

Apoptosis-related Prognostic Factors in Advanced Colorectal Cancer Determined Using Tissue Microarrays

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Abstract. *Background/Aim:* Cancer cells evade apoptosis in colorectal cancer (CRC); however, overlap between apoptosis and poor prognosis marker proteins in the invasive front of tumors has not been reported. Here, we aimed to clarify the relationship between apoptosis, apoptosis-related protein expression, and prognosis in the central and invasive front regions of CRC using tissue microarrays. *Patients and Methods:* Data of 207 patients with pathological stage 3 CRC, who underwent radical surgery between October 2010 and November 2014, were retrospectively reviewed. We assessed apoptosis using M30 CytoDEATH, CD163, and p53 immunostaining in tumor sections in the center and invasive front using tissue microarrays and correlated the results with the survival outcomes. *Results:* M30 CytoDEATH staining was negative; 134 cases (64.7%) were apoptosis-negative in the center and 103 (49.8%) were apoptosis-negative at the invasive front. CD163 positivity was observed in 16 cases (7.8%) in the center and in 36 cases (17.6%) at the invasive front; p53 positivity was observed in 33 (15.9%) and 64 (30.9%) cases in the center and invasive front, respectively. CD163 and p53 expression was not associated with survival outcomes; however, the apoptosis-negative group at the invasive front had significantly poorer survival outcomes (overall survival: $p=0.044$, relapse-free survival: $p=0.001$). We identified cases with a poor prognosis by combining apoptosis and CD163 expression. *Conclusion:* A lower apoptosis percentage at the invasive front is associated with a poorer prognosis. CRC cases with a poor prognosis can be

identified by evaluating apoptosis and CD163 expression in the invasive front.

Colorectal cancer (CRC) is the third most common cancer and second most common cause of cancer-related deaths worldwide (1). Several prognostic predictors and factors have been investigated to improve CRC prognosis. In one reported case, tumor cells repeatedly proliferated and metastasized because of mechanisms of evading apoptosis (2). Several pathways for apoptosis evasion, involving either genetic mutations or tumor microenvironment, have been reported. The tumor apoptosis pathway involves p53 mutations. p53 is a tumor-suppressor protein encoded by TP53, which is located on the short arm of chromosome 17 (3). p53 mutations help evade apoptosis and promote tumor growth (4-6). In the microenvironment, the expression of CD163, a marker of M2 macrophages, has been associated with tumor apoptosis (7). Forced CD163 expression in meningioma cells reportedly suppresses apoptosis and promotes tumor growth in nude mice (7). Furthermore, CD163 is expressed on some cancer cells and is associated with a poor clinical prognosis of breast, colorectal, renal, and bladder cancers (8-11). Additionally, high CD163 expression at the invasive front of tumors correlates with lymphovascular invasion, low histological differentiation, and lymph node metastasis (12). In contrast, there are no differences in tumor growth and metastatic potential according to the expression, as analyzed histologically, either at the invasive front of the center of the tumor, including the expression of proteins involved in tumor growth (13-15). Konishi *et al.* reported that tumor budding and the presence of poorly differentiated cell clusters, as pathological markers, at the invasive front of colon cancer are associated with poor prognosis (16). Recently, attention has been paid to protein expression and tumor biology at the invasive front of tumors. Previously, in patients with CRC, the group with a low apoptosis percentage at the invasive front showed a poorer

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Table I. List of antibodies used in immunohistochemistry.

Antibody	Clone	Dilution	Localization	Source
M30 CytoDEATH	M30	1:100	Cytoplasmic	Peviva AB, Bromma
CD163	D6UIJ	1:250	Stroma	Cell Signaling Technology
p53	DO7	1:100	Nucleus	Leica Biosystems (Nussloch, Germany)

Table II. Patient characteristics.

Characteristic	Number
Total number	207
Age, years ^a	66 (32-90)
Sex (male/female)	117/90
Tumor location (right side/left side/rectum)	47/82/78
CEA ng/ml ^a	5.3 (1.1-170.2)
CA19-9 U/ml ^a	15 (1-2,006)
Anti-p53 antibodies U/ml ^a	0.4 (0.4-2,144)
p-T stage (1a/1b/2/3/4/4b)	1/9/15/124/45/13
p-N stage (1-2a/2b)	192/15
Lymphatic invasion (-/+)	91/116
Vascular invasion (-/+)	38/169
Histological type (differentiated/others)	188/19
Adjuvant chemotherapy	146

^aData are expressed as median (range). p-: Pathological.

prognosis than the group with a high apoptosis percentage (17). The cohort included patients with early-stage cancer; this is because stage 3 is known to have a poorer prognosis than stage 1 and 2 and is thus necessary to focus on Stage 3. There are no reports examining apoptosis and the expression of p53 and CD163 together in the center or invasive front of colorectal tumors. Here, we aimed to clarify the relationship between apoptosis, p53 as an apoptosis-related protein, and prognosis in the central and invasive front regions of CRC using tissue microarrays.

Patients and Methods

Patients. We enrolled 205 consecutive patients who underwent radical surgery and were diagnosed with colorectal adenocarcinoma of pathological N stage 1-2b without distant metastasis, between October 2010 and November 2014, at the University of Tokyo Hospital. Patients who received neoadjuvant therapy were excluded.

Immunohistochemistry. Tissue microarrays were constructed for immunostaining, and a tissue arrayer (model KIN-2; Azumayaikakikai Inc., Tokyo, Japan) was used. We punched and retrieved duplicate tissue cores of 2 mm in diameter from the tissue block of each donor and arrayed them in the recipient blocks. Each array block contained 48 tissue cores obtained from the invasive front areas and central areas of 24 tumors each.

Table III. Association of marker expression in the center and invasive front.

	Center	Invasive front
Single marker		
M30 CytoDEATH-negative	134 (64.7%)	103 (49.8%)
CD163-positive	16 (7.8%)	36 (17.6%)
p53-positive	33 (15.9%)	64 (30.9%)
Double marker		
CD163-positive+M30-negative	8 (3.9%)	20 (9.7%)
p53-positive+M30-negative	11 (5.3%)	26 (12.6%)
Triple marker		
CD163- and p53-positive/ M30-negative	0 (0%)	5 (2.4%)

The tissue sections from formalin-fixed, paraffin-embedded tissue blocks were deparaffinized in xylene and rehydrated in a graded ethanol series. Antigen retrieval was performed using citrate buffer (pH 6.0) in an autoclave at 120°C for 10 min. After blocking endogenous peroxidase activity with 0.3% hydrogen peroxide in methanol for 30 min, the sections were incubated with 10% normal goat serum (Nichirei Biosciences, Tokyo, Japan) for 30 min. The sections were incubated with primary antibodies overnight at 4°C, and then with the corresponding biotin-conjugated secondary antibodies (Nichirei Biosciences) at 20°C for 20 min and peroxidase-conjugated streptavidin (Nichirei Biosciences) for 10 min. The primary antibodies used are listed in Table I. Subsequently, the sections were incubated with 3,3'-diaminobenzidine (Wako, Osaka, Japan) for 10 min and counterstained with hematoxylin.

Immunohistochemical evaluation. The results of immunostaining for each of the antibodies are shown in Figure 1. M30 positivity was identified as brown cytoplasmic staining. Cytoplasmic M30-positive cells in all cancer sections were counted under a light microscope at 200× magnification. The scoring system for the staining assay was based on a two-classification system: positive, apoptosis-positive if >50% of tumor cells were stained; negative, apoptosis-negative if <50% of tumor cells were stained from all tumor cells in each tissue core.

CD163 positivity was identified as brown coloration of the tumor stroma. The area of staining per unit area of colorectal cancer invasion was calculated, and the results were considered positive if >40% staining was observed. p53 positivity was identified as brown nuclear staining. p53-positivity was defined as >70% stained tumor cell nuclei; the null pattern was defined as having no stained cells. The samples were evaluated by investigators blinded to the clinical or pathological information.

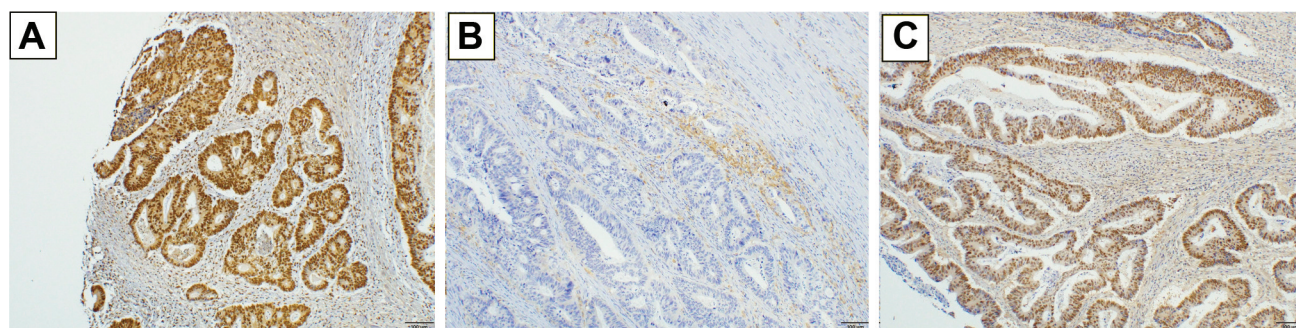


Figure 1. Immunohistochemistry. Representative images displaying positive staining for M30 CytoDEATH (A), CD163 (B), and p53 (C).

Table IV. Univariate and multivariate analyses of risk factors for overall survival.

	Univariate		Multivariate	
	p-Value	HR	95%CI	p-Value
Age (<65 vs. ≥65 years)	0.307			
Sex (male vs. female)	0.739			
p-T stage (T1-T3 vs. T4a/4b)	0.001	2.54	1.41-4.58	0.002
p-N stage (1-2a vs. 2b)	0.896			
Lymphatic invasion (negative vs. positive)	0.090	1.78	0.95-3.33	0.072
Vascular invasion (negative vs. positive)	0.146			
Histological type (differentiated vs. others)	0.040	2.25	0.99-5.07	0.051
Adjuvant chemotherapy (+ vs. -)	0.014	1.98	1.08-3.60	0.026
M30 CytoDEATH in the centre (positive vs. negative)	0.977			
M30 CytoDEATH at the invasive front (positive vs. negative)	0.044	1.94	1.05-3.60	0.035
CD163 in the centre (negative vs. positive)	0.163			
CD163 at the invasive front (negative vs. positive)	0.142			
p53 in the centre (negative vs. positive)	0.807			
p53 at the invasive front (negative vs. positive)	0.758			

HR: Hazard ratio; CI: confidence interval; p-: pathological.

Surveillance after surgery and outcome measures. All patients underwent a standardized follow-up schedule that included carcinoembryonic antigen level assessment every 3 months, chest-to-pelvic computed tomography every 6 months, and colonoscopy every 12 months. Clinical outcomes were evaluated by assessing the overall survival (OS) and relapse-free survival (RFS). OS was defined as the interval between the date of surgery and date of death from any cause. RFS was measured from the date of surgery to the date of first recurrence, including local and distant recurrences and death from any cause.

Statistical analyses. Patient characteristics are summarized using descriptive statistics, and comparative analysis was performed using the chi-square test to compare the immunostaining results. Survival curves were generated using the Kaplan-Meier method and compared using the log-rank test. Variables with $p < 0.1$ in the univariate analyses were subjected to multivariate Cox proportional-hazards analyses. All analyses were performed using JMP Pro 15.0 software (SAS Institute, Inc., Cary, NC, USA). This study was approved by the Ethics Committees of the University of Tokyo [No. 3252-(15)]. Consent to participate in the study was obtained from all patients using the opt-out method.

Results

Patient characteristics are shown in Table II. Tumors were localized to the right colon in 47 patients, left colon in 82, and rectum in 78. The pathological N stages 1-2a and 2b were noted in 192 and 15 patients, respectively. Postoperative adjuvant chemotherapy was administered to 146 patients.

The immunostaining results are shown in Table III. M30 CytoDEATH staining was negative, or apoptosis-negative, in 134 (64.7%) patients in the center and 103 (49.8%) patients at the invasive front. CD163 positivity was observed in 16 (7.8%) patients in the center and 36 (17.6%) patients at the invasive front, and p53 positivity was observed in 33 (15.9%) and 64 (30.9%) patients in the center and invasive front, respectively. The positive expression of all proteins was more frequently observed in the invasive front. When the markers were combined and examined, eight patients were noted to be CD163-positive; apoptosis-negative results

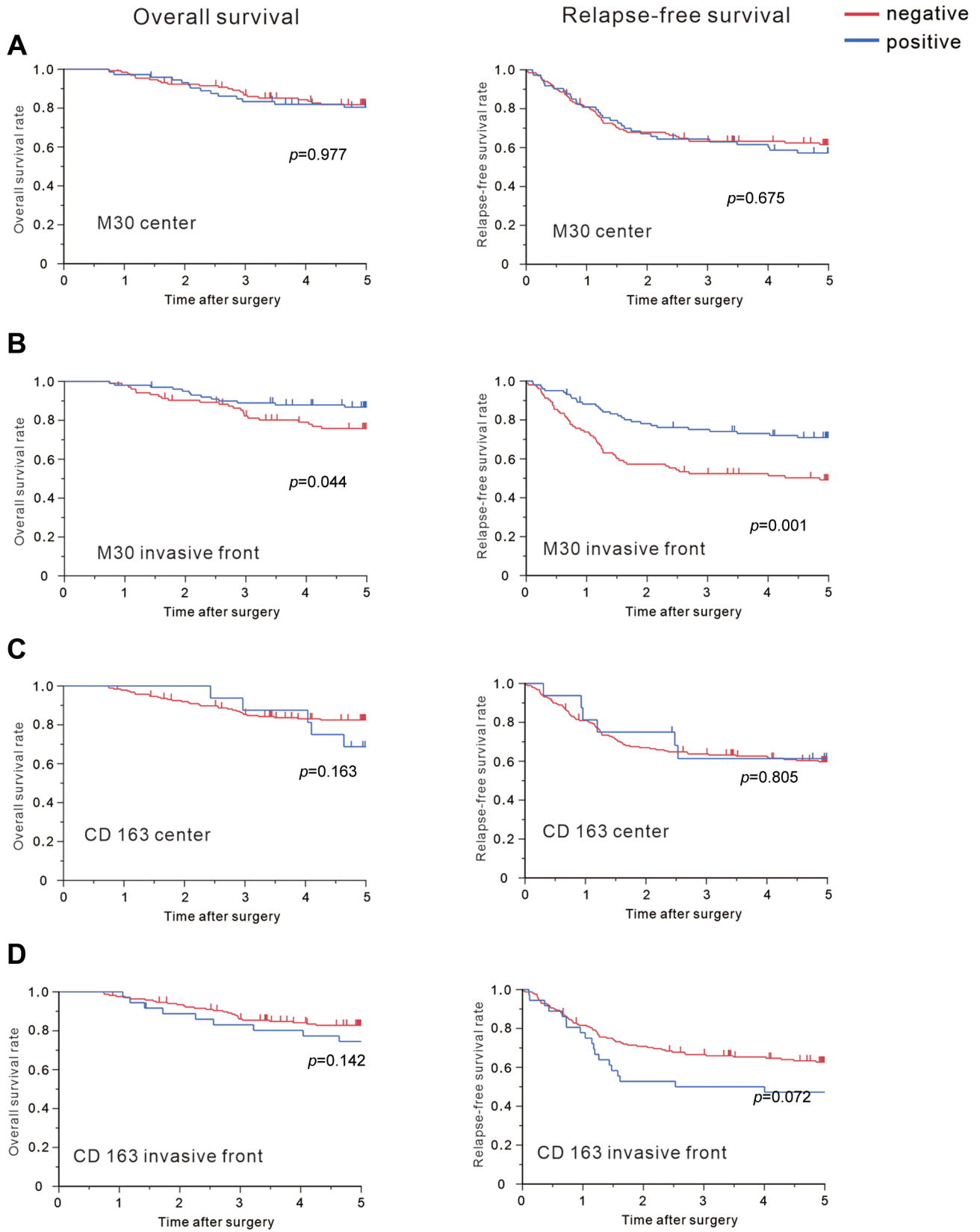


Figure 2. Continued

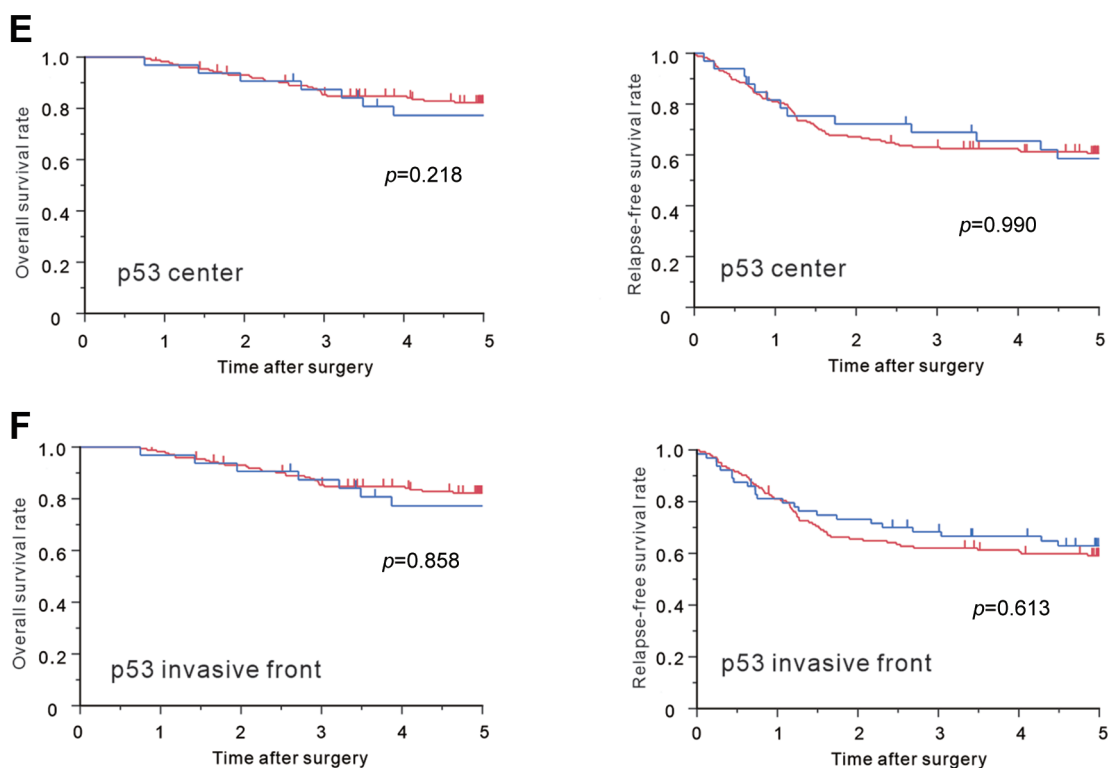


Figure 2. Overall survival (OS) and relapse-free survival (RFS) rate curves according to each marker in the center and invasive front. Red line represents patients with negative marker expression results. Blue line represents patients with positive marker expression results. OS and RFS in cases of (A) M30 CytoDEATH positivity in the center, (B) M30 CytoDEATH positivity at the invasive front, (C) CD163 positivity in the center, (D) CD163 positivity at the invasive front, (E) p53 positivity in the center, and (F) p53 positivity at the invasive front.

were noted in 8 (3.9%) and 20 (9.7%) patients in the center and invasive front, respectively. Furthermore, 11 (5.3%) and 26 (12.6%) patients tested p53-positive and apoptosis-negative, respectively. There were no cases of marker expression in the center, whereas there were five cases (2.4%) positive for both CD163 and p53, and apoptosis-negative in the invasive front.

The survival outcomes based on each marker expression are shown in Figure 2. The 5-year OS and RFS were not different depending on the number of apoptotic cells in the center (81.8% vs. 80.5%, $p=0.977$ and 61.5% vs. 57.2% $p=0.675$) (Figure 2A). However, in the invasive front, the apoptosis-negative group had significantly poorer OS and RFS (75.8% vs. 86.8% $p=0.044$ and 49.2% vs. 71.0%, $p=0.001$) (Figure 2B). CD163 expression at the invasive front tended to correlate with shorter RFS, and CD163 and p53 expression in either the center or invasive front did not significantly affect OS and RFS (CD163 center, 82.5% vs. 68.8%, $p=0.163$; 59.8% vs. 61.4%, $p=0.805$; invasive front, 82.8% vs. 74.5%, $p=0.142$ and 62.7% vs. 47.2%, $p=0.072$; p53 center, 82.2% vs. 77.3%, $p=0.218$ and 60.6% vs. 58.6% $p=0.990$; invasive front, 80.4% vs. 84.1%, $p=0.858$ and 59.1% vs. 62.9% $p=0.613$) (Figure 2C-F). Figure

3 shows the comparison of prognosis of patients that express apoptosis markers and CD163 or p53 in the invasive front of the tumor. The group that was apoptosis-negative and positive for CD163 or p53 had the poorest prognosis in terms of both OS and RFS (CD163-positive+apoptosis-negative, 65.0% and 35.0%; p53-positive+apoptosis-negative, 73.9% and 43.0%). Although not shown in Figure 3, in the invasive front, CD163-positive, p53-positive, and apoptosis-negative cases had the poorest prognosis, with 5-year OS and RFS of 71.5% and 45.0%, respectively.

The univariate and multivariate analysis results for the associations between clinicopathologic variables and OS are shown in Table IV. Pathological (p) T4a/4b, no adjuvant chemotherapy, and apoptosis-negative at the invasive front were independent risk factors [hazard ratio (HR)=2.54, $p=0.002$, HR=1.98, $p=0.026$, and HR=1.94, $p=0.035$]. The results of the univariate and multivariate analyses for the associations between clinicopathologic variables and RFS are shown in Table V. pT4a/4b, no adjuvant chemotherapy, and apoptosis-negative at the invasive front were found to be independent risk factors (HR=2.15, $p=0.001$, HR=1.59, $p=0.046$, and HR=2.24, $p=0.001$).

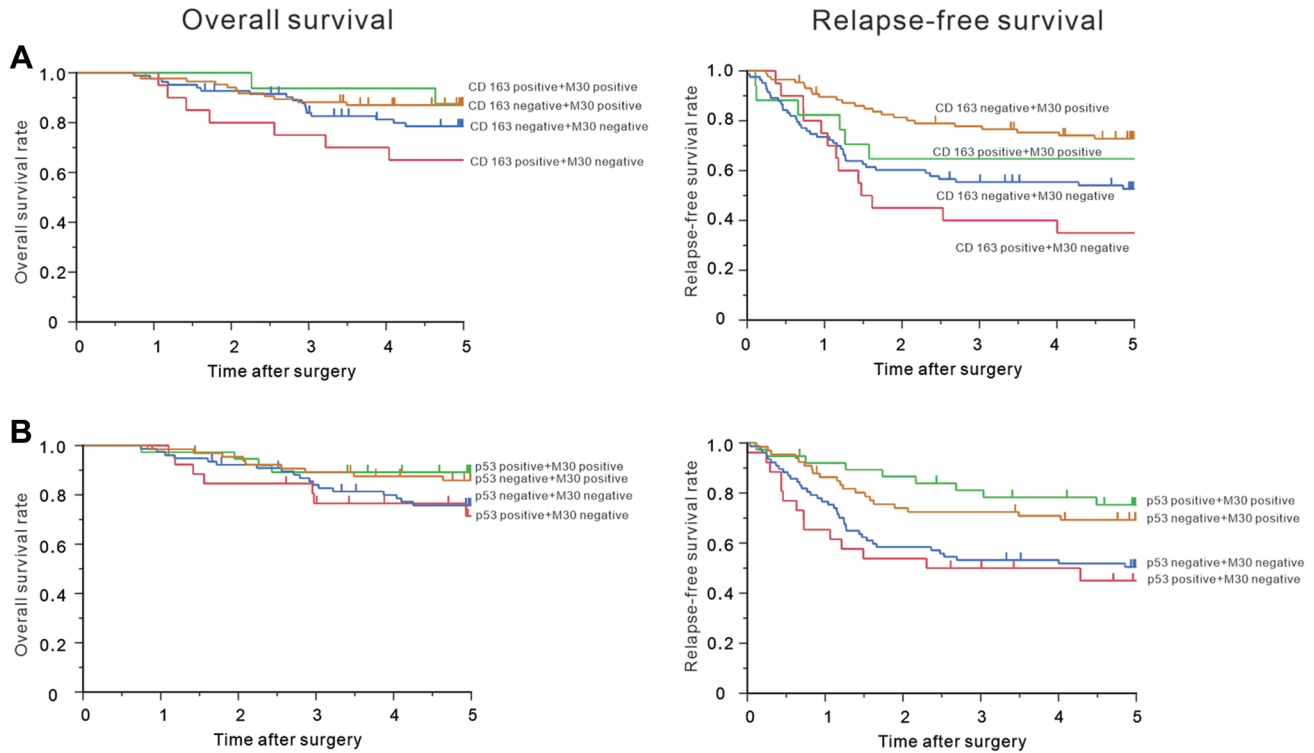


Figure 3. Overall (OS) and relapse-free survival (RFS) rate curves according to each double marker at the invasive front. Red line represents patients with negative marker expression results. Blue line represents patients with positive marker expression results. OS and RFS in cases of (A) with CD163+M30 CytoDEATH positivity and (B) p53+M30 CytoDEATH positivity.

Table V. Univariate and multivariate analyses of risk factors for relapse-free survival.

	Univariate		Multivariate	
	p-Value	HR	95%CI	p-Value
Age (<65 vs. ≥65 years)	0.893	–	–	–
Sex (male vs. female)	0.727	–	–	–
p-T stage (T1–T3 vs. T4a/4b)	0.001	2.15	1.37-3.38	0.001
p-N stage (1–2a vs. 2b)	0.894	–	–	–
Lymphatic invasion (negative vs. positive)	0.156	–	–	–
Vascular invasion (negative vs. positive)	0.087	1.80	0.92-3.50	0.085
Histological type (differentiated vs. others)	0.739	–	–	–
Adjuvant chemotherapy (+ vs. -)	0.016	1.59	1.01-2.51	0.046
M30 CytoDEATH in the centre (positive vs. negative)	0.675	–	–	–
M30 CytoDEATH at the invasive front (positive vs. negative)	0.001	2.24	1.41-3.55	0.001
CD163 in the centre (negative vs. positive)	0.805	–	–	–
CD163 at the invasive front (negative vs. positive)	0.072	1.38	0.81-2.33	0.232
p53 in the centre (negative vs. positive)	0.309	–	–	–
p53 at the invasive front (negative vs. positive)	0.488	–	–	–

HR: Hazard ratio; CI: confidence interval; p-: pathological.

Discussion

To our knowledge, this is the first study to report an overlap of apoptosis and apoptosis-related protein expression at the

invasive front of patients with stage 3 CRC. A low apoptosis rate at the invasive front was associated with a poor prognosis and CD163-positive status in the apoptosis-negative group was associated with a worse prognosis.

The tumor invasive front is the most important area for the infiltration of cancer cells and immune response of hosts to cancer. The biological characteristics of cancer cells at the invasive front might reflect the invasive ability of cancer tissues. Concurrent with these facts, here, the group with a low rate of apoptosis at the invasive front showed predominantly lower OS and RFS than the group with a high degree of apoptosis. This result indicates a correlation between the degree of apoptosis in the invasive front and tumor progression. While no correlation was found between the degree of apoptosis in the central region and clinical outcomes, apoptosis-negative status in the invasive front was found to be an independent risk factor in the multivariate analysis of OS and overall recurrence, suggesting that evaluating apoptosis at the invasive front region rather than the central region is important.

Regarding apoptosis of cancer cells, several mechanisms by which tumor cells continuously evade apoptosis and proliferate have been reported (18). Among them, *TP53* dysfunction is known to suppress cancer cell apoptosis (4-6). *TP53* is a transcription factor that induces cell cycle arrest, senescence, and apoptosis under cellular stress, and patients with mutant *TP53* are often resistant to current therapies (5). Our study showed no correlation between prognosis and *TP53* mutation regardless of the apoptotic status. Data regarding the prognostic role of *TP53* mutations in CRC is heterogeneous. Studies have reported that *TP53* mutations are an adverse prognostic factor (19, 20), whereas other studies have not found a relationship with outcomes (21, 22). The discrepancy in the findings of association between *TP53* mutation and prognosis can be attributed to various factors, including the differences in mutation location and tumor site, and the administration or non-administration of chemotherapy (23).

Furthermore, CD163 expression suppresses apoptosis and is associated with a poor clinical prognosis (7, 8). Interestingly, increased CD163-positive macrophage infiltration at the tumor invasive front is significantly associated with epithelial–mesenchymal transition (EMT), mesenchymal circulating tumor cell ratio, and dismal prognosis in CRC (24). Additionally, high CD163 expression at the invasive front of tumor is associated with less E-cadherin and more vimentin expression, an indicator of EMT (24). Here, the expression or non-expression of CD163 did not contribute to OS outcomes; however, CD163 expression at the invasive front tended to correlate with shorter RFS. Notably, a combined evaluation of CD163 expression and apoptosis identified a group with a poorer prognosis than the group with low apoptosis. This poor prognosis in the CD163-positive and low apoptosis group is thought to be influenced by the apoptosis-inhibiting effect and EMT-promoting effect of M2 macrophages. Furthermore, these results suggest that a combined evaluation of factors associated with a poor prognosis, rather than their individual evaluation, may be a

more powerful predictor of poor prognosis in patients with advanced CRC with lymph node metastases.

The present study has certain limitations. First, it was a single-facility study. Second, the post-operative adjuvant chemotherapy rate was low (70.5%). The Japanese guidelines recommend postoperative adjuvant chemotherapy for patients with pathologic stage 3 or higher disease (25). The administration of postoperative adjuvant chemotherapy in elderly patients is recommended in selected cases (26). As the study population included elderly patients, the decision to administer adjuvant chemotherapy was made on a case-by-case basis; hence, adjuvant chemotherapy was not found to be an independent risk factor for OS and RFS, and the low rate of adjuvant chemotherapy administration may have affected the survival outcomes.

Our findings revealed that a lower apoptosis rate at the invasive front is associated with a poorer prognosis. Moreover, identifying cases with a poorer prognosis by evaluating apoptosis and CD163 expression as poor prognostic markers at the invasive front was possible.

Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

All Authors (Kosuke Ozaki, Kazuhito Sasaki, Hiroyuki Abe, Hiroaki Nozawa, Koji Muroto, Shigenobu Emoto, Tetsuo Ushiku, and Soichiro Ishihara) contributed to: A) conception and design, or acquisition of data, or analysis and interpretation of data; B) Drafting the article or revising it critically for important intellectual content; C) Final approval of the version to be published.

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