Myofibroblasts of the muscle layer stimulate the malignant potential of colorectal cancer

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Abstract. Myofibroblasts of colorectal cancer (CRC) have been associated with histopathological factors such as lymph node metastasis, liver metastasis and local recurrence. However, few studies have assessed the association between these malignant traits and the myofibroblast distribution in CRC. We aimed to evaluate the relationship between clinical factors and myofibroblast distribution around CRC invasive lesions. The study included 121 cases of pT3 CRC that were diagnosed at stage II or III. Myofibroblast density of the following three histological layers was measured: the submucosa (SM), muscularis propria (MP) and subserosa (SS). We analyzed the relationship between the clinicopathological factors and myofibroblast density by studying the histopathological features of the three layers. The myofibroblast density of the MP layer was significantly higher in the groups with high-frequency lymphatic and venous invasion than the groups with low-frequency lymphatic ($P<0.001$) and venous ($P<0.01$) invasion, respectively. In the positive lymph node metastasis group, the myofibroblast density at the MP layer was significantly higher than that in the negative lymph node metastasis group ($P<0.001$). The high myofibroblast density group at the MP layer was significantly associated with poor overall survival ($P<0.003$). Our study indicated that myofibroblasts are a type of cancer-associated fibroblasts and that the myofibroblast distribution contributes to the malignant potential of CRC. Furthermore, we demonstrated that myofibroblasts present at the MP layer play an important role in the malignant potential and poor prognosis of patients with CRC.

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Abbreviations: CRC, colorectal cancer

Key words: colorectal cancer, myofibroblast, muscularis propria, prognosis

Introduction

Colorectal cancer (CRC) is the most common malignancy of the colon and rectum and the third most common cause of cancer-related death among men and women worldwide (1). Outcome prediction based on tumor stage reflected by the tumor node metastasis (TNM) system of the Union for International Cancer Control (UICC) is currently regarded as the standard prognostic parameter (2,3). Venous and lymphatic vessel invasion are also important malignant factors of CRC (3-5). Both lymphangiogenesis and angiogenesis also play important roles as poor prognostic factors in tumorigenesis (6-8). In addition, the extracellular matrix (ECM) influences cancer proliferation, activities of invasion and metastasis by stimulating angiogenesis and lymphangiogenesis (9,10).

In contrast, the relationship between CRC and myofibroblasts in the tumor microenvironment has recently attracted considerable attention. Myofibroblasts are not only known as a principal cellular component in the granulation tissue of healing wounds but are also one of the cancer stromal cells that constitute the ECM (11,12). The myofibroblasts in the stroma of CRC serve an important function in promoting the desmoplastic reaction and influencing tumor invasion, microvessel density around the invasive lesion and metastatic carcinomas (13-15). Moreover, myofibroblast activation in tumor metastatic lymph nodes influences the microenvironment supporting CRC metastasis (16).

With regard to both the tumor growth and spreading of CRC, three histological layers of the colorectum, the submucosa (SM), muscularis propria (MP) and subserosa (SS), may play important functions in the mechanical and physiological protection against invasive growth. MP is exclusively composed of smooth muscle bundles and comprises tight connective tissue, whereas SM and SS are mainly composed of loose connective tissue (17,18). However, it is unclear how myofibroblasts are distributed around the CRC invasive border of these three layers as well as how the distribution is related to the malignant potential of CRC.

In the present study, we measured the myofibroblast density of each colorectal layer using imaging analysis and investigated the association between myofibroblast distribution and

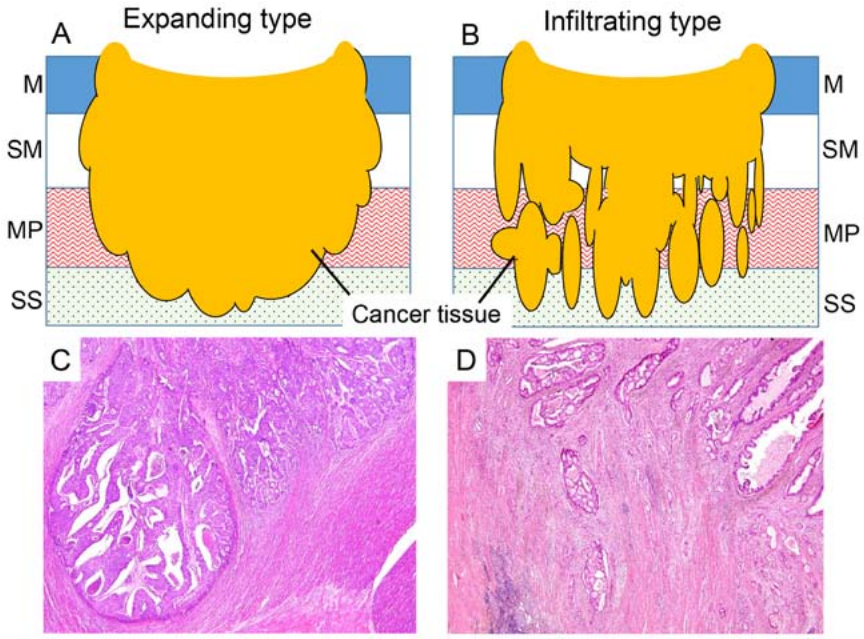

Figure 1. The expanding and infiltrating types of colorectal cancer, which invaded through the mucosa to the subserosa. The expanding type was recognised as the overall pushing growth type of adenocarcinoma and the invasive margin was clear (A). The infiltrating type was recognised as the widespread streaming form of adenocarcinoma (B). Histology of the expanding type (C) and the infiltrating type (D) around the tumor invasive lesion in the muscularis propria layer. M, mucosa; SM, submucosa; MP, muscularis propria; SS, subserosa.

clinicopathological factors such as lymph node metastasis and venous invasion. Furthermore, we showed the relationship between the myofibroblast distribution and overall survival of patients with CRC.

Materials and methods

Patients. One hundred and twenty-one patients with advanced CRC, defined as adenocarcinoma, which had invaded the SS layer of the colorectal wall (pT3), underwent surgical resection from January 2008 to December 2009 at Hirosaki University Hospital. The clinical stages of these patients were stage II or III according to the TNM classification of the UICC (2). Survival data were obtained from hospital medical charts. Cancer-specific survival was measured from the date of surgery until the date of death from CRC. None of the patients were treated with neoadjuvant chemotherapy, and none of them had synchronous multiple CRCs or synchronous metastasis to other organs.

Pathological analysis. We used surgically resected specimens that were fixed with 10% formalin, embedded in paraffin and stained with hematoxylin and eosin (H&E) for pathological evaluation. Degrees of lymphatic vessel invasion were classified as 0, no invasion; 1, mild invasion; 2, moderate invasion and 3, severe invasion. The modes of invasive growth pattern were classified into two groups, namely expanding type, the overall pushing growth type of adenocarcinoma with a clear invasive margin; and infiltrating type, a widespread streaming form of adenocarcinoma with an unclear borderline of the invasive front (Fig. 1). To evaluate the myofibroblast distribution of each case, we selected the paraffin-embedded specimen that showed three invasive lesions in each histological layer (SM, MP and SS) as diagnosed by H&E staining (Fig. 2).

Immunohistochemistry. For immunohistochemical examination concerning the myofibroblast distribution of each case, the paraffin-embedded specimen which was described in 'Pathological analysis' was a representative specimen of each case, and we used serial 4-μm sections for the immunohistochemical analysis. The sections were mounted on saline-coated glass slides. The antibodies used included α-smooth muscle actin (α-SMA, 1:100, clone 1A4) and desmin (1:100, clone D-33) (both from Dako, Glostrup, Denmark). Immunostaining for α-SMA and desmin was performed using the standard avidin-biotin-peroxidase complex method with an automated immunostainer (Benchmark XT; Ventana Medical System, Tucson, AZ, USA). The signature characteristic of myofibroblasts is an α-SMA-positive and desmin-negative pattern, whereas that of smooth muscle is an α-SMA-positive and desmin-positive pattern.

Image analysis. We used imaging analysis to investigate the myofibroblast density. All cases had an invasive lesion of the three colorectal walls: SM, MP and SS. To obtain the images, we used an Olympus microscope BX50 with a U PlanApo objective lens (x4 magnification), DP Control software and a DP-70 digital camera (all from Olympus, Tokyo, Japan). We applied ImageJ software (National Institutes of Health, Bethesda, MD, USA) to view and analyze our obtained images (19). We captured images of α-SMA and desmin (Fig. 3A and D), and these images were binarised (Fig. 3B and E). The binarised images showed that the positively and negatively immunostained lesions were black and white, respectively. We made a subtraction image by pasting the binarised images of desmin onto the binarised images of α-SMA using the subtraction mode in ImageJ software (Fig. 3F). The subtraction images were shown as the value of α-SMA minus that of desmin, and we could interpret the subtraction images showing myofi-

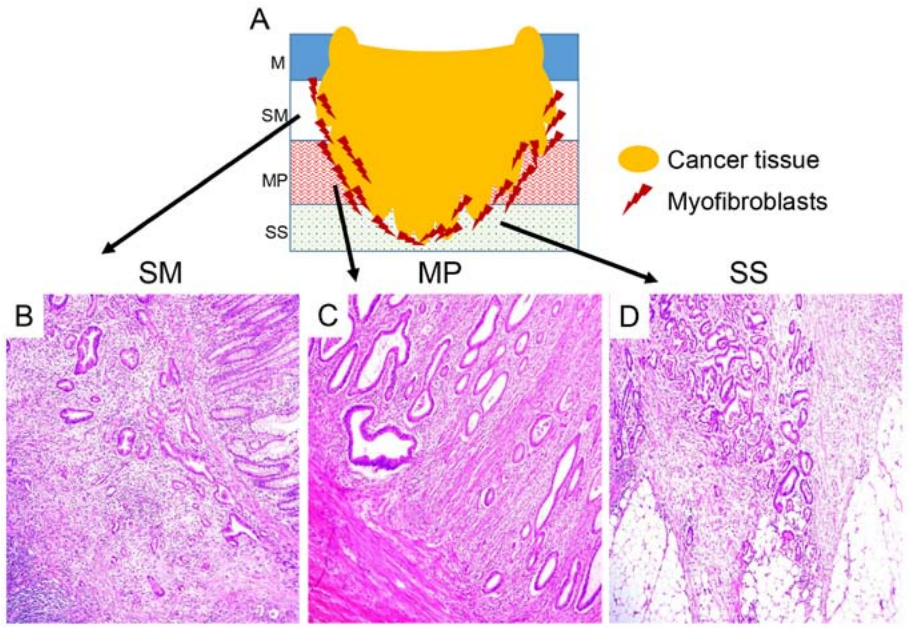

Figure 2. Representative case of the myofibroblast distribution in each invasive level of colorectal cancer. Myofibroblasts exist around the invasive front of each colorectal histological wall level (A). We identified the colorectal cancer invasive lesions using hematoxylin and eosin staining and selected the paraffin-embedded specimen that showed three invasive lesions in each histological layer (SM, MP and SS) (B-D). M, mucosa; SM, submucosa; MP, muscularis propria; SS, subserosa.

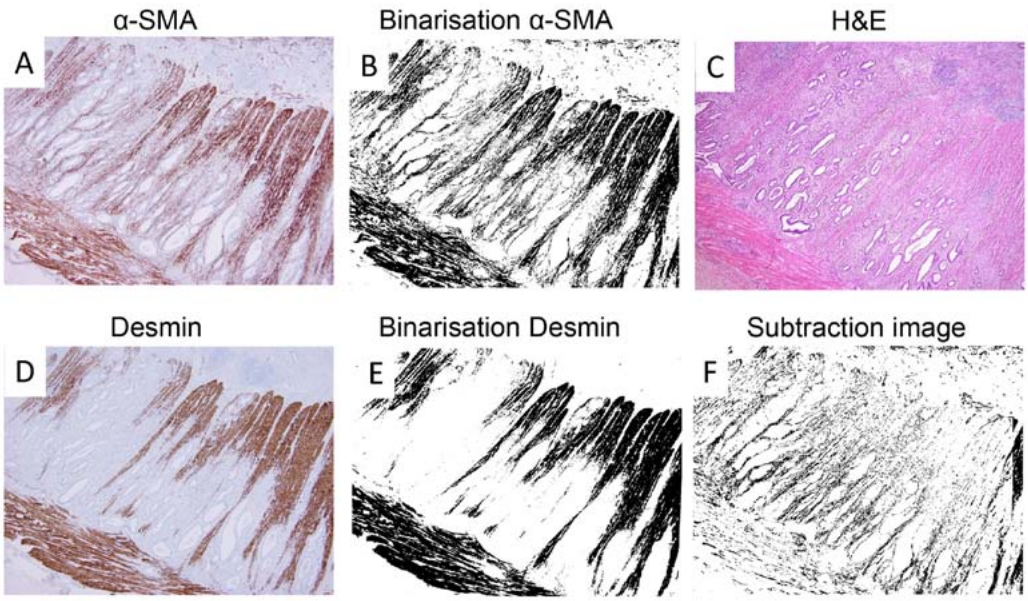

Figure 3. Representative case of an invasive lesion of the muscularis propria layer: α-SMA (A), the binarisation image of α-SMA (B), hematoxylin and eosin staining (C), desmin (D), the binarisation image of desmin (E) and subtraction image (α-SMA-desmin) (F). α-SMA, α-smooth muscle actin.

broblasts in the representative sections of each case. From all 121 cases, we obtained subtraction images of the three colorectal wall layers (SM, MP and SS) and measured the myofibroblast density in 1x1 mm^2 areas in the invasive border of each layer. We selected a hot spot myofibroblast density area from each invasive layer.

Statistical analysis. All values are presented as the means ± standard error of the mean. Chi-square tests were performed for non-continuous variables, while the Mann-Whitney test and Welch t-tests were used for comparing continuous variables. Survival curves were constructed using the Kaplan-Meier method, and differences in survival were evaluated using the log-rank test. The relative prognostic factors were analysed with a Cox proportional hazards regression model. Differences were considered as statistically significant if the P-value was <0.05. Statistical analysis was performed with R (http://www.r-project.org) and Microsoft Excel software (Microsoft Corporation, Redmond, WA, USA).

Table I. Histopathological characteristics of the 121 cases.

Variables	No. of patients
Age in years, median (range)	67.4 (26-93)
Gender	
Male	66
Female	55
Location	
Colon	77
Rectum	44
Histological type	
Well, mod	110
Por, muc	11
Invasive type	
Expanding	57
Infiltrating	64
Lymphatic invasion	
Low (ly0 or ly1)	80
High (ly2 or ly3)	41
Venous invasion	
Low (v0 or v1)	90
High (v2 or v3)	31
Lymph node metastasis	
Negative	64
Positive	57

Well, well-differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; por, poorly differentiated adenocarcinoma; muc, mucinous adenocarcinoma; ly, lymphatic invasion; v, venous invasion.

Results

Clinicopathological characteristics. The clinicopathological characteristics of the 121 CRC cases are summarised in Table I. The series consisted of 66 men and 55 women, with a median age of 67.5 years (range, 26-93 years). The carcinomas were located in the colon (77 cases) and rectum (44 cases). One hundred and ten carcinomas were diagnosed as well and moderately differentiated adenocarcinoma, and 11 carcinomas were diagnosed as poorly differentiated and mucinous adenocarcinoma. In terms of the CRC invasive pattern, 57 cases were the expanding type, and 64 cases were the infiltrating type. Eighty cases and 41 cases had low and high degrees of lymphatic invasion, respectively. In contrast, the numbers of cases with low and high degrees of venous invasion were 90 and 31 cases, respectively. Furthermore, the numbers of cases with negative and positive lymph nodes were 64 and 57 cases, respectively.

Myofibroblast distribution in the invasive lesion at each colorectal wall stratified by expanding type vs. infiltrating type. We measured the myofibroblast density around the invasive front of each layer (SM, MP and SS) for the expanding and infiltrating types (Fig. 4A). In 57 cases of the expanding type, the mean myofibroblast densities for each wall of the invasive lesion were 11.03±0.88% (SM), 11.62±0.50% (MP) and 19.24±1.34% (SS). Meanwhile, in 64 cases of the infiltrating type, the mean myofibroblast densities were 13.60±0.79% (SM), 20.52±0.62% (MP) and 22.40±1.07% (SS). Significantly more myofibroblasts were located around these three invasive layers in the infiltrating type than the expanding type ($P<0.05$).

Association between the distributions of myofibroblast density and lymphatic vessel invasion. To investigate the association between the myofibroblast distribution and the degree of lymphatic vessel invasion, we stratified the 121 cases of CRC into either a low lymphatic vessel invasion (ly0 and ly1) group or a high lymphatic vessel invasion (ly2 and ly3) group. We analysed the myofibroblast distribution around the invasive front of each layer (Fig. 4B). The mean myofibroblast densities in the three layers within the low lymphatic vessel invasion group (n=80) were 11.73±0.77% (SM), 13.89±0.57% (MP) and 19.99±1.06% (SS). In contrast, the mean myofibroblast densities in the high lymphatic vessel invasion group (n=41) were 14.68±1.39% (SM), 21.48±1.05% (MP) and 24.76±1.86% (SS). The myofibroblast density was significantly higher in the group with high degree lymphatic vessel invasion than that noted in the group with low degree lymphatic vessel invasion in the MP layer ($P<0.001$) and the SS layer ($P=0.04$), respectively. On the other hand, there was no significant difference between the low and high venous vessel invasion group in regards to the myofibroblast density of the SM layer ($P=0.103$).

Association between the myofibroblast density distribution and venous vessel invasion. To investigate the association between the myofibroblast distribution and the degree of venous vessel invasion, we stratified the 121 cases into a low venous vessel invasion (v1 and v2) group and a high venous vessel invasion (v2 and v3) group and analysed the myofibroblast distribution around the invasive front of each layer (Fig. 4C). The mean myofibroblast densities in the low venous invasion group (n=90) were 11.99±0.79% (SM), 15.40±0.70% (MP) and 21.54±1.10% (SS), while the mean myofibroblast densities in the high venous invasion group (n=31) were 14.85±1.47% (SM), 19.54±1.09% (MP) and 24.70±1.91% (SS). There was a significant difference in the myofibroblast density of the MP layer between the low and high venous invasion groups ($P<0.01$). There were not significant differences between the two groups in regards to the myofibroblast density of the SM layer ($P=0.07$) and SS layer ($P=0.06$).

Association between the myofibroblast distribution and lymph node metastasis. We stratified the 121 CRC cases into a lymph node metastasis-negative group and -positive group and investigated the myofibroblast distribution of the three invasive walls (Fig. 4D). The mean myofibroblast densities of the three walls within the lymph node metastasis-negative group (n=64) were 12.24±0.98% (SM), 14.12±0.63% (MP) and 20.73±1.16% (SS). The mean myofibroblast densities in the lymph node metastasis-positive group (n=57) were 13.28±1.01% (SM), 19.01±0.97% (MP) and 22.61±1.56% (SS). The lymph node-positive group had higher myofibroblast densities for all of the invasive layers than the lymph node-negative group.

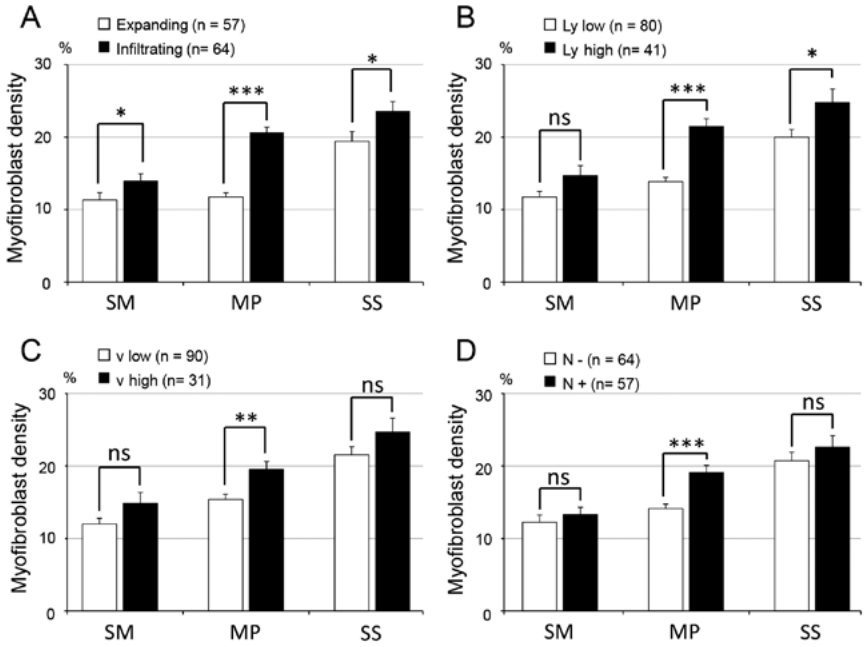

Figure 4. The mean myofibroblast density in each invasive level of colorectal cancer. The association between the invasive type and myofibroblast distribution (A). The association between the lymphatic invasion and myofibroblast distribution (B). The association between venous invasion and myofibroblast distribution (C). The association between lymph node metastasis and myofibroblast distribution (D). Values are given as the mean ± standard error of the mean. *P<0.05, **P<0.01 and ***P<0.001. ns, not significant. SM, submucosa; MP, muscularis propria; SS, subserosa.

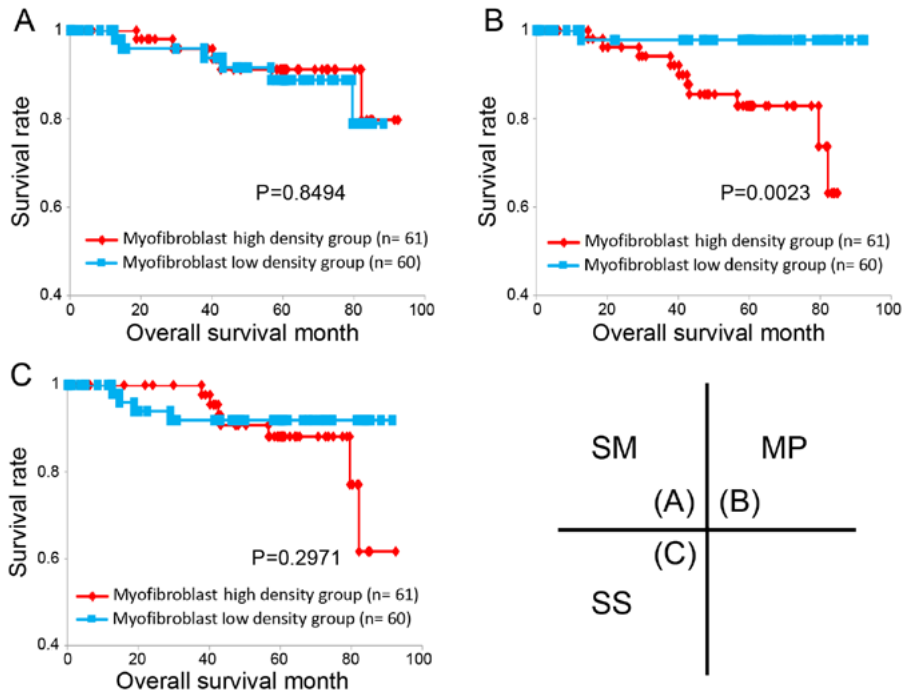

Figure 5. The association between the myofibroblast density of each invasive wall level and overall survival of patients with colorectal cancer. There was no significant difference between the high and low myofibroblast density groups in both the SM and SS invasive wall levels (A and C). There was a significant difference between the high and low myofibroblast density groups in the MP invasive wall level (B). SM, submucosa; MP, muscularis propria; SS, subserosa.

Furthermore, there was a significant difference between the lymph node metastasis-positive and -negative groups relating to the myofibroblast density of the MP layer (P<0.001). There were no significant differences between the two groups in regards to the myofibroblast density of the SM layer (P=0.33) and the SS layer (P=0.35).

Association between the myofibroblast density distribution and patient overall survival. To investigate the association between the myofibroblast distribution and overall survival, we stratified the 121 cases of CRC into either a low myofibroblast density group or a high density group in each invasive layer and compared the high and low groups regarding the overall survival

Table II. Univariate and multivariate analyses of prognostic factors of survival.

Variables	n (%)	Univariate analysis P-value	Multivariate analysis P-value
SM myofibroblast density		0.728	-
Low	61 (50.5)		
High	60 (49.5)		
MP myofibroblast density		0.025	0.332
Low	61 (50.5)		
High	60 (49.5)		
SS myofibroblast density		0.303	-
Low	61 (50.5)		
High	60 (49.5)		
Histological type		0.998	-
Well, mod	110 (90.9)		
Por, muc	11 (9.1)		
Invasive type		0.027	0.488
Expanding	57 (47.1)		
Infiltrating	64 (52.9)		
Lymphatic invasion		0.028	0.258
Low (ly0 or ly1)	80 (66.1)		
High (ly2 or ly3)	41 (33.9)		
Venous invasion		0.392	-
Low (v0 or v1)	90 (74.4)		
High (v2 or v3)	31 (25.6)		
Lymph node metastasis		0.319	-
Negative	64 (52.9)		
Positive	57 (47.1)		

SM, submucosa; MP, muscularis propria; SS, subserosa; well, well-differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; por, poorly differentiated adenocarcinoma; muc, mucinous adenocarcinoma. Mode of invasive type, as described in Materials and methods: ly, lymphatic invasion; v, venous invasion.

of the patients. The cut-off point between the two groups was set at the median value of the myofibroblast density in each invasive layer; the median values of the myofibroblast density were 11.52% in the SM layer, 16.19% in the MP layer and 21.48% in the SS layer. In only the MP level, but not the SM and SS layers, patients with high myofibroblast densities showed a significantly reduced overall survival (P<0.003; Fig. 5). To clarify the potential indicators, we analysed various pathological factors that were recorded in this study (Table II). Univariate analysis revealed that the following factors were correlated with poor prognosis: myofibroblast density of MP [relative risk (RR), 10.504; 95% confidence interval (CI), 1.344-82.09; P=0.025], invasive type (RR, 10.190; 95% CI, 1.302-79.75; P=0.027) and lymphatic invasion (RR, 4.4291; 95% CI, 1.175-16.7; P=0.028). In the multivariate analysis, there was no significant difference among the myofibroblast density of the MP layer, the invasive type and lymphatic invasion.

Discussion

In the present study, we evaluated the association between clinicopathological characteristics of CRC and the myofibroblast distribution of three invasive layers using image analysis. We revealed that the myofibroblast density of MP plays an important role in CRC malignant behaviors, such as lymphatic invasion, venous invasion and lymph node metastasis, which can result in short overall survival of the patients.

We found that as the invasion of the CRC became deeper, the number of myofibroblasts increased around the invasive lesions, and the infiltrating growth type had a significantly higher density of myofibroblasts than that noted in the expanding type. Previous studies identified that the infiltrating type of CRC carries a high risk of liver metastasis and a worse prognosis compared with the expanding type (20-22). Myofibroblasts are a type of cancer-associated fibroblasts (CAFs) and are involved in desmoplastic reactions (23). CAFs actively associate with neoplastic cells and form the ECM of cancer lesions that promote cancer growth, angiogenesis and survival (24). CAFs interact with adjacent cancer cells through soluble factors or direct cell-cell adhesion to promote cancer cell invasion (25). In malignancy of CRC, myofibroblasts also promote CRC invasion and metastasis as they proliferate around the invasive lesion and alter the adhesive and migratory properties of CRC cells (15,26). A previous study showed that myofibroblasts co-cultured with CRC cells may be involved in the invasiveness of CRC, even when the expression of E-cadherin, which is understood to be an adhesion molecule, prevents tumor cell invasiveness *in vitro* (27). Therefore, we suggest that it is possible that the large quantity of myofibroblasts which play a role as CAFs may alter both the adhesive and migratory properties of CRC cells and consequently aid CRC invasion into the deep colorectal layers. Moreover, our study indicated that the association between the infiltrating type, which is regarded as a malignant factor and myofibroblasts is stronger than the association between the expanding type and myofibroblasts.

Our results showed that the myofibroblast density of the MP layer was significantly higher in the group with a high frequency of lymphatic vessel and venous invasion compared with that in the group with a low frequency of lymphatic vessel and venous invasion. Furthermore, the lymph node-positive group had a significantly higher myofibroblast density in the MP layer than that of the lymph node-negative group. The lymphatic and venous vessels exist in three colorectal layers (SM, MP and SS), despite the differences in their histological structures. The distribution of lymphatic and venous vessels in normal colonic tissue tends to increase in frequency with depth throughout the wall (28). The functions of α-SMA-positive myofibroblasts may be associated with promoting the ECM of tumor cells and lymphogenesis of the metastatic microenvironment in oral tongue squamous cell carcinoma (29). With respect to CRC, proliferation of myofibroblasts in the peri-tumoral areas was predicted to play an important role in lymphangiogenesis and was also found to be associated with

lymph node metastasis (15). A previous study indicated that the CRC-invading MP layer may result in a greater ability to induce angiogenesis in adjacent normal tissue (30). Another study showed that the morphological mode of tumor invasion in the MP layer was associated with hematogenous metastasis of CRC (31). Our study predicted that compared to myofibroblasts of the other layers, myofibroblasts of the MP layer change the morphological mode of tumor invasion in CRC and increase the number of lymphatic and venous vessels that are invaded by CRC cells. Therefore, myofibroblasts of the MP layer are associated with the malignant potential of CRC, including lymph node metastasis.

The results of the univariate analysis revealed that myofibroblasts in the MP layer were significantly correlated with poor patient prognosis; however, the multivariate analysis using Cox proportional hazards model showed that a high myofibroblast density of MP was not an independent prognostic factor for overall survival. We suspected that the reason for this was that myofibroblasts of the MP layer may be strongly associated with the invasive growth pattern and lymphatic invasion.

In conclusion, we revealed that the myofibroblast distribution contributes to the malignant potential of CRC. Furthermore, we showed that myofibroblasts around the MP layer play an important role in the malignant potential and poor prognosis of CRC patients.

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