMP-3 did not yield meaningful results because they reacted below our detection level; therefore, their effects could not be studied. Given that the TME is dynamic and subject to changes over time, we utilized freshly frozen tissue samples to capture the most current state of the tumor and its surrounding environment. It is possible that certain aspects of the microenvironment may not have been fully captured because the freeze-thaw process can alter the expression of certain biomolecules. However, the use of frozen tissue samples is a widely accepted method for studying gene expression and protein levels and has yielded reliable results in various research settings [39].

## 4. Materials and Methods

### 4.1. Study Population

Patients with CRC who underwent curative surgical resection between December 2014 and January 2017 were included in this study. Patients who underwent bypass surgery without curative resection for palliative purposes, patients with cancers of other organs, and patients with a previous history of CRC were excluded from the study. Tumor tissues were prospectively obtained during the index surgical procedure. Clinical, hematological, and pathological information were extracted from the patients' medical records. Informed consent to participate in this study was obtained from all patients. The study protocol was approved by the Institutional Review Board of Wonju Severance Medical Center (CR:318334), and the study was conducted in accordance with the tenets of the Declaration of Helsinki.

### 4.2. Tissue Sample Preparation

Tissue samples from both tumor and non-tumor areas of the resected specimen were collected and immediately placed in an ice-cold radioimmunoprecipitation assay buffer that contained protease inhibitor cocktails (Sigma Chemical Co., St. Louis, USA). The tissue samples were homogenized at 14000 rpm for 10 min and centrifuged for 5 min. The resultant supernatant was collected and stored at  $-80^{\circ}\text{C}$  until it was ready for further analysis.

### 4.3. Bio-plex Multiplex Immunoassay System

The concentrations of immune-related markers (APRIL/TNFSF13, BAFF, LAG-3, PD-1, PD-L1, and CTLA-4) and inflammatory markers (CHIT, MMP-3, osteocalcin, pentraxin-3, sTNF-R1, and sTNF-R2) in colon tumor and non-tumor tissues were measured using the Milliplex® map human immuno-oncology checkpoint protein magnetic bead panel 96-well plate assay (Millipore Corporation, Billerica, USA), which is a luminex-based multiplex technology. The Bio-Plex assays (Bio-Rad, Hercules, CA, USA) contained standard concentrations of each analyte, and the calculated standard curves allowed for precise definition of the concentration of the protein of interest. The assay was performed according to the manufacturer's instructions. Lyophilized cytokine standard was resuspended in standard diluents, and serial dilution of the standard (30  $\mu\text{L}$ ) was performed to generate the standard curves for each protein of interest. The bead mixture was added to the standard or sample and the plate was incubated overnight (16-18 h) at  $4^{\circ}\text{C}$ . The sample was washed three times with wash buffer/well using an automatic washer for magnetic beads. The detection antibody was then added to the plate and incubated at room temperature for 1 h. Streptavidin-phycoerythrin mix was added, and the sample was incubated at room temperature for 30 min. After washing, the assay buffer was added and analyzed using the Luminex 200 Bio-Plex instrument (Bio-Rad, Hercules, CA, USA).

### 4.4. Surgery and Pathological Examination

Complete mesocolic excision and total mesorectal excision, which are standard surgical approaches for colon and rectal cancer, respectively, were performed. The excised tissue was immediately transported to the pathology department, where a pathologist extracted normal and tumor tissue sections. Thorough histopathological examination was conducted following standardized guidelines, and the resulting histological data were recorded.

### 4.5. Statistical Analyses

Categorical variables were analyzed using the chi-square test and are described as frequencies and percentages. Fisher's exact test was performed if the frequency of the data was  $<5$ . The normality of all continuous data was tested using the Shapiro-Wilk test. Continuous variables were analyzed using Student's t-test and are expressed as mean values and standard deviations. Non-normally distributed data were analyzed using the Mann-Whitney U test and are described as medians and

interquartile ranges. DFS was defined as the period from the date of the index surgery to the date of recurrence or death. OS was defined as the period from the date of the index surgery to the date of death. Survival analysis was performed using the Kaplan-Meier curve with the log-rank test. All statistical analyses were performed using R statistical software (version 4.1.0; R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was set at  $p < 0.05$ .

## 5. Conclusions

In conclusion, this study confirmed that various immune-related and inflammatory markers, such as APRIL/TNFSF13, BAFF, and MMP-3, are overexpressed in colorectal tumor tissues and are related to the unfavorable clinicopathological features and poor prognosis of CRC. The results of this study suggest that these markers could potentially serve as diagnostic or prognostic biomarkers for CRC.

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