Stromal Myofibroblasts Predict Disease Recurrence for Colorectal Cancer

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Abstract

Purpose: Myofibroblasts, which are specifically differentiated fibroblasts, are thought to play a central role in the desmoplastic reaction, a dynamic stromal change closely associated with cancer development. Although fundamental studies suggest that myofibroblasts may either facilitate or inhibit cancer progression, cumulative evidence supports their role in promoting tumor progression. The aim of this study was to assess the value of myofibroblasts in the cancer stroma as an indicator of disease recurrence after colorectal cancer surgery.

Experimental Design: Using computer-assisted image analysis, we quantified myofibroblasts in the cancer-associated stroma of 192 colorectal cancers using α-smooth muscle actin as a marker.

Results: The cancer-associated stroma contained various numbers of myofibroblasts (0.35-19.0%; mean, 5.55 ± 3.85%). Tumors with abundant myofibroblasts were associated with shorter disease-free survival rate ($P = 0.001$) for stage II and III colorectal cancer. Multivariate analysis indicated that α-smooth muscle actin was a significant prognostic factor comparable with lymph node metastasis and superior to other tumor and stromal components, including histology of the tumor invasive front, peritumoral lymphocytic infiltration, and Crohn's-like lymphoid reaction. Moreover, colorectal cancers with synchronous liver metastasis generally displayed an active desmoplastic reaction, which was retained in the metastatic lesion to a similar extent.

Conclusions: The results suggest that the abundance of myofibroblasts in cancer-associated stroma may be a useful indicator of disease recurrence after curative colorectal cancer surgery.

Two decades ago, Dvorak (1) proposed the concept that tumors are wounds that do not heal. Since then, cancer research has focused on malignant transformed cells with regard to gene abnormalities, epigenetic changes, and altered gene expression. However, our comprehension of cancer-associated stroma, which is produced in association with cancer progression, is rather limited in comparison (2, 3). Cancer-associated stroma is a complex medium where a variety of interactions between tumor and host tissue cells take place. Tumor cells proliferate and invade the stroma where host immune cells congregate around tumor nests and tumor angiogenesis is promoted (4). Because the dynamic changes in the cancer-associated stroma resemble a wound-healing reaction (1), it is termed a desmoplastic reaction.

The desmoplastic reaction is thought to be supported mainly by the activation of host fibroblasts referred to as "myofibroblasts" (5–7). Myofibroblasts are differentiated host fibroblasts that express α-smooth muscle actin (α-SMA) as cytoplasmic microfilaments, and desmin to a limited extent, whereas quiescence host resident fibroblasts express vimentin as intermediate filament proteins (8). Myofibroblasts produce an extracellular matrix enriched in type III and V collagen, which is considered to be responsible for the hard consistency of many carcinomas (9).

Several fundamental studies on myofibroblasts have been conducted to clarify the role of cancer-associated desmoplastic reactions. Although a few studies showed that myofibroblasts might have a protective role against a subset of tumor cells (10, 11), other experiments suggested that myofibroblasts might have a supportive or facilitating role in tumorigenesis and progression of carcinomas of the prostate, breast, and keratinocytes (12–14). Thus, the clinical value of myofibroblasts has been flagged as a potentially important marker with respect to diagnosis, treatment, and prognosis of cancer (15). However, to date, a quantitative study of the value of myofibroblasts has not been undertaken.

To elucidate the clinical and biological relevance of the presence of myofibroblasts in cancer-associated stroma, we quantified myofibroblasts in the tumors from 192 patients with colorectal cancer using computer-assisted image analysis with α-SMA as a marker for myofibroblasts. Comparative analyses with collagen deposits in the cancer-associated

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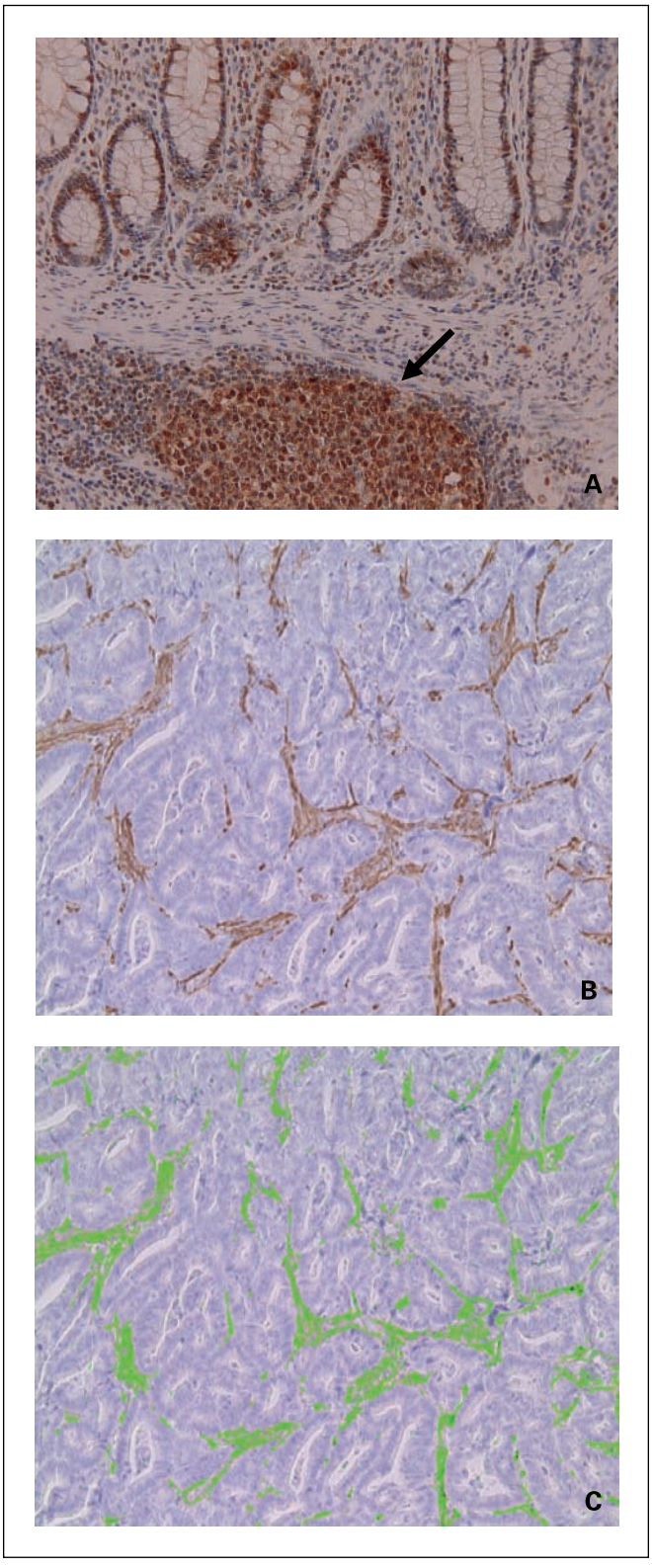


Fig. 1. *A,* representative sections stained with PCNA as a tissue quality control. Clear immunoreactivity was observed in the proliferative zone of colonic epithelium or germinal centers (*arrow*) of the lymphoid follicle. Magnification, ×100. *B,* immunostaining of α-SMA in a colorectal cancer tissue section. *C,* computer-assisted image for α-SMA expression. The expression level in this sample was determined to be 5.77%. Human cirrhotic liver tissue was used as a positive control for myofibroblasts (22). Magnification, ×50.

stroma suggest a specific role for myofibroblasts in tumor progression. For assessment of the prognostic power of the presence of myofibroblasts, we also evaluated other stromal constituents, host protective immune systems, such as Crohn's-like lymphoid reaction (CLR), peritumoral lymphocytic infiltration (PLI), and the histology of the invasive tumor front, as well as conventional clinical pathologic variables.

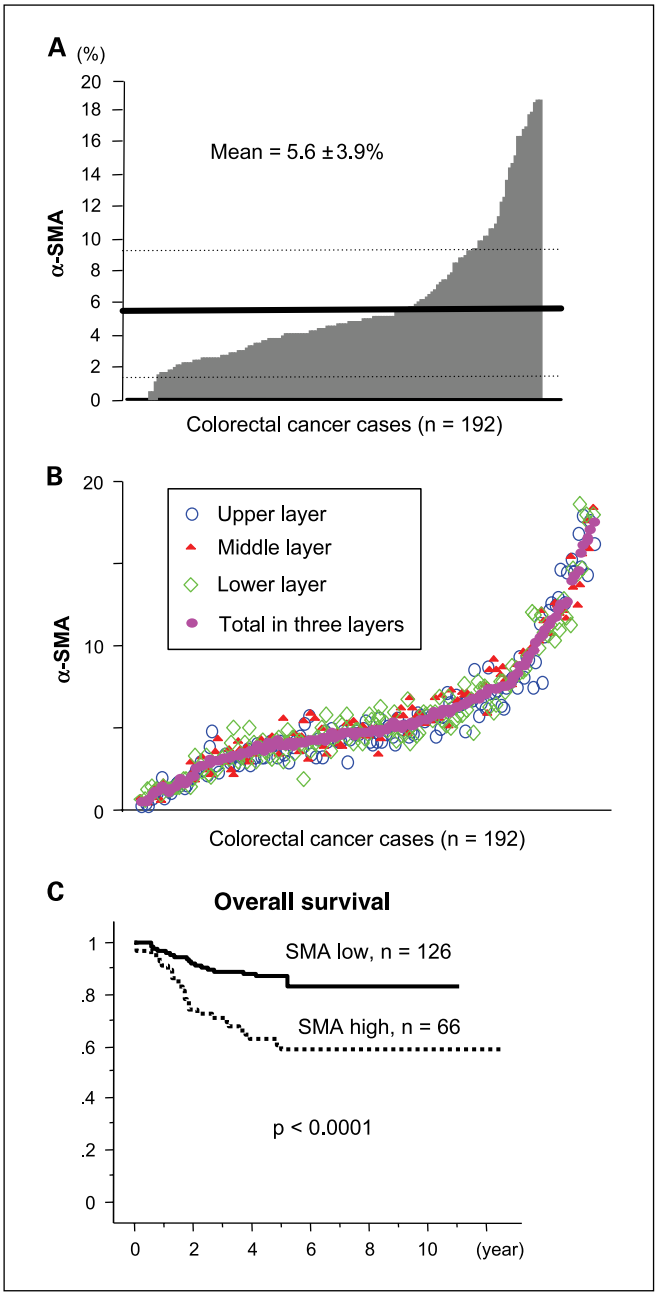


Fig. 2. *A,* expression of α-SMA in 192 stage I to IV colorectal cancer cases. Dotted line, ±SD range. *B,* α-SMA expression in the upper, middle, and lower layers of the cancer body. No significant difference was found between the averages of expression in the three layers. *C,* overall survival rate. When the cutoff was set at the mean expression value (5.55%), the 5-y disease-free survival rate of the high-expression group was significantly shorter than the low-expression group ($P < 0.0001$).

Table 1. α-SMA expression and clinicopathologic characteristics

Clinicopathologic characteristic		*n*	α-SMA expression		*P*
			High	Low	
Age (y)*		192	61.2 ± 9.83	63.5 ± 10.4	0.146
Tumor size (cm)*		192	4.6 ± 2.0	5.1 ± 1.9	0.096
Gender	Male	112	40	72	0.644
	Female	80	26	54	
Tumor site	Colon	111	42	69	0.237
	Rectum	81	24	57	
Degree of differentiation	Well	84	32	52	0.339
	Mod/Poor	108	34	74	
Depth of invasion	~mp	41	15	26	0.737
	ss~	151	51	100	
Lymph node metastasis	Absent	109	35	74	0.449
	Present	83	31	52	
Lymphatic invasion†	Absent	71	30	41	0.078
	Present	121	36	85	
Venous invasion	Absent	149	56	93	0.081
	Present	43	10	33	
	Total	192	66	126	

Abbreviations: Well, well-differentiated adenocarcinoma; Mod, moderately differentiated adenocarcinoma; Poor, poorly differentiated carcinoma (this category included five cases of poorly differentiated adenocarcinoma, five cases of mucinous carcinoma, and one case of signet ring cell carcinoma); mp, muscularis propria; ss, subserosa.
*Data are the mean ± SD.
†Determined by the presence of tumor cells in lymphatic ducts.

Materials and Methods

Patients and tissue samples. We randomly selected 192 patients who underwent surgery between 1991 and 1996, without knowledge of clinicopathologic features except for clinical stage. The tissue samples included 24 stage I, 79 stage II, 66 stage III, and 23 stage IV carcinomas according to the International Union Against Cancer tumor-node-metastasis classification (16). Fourteen stage IV patients had liver metastectomies because of synchronous metastasis to liver. The mean age of the patients was 62.7 ± 10.2 years (±SD), and the group consisted of 80 females and 112 males. The tumors were resected from either the colon (n = 111) or rectum (n = 81). The mean follow-up period was 59.7 ± 30.6 months. Postoperatively, stage III and IV patients received 5-fluorouracil–based chemotherapy, whereas stage I and II patients principally received no chemotherapy. The study protocol was approved by the Human Ethics Review Committee of the Graduate School of Medicine, Osaka University (Osaka, Japan).

H&E staining and immunohistochemistry. Tissue sections (4 μm thick) were prepared from formalin-fixed paraffin-embedded blocks and stained with H&E solution. Immunostaining was done using the Vectastain avidin-biotin complex method peroxidase kit (Vector Laboratories, Burlingame, CA) as described previously by our laboratories (17, 18). The dilutions of monoclonal antibodies used were as follows: (*a*) anti-human α-SMA (clone 1A4; DAKO, Carpinteria, CA), 1:50; (*b*) anti–proliferating cell nuclear antigen (PCNA; PC10; Novocastra, Newcastle upon Tyne, United Kingdom), 1:50. Nonimmunized mouse IgG (Vector Laboratories) was used as a substitute for the primary antibody in negative controls.

Computer-assisted image analysis. After staining for α-SMA in colorectal cancer tissue, the sections were viewed under a microscope equipped with a charge-coupled device color camera (Olympus Corp., Tokyo, Japan). A random selection of five fields each in the shallow, middle, and deep layers of the cancer body (a total of 15 fields per specimen) was assessed for α-SMA expression at high-power magnification. In assessment of deep layers, stroma area facing to the tumor frontier was mainly taken. The α-SMA expression in cancer-associated stroma was quantified as the relative percentage of the α-SMA area to the selected field area with an imaging processor, MacSCOPE software (Mitani Corp., Fukui, Japan), as described previously (19). The muscle layer was avoided for this assessment because muscle fibers exclusively express α-SMA. The α-SMA staining was also done in the tumors and liver metastases of stage IV patients who were eligible for liver metastectomies to compare levels of α-SMA expression in metastatic lesions with that in the tumor.

Collagen staining. Collagen staining was done using a collagen staining kit (Collagen Research Center, Tokyo, Japan) as described previously (18). Briefly, paraffin-embedded tissue sections were prepared in 4-μm thickness, deparaffinized in xylene, and rehydrated. After washing twice with PBS for 10 min each, the slides were incubated for 1 h at room temperature with staining solution A, which specifically reacts with collagen. The slides were then washed thrice with PBS and once with distilled water for 5 min each. The tissue fraction containing collagen is stained red. The quantification of collagen deposits in the cancer-associated stroma was executed by computer-assisted image analysis as well.

Assessment of CLR, PLI, and tumor growth characteristics. The density of the CLR, defined as lymphoid aggregates surrounding the periphery of the invasive carcinoma, was assessed as described previously (20). PLI was regarded as "conspicuous" when lymphocytes were scattered in a distinctive connective tissue mantle or cap at the invasive margin of the growth (21). Tumor growth characteristics were classified as expanding or infiltrating according to Jass et al. (21).

Statistical analysis. Statistical analysis was done using the StatView J-5.0 program (Abacus Concepts, Inc., Berkeley, CA). The Kaplan-Meier method was used to estimate tumor recurrence or death from colorectal cancer, and the log-rank test was used to determine the statistical significance. A Cox proportional hazards model was used to assess the risk ratio under simultaneous contributions from several covariates. Associations between discrete variables were assessed using the χ^2 test. Mean values were compared using the Student's t test. All data were expressed as the mean ± SD. P values of <0.05 were accepted as statistically significant.

Results

PCNA expression as quality control of the blocks. To verify the quality of the paraffin blocks, we stained the nontumor mucosa adjacent to the tumor with PCNA antibody. Intense immunoreactivity for PCNA was observed in the proliferative zone of the colonic epithelium or the germinal center of the lymphoid follicles in all samples tested (Fig. 1A). Cancer tissues generally expressed PCNA at variable levels (data not shown).

Expression of myofibroblasts in cancer-associated stroma and its prognostic value. Stromal myofibroblasts in 192 colorectal cancer samples were quantified using a computer-assisted image analysis as described in Materials and Methods. A representative photograph stained with α-SMA and the corresponding photo image treated with an imaging processor are shown in Fig. 1B and C, respectively (22). The α-SMA expression was found mostly in myofibroblasts, with a little in vascular pericytes. Tumor cells were negative for staining. When five random fields from the upper, middle, and lower layers of the cancer body were assessed for α-SMA expression (a total of 15 fields was measured), the α-SMA scores in all layers varied widely from 0.35% to 19.0% (mean, 5.55 ± 3.85%; Fig. 2A). However, overall, no significant difference was found between the averages of the three layers (Fig. 2B). The above evaluation of α-SMA was executed separately by two of the investigators (I.S. and C.Y.N.) with similar results. When the colorectal cancer cases were divided into two groups, a high α-SMA group (n = 66) and a low α-SMA group (n = 126) according to α-SMA expression at a cutoff point at the mean value of 5.55%, the patients with high α-SMA expression had a significantly poorer overall survival rate ($P < 0.0001$; Fig. 2C). When the colorectal cancers were divided according to the median value (4.36%), which equally divided colorectal cancer cases, a similar significant difference was obtained with regard to overall survival (data not shown). When α-SMA expression was compared with the various clinical and pathologic variables listed in Table 1, no significant associations were found.

Prognostic value of α-SMA for predicting disease recurrence of stage II and III colorectal cancers. We then analyzed 145 stage II and III colorectal cancer cases because these intermediate stages display a relatively variable prognosis, which is in contrast to the generally good or extremely poor prognosis for patients with stage I or IV cancers, respectively. Disease-free survival reflects the nature of cancer more specifically than does overall survival because the latter does not distinguish patients with recurrent disease from those without recurrence (23). Moreover, disease recurrence is a practical concern after curative surgery; therefore, further analyses were done based on disease recurrence. As shown in Fig. 3A, the patients with stage II and III cancers with high α-SMA expression had a shorter disease-free survival rate (P = 0.0004). The relationship between high α-SMA and shorter disease-free survival was maintained when

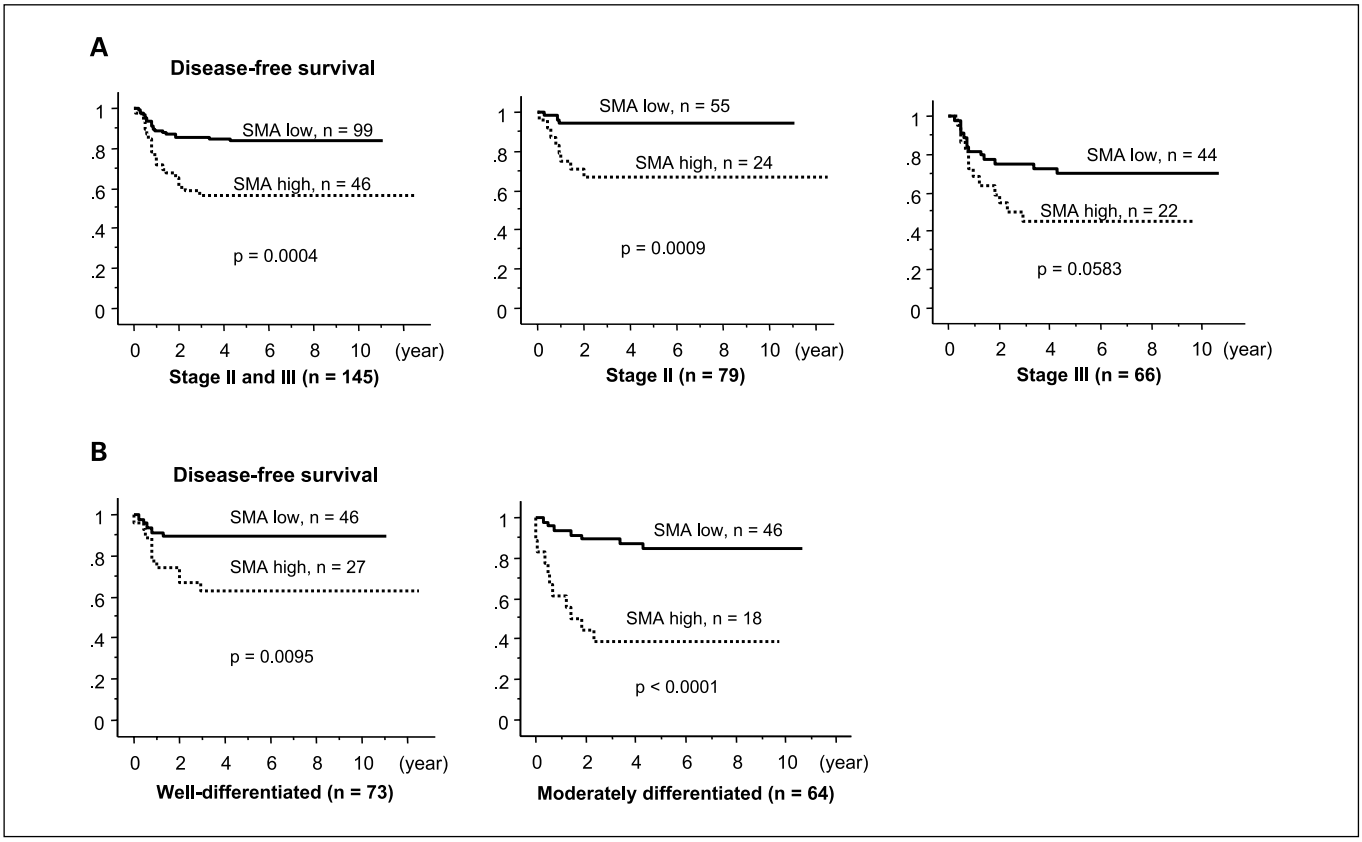


Fig. 3. Disease-free survival rate in stage II and III colorectal cancers. *A,* the 5-y disease-free survival rate of the high-expression group was significantly shorter compared with the low-expression group (P = 0.0004). A similar significant difference was maintained in stage II colorectal cancer. *B,* disease-free survival of well-differentiated adenocarcinoma and moderately differentiated adenocarcinoma. Significance was noted in each category. The number of poorly differentiated carcinomas (n = 11) was too small to be appropriately evaluated (data not shown).

analyzed separately in stage II (without lymph node metastasis) colorectal cancer alone (P = 0.0009), and marginal insignificance was noted in stage III (with lymph node metastasis) alone (P = 0.058). With regard to histologic grade in stage II and III colorectal cancers, high α-SMA expression was associated with poor prognosis within the categories of well-differentiated or moderately differentiated adenocarcinoma (P = 0.0095 and P < 0.0001, respectively; Fig. 3B).

Other components in cancer-associated stroma and tumor characteristics. We then assessed several other components of cancer-associated stroma with potential prognostic value in the same series of stage II and III colorectal cancers. These included the presence of conspicuous PLI (21) and a CLR at the advancing edge of the tumor (20). Conspicuous PLI and CLR were noted in 102 (70.3%) colorectal cancers and 53 (36.6%) colorectal cancers, respectively (Fig. 4A and B; Table 2). Based on the Jass classification (21), tumors were classified into two groups: expanding type (n = 68) and infiltrating type (n = 77), respectively (Fig. 4C and D). When the above stromal and tumor variables were evaluated for their relationship to α-SMA expression, a significant correlation was noted between α-SMA expression and infiltrating type (P = 0.046) but not others (data not shown). We then assessed the relationships between the clinicopathologic characteristics listed in Table 1. An inverse association was found between PLI and lymph node metastasis (P = 0.007). In addition, a strong association was noted between PLI and CLR (P < 0.0001). Associations were also found between infiltrating tumor margin and lymphatic invasion (P = 0.005).

Table 2 shows the results of the univariate analysis with regard to disease-free survival. Among conventional clinical and pathologic variables, lymph node metastasis was indicative of shorter disease-free survival (P = 0.001). The presence of conspicuous CLR or PLI was associated with a better prognosis (P = 0.036 and 0.004, respectively). Histologic features of the tumor margin also conferred a significant statistical result (P = 0.045), with the expanding type correlated with better prognosis. When the covariates with statistical significance were examined together with α-SMA expression, multivariate analysis showed that α-SMA expression and lymph node metastasis were retained as independent indicators of disease recurrence. The relative risk of recurrence in the high α-SMA group was 2.6-fold that of the low α-SMA group (P = 0.004), similar to that of lymph node metastasis (2.7-fold; P = 0.005; Table 3).

Collagen deposits in cancer-associated stroma. To address our concern that the stromal area itself might be indicative of disease recurrence, we also evaluated collagen deposits in the stromal area. One hundred samples were randomly selected from the stage II and III colorectal cancers, and collagen deposits in the cancer-associated stroma were determined by computer image analysis (Fig. 5). The percentage of collagen deposits was usually higher than the α-SMA score in each colorectal cancer case, ranging widely from 3.08% to 52.7% (mean, 19.7 ± 9.17%). No significant relationship was noted

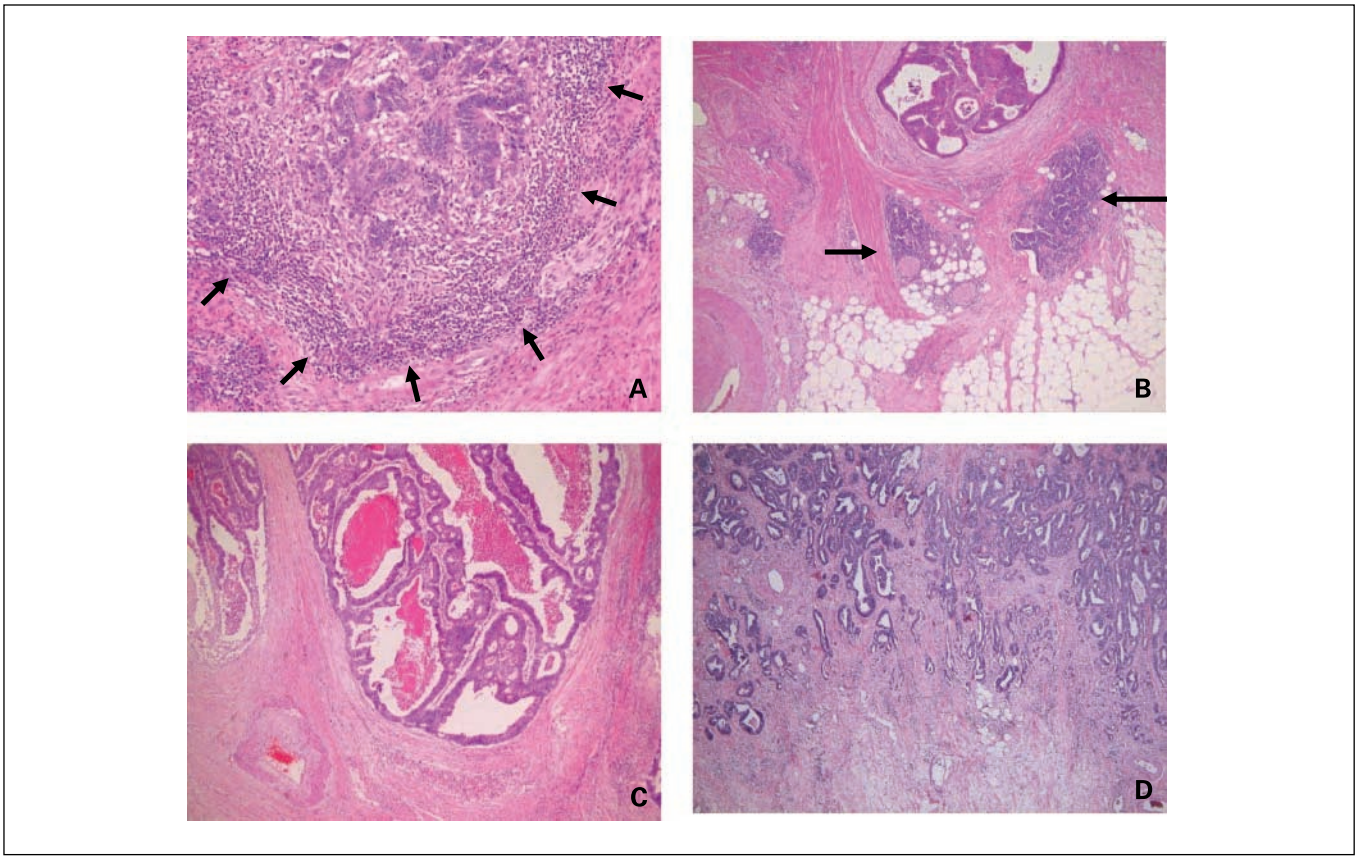


Fig. 4. Representative photographs. *A,* PLI. Arrows, conspicuous PLI is present. *B,* arrows, CLR at the advancing edge of the tumor. Histology of expanding (*C*) and infiltrating (*D*) types of the tumor front. Magnifications, ×50 (*A*, *C*, and *D*) and ×25 (*B*).

Table 2. Results of univariate survival analysis in stage II and III colorectal cancers

Characteristic	Category	*n*	Disease-free survival (%)	*P*
Age (y)	<63	72	73.6	0.708
	≥63	73	76.7	
Tumor size (cm)	≥4.9	81	75.3	0.952
	<4.9	64	75.0	
Gender	Male	81	77.8	0.502
	Female	64	71.9	
Tumor site	Colon	82	72.0	0.379
	Rectum	63	79.4	
Degree of differentiation	Well	72	80.6	0.144
	Mod/Poor	73	69.9	
Depth of invasion	~mp	14	64.3	0.208
	ss~	131	76.3	
Lymph node metastasis	Absent	79	86.1	0.001
	Present	66	62.1	
Lymphatic invasion	Absent	59	79.7	0.281
	Present	86	72.1	
Venous invasion	Absent	112	75.9	0.737
	Present	33	72.7	
Tumor margin	Expansive	68	82.4	0.045
	Infiltrating	77	68.8	
CLR	Conspicuous	53	84.9	0.036
	Inconspicuous	92	69.6	
PLI	Conspicuous	102	81.4	0.004
	Inconspicuous	43	60.5	

between collagen deposits and α-SMA expression (Fig. 5). In contrast to the clear predictive value of α-SMA for disease recurrence ($P = 0.0047$), collagen deposits did not exhibit such a feature (Fig. 5C). A clinical and pathologic survey indicated that collagen deposits correlated with deeper invasion ($P = 0.013$) and high-grade differentiation ($P = 0.045$) but not with the other variables (data not shown).

α-SMA expression in liver metastasis. To explore whether myofibroblast production could occur through a cancer-stroma interaction, we measured α-SMA levels in the primary colorectal cancers and synchronous liver metastasis from 14 stage IV patients. Hepatocytes did not express α-SMA, whereas liver metastasis produced cancer-associated stroma and expressed α-SMA (Fig. 6A and B). All the colorectal cancer samples except one expressed rather high levels of α-SMA, and a comparative analysis between each primary tumor and a corresponding metastatic lesion showed that both displayed similar α-SMA values (Fig. 6C).

Discussion

Cancer cells create a new tissue growth with a surrounding stroma that seems to provide a new avenue for the cancer to expand. However, the actual events between the tumor and stroma for this interaction to occur remain obscure (4, 24). A few earlier studies showed that a pronounced desmoplastic reaction was associated with an unfavorable prognosis in breast and colon carcinomas, whereas such significance was not observed in rectal cancer (25–27). One of the reasons for the controversy may be that the desmoplastic reaction was evaluated by the relative amount of "fibrous tissue" in these studies. At present, cumulative evidence supports the notion that myofibroblasts play a major role in the establishment of cancer-associated stoma (4, 6, 7, 15). It is reported that myofibroblasts appear during the wound-healing process and also during tumor progression and disappear with tissue reconstruction or tumor regression (1, 28). These findings suggest that the presence of myofibroblasts may be a sensitive marker of dynamic activity of the desmoplastic reaction rather than fibrous tissue per se. Consistently, the present study also revealed for the first time the superior prognostic value of myofibroblasts compared with stromal collagen deposits in colorectal cancer.

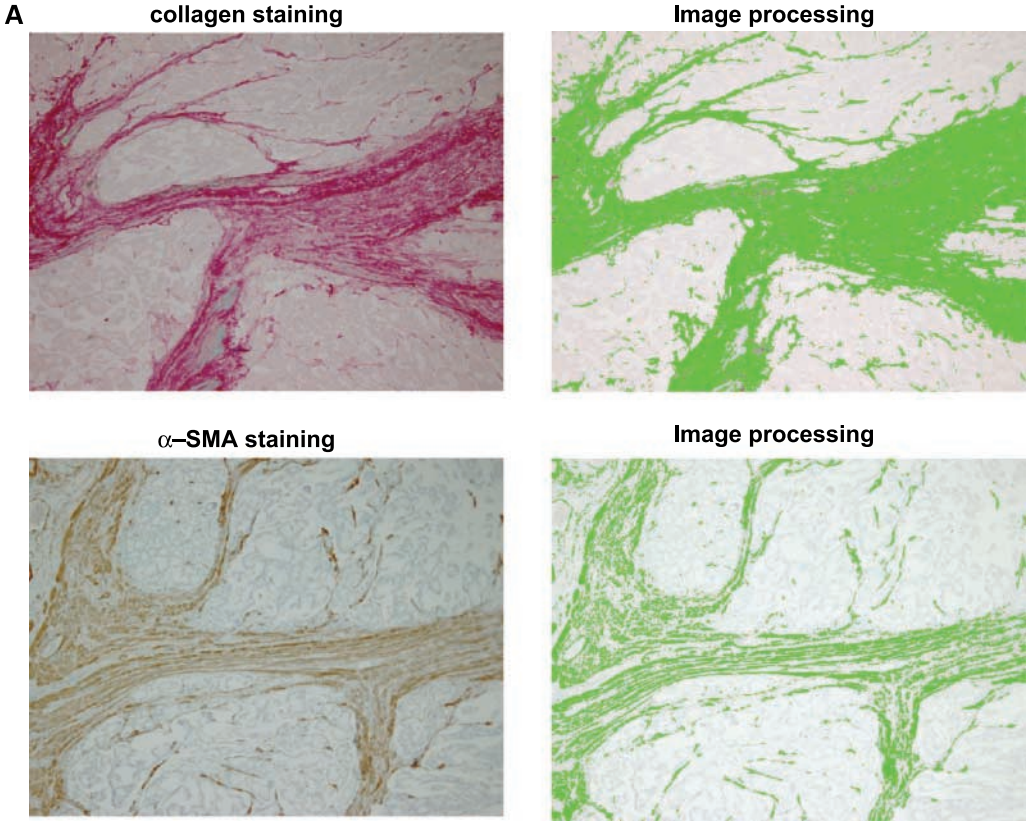
We found that α-SMA expression was a universal indicator of poor prognosis in the entire series of colorectal cancers, as well as in stage II colorectal cancer alone, and that it identified patients at high-risk for disease recurrence within the categories of well-differentiated or moderately differentiated adenocarcinomas. Taken together, these findings suggest that stromal myofibroblasts may have certain features that facilitate cancer progression irrespective of cancer spread to lymph nodes or tumor differentiation. In 2004, American Society of Clinical Oncology concluded that direct evidence did not support the routine use of adjuvant chemotherapy for patients with stage II colon cancer, although they acknowledged that the relative benefit in stage III disease might serve as an indirect evidence of benefit for stage II disease in considering the use of adjuvant chemotherapy for those patients with high-risk stage II disease

Table 3. Results of multivariate analysis

	P	Risk ratio (95% confidence interval)
α-SMA	0.004	2.65 (1.36-5.14)
Lymph node metastasis	0.006	2.75 (1.34-5.62)
Tumor margin	0.103	1.79 (0.89-3.62)
PLI	0.240	1.54 (0.75-3.17)
CLR	0.193	1.77 (0.75-4.17)

(29). In this regard, the ability to predict for disease recurrence in stage II disease is of particular clinical relevance because definitive markers, such as lymph node metastasis in stage III, have not been identified thus far.

The precise mechanism for why myofibroblast could be an independent prognostic factor is not clear at present. However, there is evidence that myofibroblasts modulate various aspect of tumor progression (24). For example, they are activated by



B

α-SMA(5.55%)	collagen (cut off 19.7%) high	low	
high	11	19	N.S.
low	37	33	
	48	52	

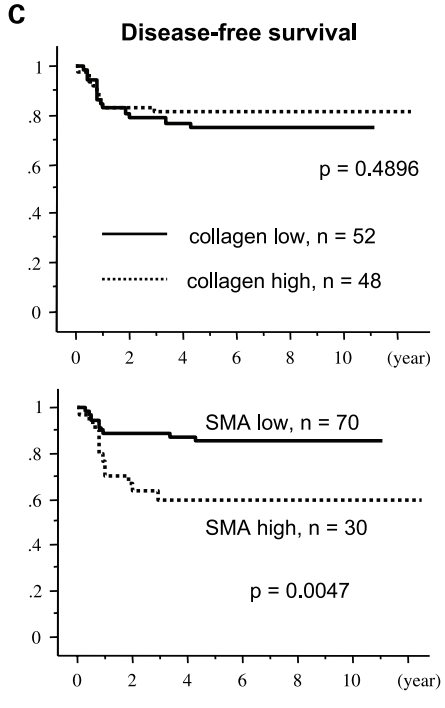


Fig. 5. Assessment of collagen deposits in the cancer-associated stroma. *A,* collagen deposits were more abundant than myofibroblasts. The mean value of collagen deposits was 19.7%. *B,* no association was noted between collagen deposits and α-SMA expression in stage II and III colorectal cancer tissues. *C,* the abundance of α-SMA, but not collagen deposits, affected survival.

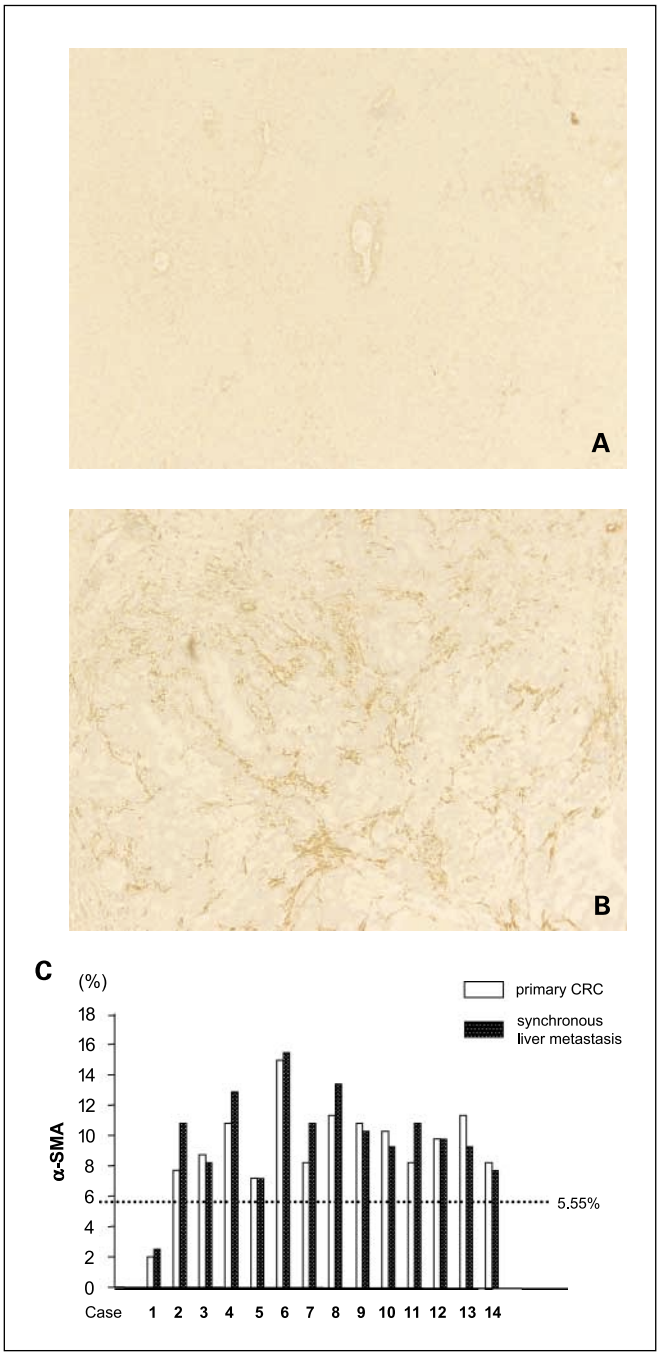


Fig. 6. Immunostaining of α-SMA in liver metastasis. *A,* hepatocytes did not express α-SMA. *B,* liver metastasis produced cancer-associated stroma and expressed α-SMA. Magnifications, ×25 (*A*) and ×50 (*B*). *C,* α-SMA expression in paired primary colorectal cancer and synchronous liver metastatic lesions. Note similar expression levels of α-SMA in each of the paired lesions, with high levels of α-SMA in 13 of 14 cases.

cytokines, such as transforming growth factor β produced by tumor cells, and in turn produce cytokines or growth factors that stimulate tumor cells leading to tumor cell proliferation or tumor invasion (30, 31). In addition, myofibroblasts express various cytokines and growth factors, such as vascular endothelial growth factor and its receptors, suggesting a role in tumor angiogenesis (32). A coculture system also revealed that tumor-derived fibroblasts might inhibit tumor cell death (33). It was also suggested that fibroblasts might produce proteinases, which are implicated in tumor invasion (34). Considered together, these findings suggest that the presence of myofibroblasts reflects an increased malignant behavior of the tumor.

To estimate the relative power of α-SMA as a prognostic factor, we evaluated several stromal and tumor-associated factors reported to be of prognostic value (20, 21). We also assessed host protective immune systems, including PLI and CLR. Notably, PLI and CLR displayed a positive relationship, and PLI was negatively associated with lymph node metastasis. The finding that both PLI and CLR displayed prognostic value in the univariate survival analysis suggested that the host immune system may, at least in part, play a protective role against colorectal cancer. However, the prognostic value of PLI and CLR disappeared in the multivariate analysis. In the multivariate analysis, α-SMA expression and lymph node metastasis were retained as significant prognostic factors. Although lymph node metastasis reflects cancer expansion, and thus serves as a powerful prognostic factor for colorectal cancer (16, 35, 36), our results identified the presence of stromal myofibroblast as a strong independent prognostic factor similar to lymph node metastasis.

The character of an individual cancer is thought to reflect the accumulation of numerous gene alterations. Recent DNA microarray analyses provide a great deal of information revealing "cancer personalities." It is postulated that cancers may produce the optimal microenvironment that favors its survival by evolving the surrounding stroma (4), possibly in large part through paracrine signaling between tumor cells and stromal fibroblasts (37). Our results showing a high concordance of α-SMA scores in pairs of primary tumors and their corresponding liver metastasis (Fig. 6) may support the hypothesis that individual cancers produce their own optimal microenvironment. In the context of a highly significant prognostic factor, myofibroblasts may reflect the malignant personality of cancer cells.

In conclusion, despite the retrospective nature of the study, it is apparent from the results that myofibroblasts may be a useful diagnostic tool in clinical practice and our data warrant a large-scale prospective study to establish the clinical utility of stromal myofibroblasts as prognostic indicators.

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