Cancer-associated fibroblasts correlate with poor prognosis in rectal cancer after chemoradiotherapy

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Abstract. Cancer-associated fibroblasts (CAFs) in the stroma play an important role in influencing the proliferation, invasion and metastasis of cancer cells. Fibroblast activation protein-α (FAP-α) is known as a marker of CAFs, while stromal cell-derived factor-1 (SDF-1) is primarily expressed by CAFs. Herein, we investigated whether the expression levels of these genes are associated with clinical outcome after pre-operative chemoradiotherapy (CRT) in rectal cancer patients. We obtained total RNA from residual cancer stroma using microdissection from a total of 52 rectal cancer specimens from patients who underwent pre-operative CRT, we performed transcriptional analyses, and the serum protein concentrations in 40 matched microdissected specimens were measured by enzyme-linked immunosorbent assay. Additionally, we sought to clarify the location of FAP-α and SDF-1 expression using immunohistochemical staining. Of the 52 patients, 15.6 and 36.8% showed detectable FAP-α and SDF-1 mRNA expression, respectively. A significant correlation was observed between stromal FAP-α and SDF-1 mRNA levels. Moreover, there was a significant correlation between stromal SDF-1 gene expression levels and serum protein levels. Patients who developed distant recurrences after CRT had positive expression of both genes ($P<0.05$). The positive expression of both genes was also associated with poor probability of recurrence-free and overall survival ($P<0.05$). Patients with elevated serum SDF-1 levels had equally poor overall survival as those with positive stromal SDF-1 gene expression ($P<0.05$). In immunohistochemistry, both FAP-α and SDF-1 expression was observed in certain activated fibroblasts. In conclusion, FAP-α and SDF-1 expression was shown to be involved in tumor re-growth and recurrence in rectal cancer patients treated with pre-operative CRT.

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Introduction

Rectal cancer is one of the most common cancers in Japan as well as in the western world. The introduction of pre-operative chemoradiotherapy (CRT) and total mesorectal excision (TME) in the management of rectal cancer has significantly decreased local recurrence rates and has improved patient survival. However, the rate of distant recurrent relapse remains as high as 15 to 20% in rectal cancer patients treated with pre-operative CRT followed by TME (1-3). The identification of predictive markers for distant recurrence should improve both clinical outcome and potential treatment stratification for such patients.

Over the years, the importance of the cancer stroma surrounding the tumor in the proliferation, invasion and metastasis of cancer cells has been increasingly recognized (4-7). For example, Finak *et al* investigated stromal gene expression in primary breast cancer and constructed a stroma-derived prognostic predictor, which was independent from all standard prognostic factors (8). This result suggests the importance of the cancer stroma in tumor progression, and underscores the need for useful prognostic tumor stroma markers. Among stromal cells, cancer-associated fibroblasts (CAFs), which play an important role in tumor progression and regulate the microenvironment, have recently attracted attention.

Fibroblast activation protein-α (FAP-α), also known as seprase, is a cell-surface serine protease. FAP-α is highly expressed in CAFs, but not in normal human tissue (9-11). Therefore, FAP-α is recognized as a marker for CAFs. FAP-α expression has been reported to be associated with tumor growth through the promotion of angiogenesis in breast cancer (12). Additionally, Lee *et al* reported that FAP-α expressed by CAFs has the potential to function as a tumor rejection antigen in many cancers in both *in vitro* and *in vivo* studies (13).

Stromal cell-derived factor-1 (SDF-1), also known as the chemokine CXCL12, is primarily expressed by CAFs, and the expression of SDF-1 has been reported to be associated with the migration, invasion and proliferation of tumor cells (7,14,15). SDF-1 is expressed by stromal cells, such as fibroblasts and endothelial cells (16). In colorectal cancer, elevated SDF-1 expression is associated with metastasis and poor prognosis (17,18).

We investigated whether stromal FAP-α and SDF-1 expression levels are associated with clinical outcome, and whether these mRNA levels are correlated with their serum levels in patients with rectal cancer after CRT.

Materials and methods

Patients and specimens. From 2001 to 2008, 52 patients with rectal cancer (clinical stage II/III based on the TNM Classification established by the International Union Against Cancer) underwent pre-operative CRT followed by surgery at our institute. Post-CRT formalin-fixed, paraffin-embedded (FFPE) specimens were available for use in this study. Peripheral venous blood samples were obtained from 40 patients matched to microdissected specimens after CRT. Serum samples were allowed to clot and serum was stored at -80˚C until use. All patients signed informed consent forms for their tissues to be used in this study.

5-Fluorouracil-based chemoradiotherapy regimen. The chemoradiotherapy regimen included 4 cycles of 5-fluorouracil (5-FU) given as a 24-h, 600-mg/m^2 continuous infusion, and tegafur-uracil (UFT) given 400 mg/m^2 orally for 5 days, with concurrent 20-45 Gy radiation, followed by resection. This regimen was based on the previously tested combination of continuous-infusion 5-FU plus UFT. The time interval between pre-operative CRT and surgery was 2-3 weeks in surgical patients (19). A total of 42 patients received short-course radiation at 20 Gy in 4 fractions over 1 week. The remaining 11 patients received conventionally fractionated radiation at a dose of 45 Gy in 25 fractions for 4 weeks. All patients underwent standard surgery including TME, and received 5-FU-based adjuvant chemotherapy after surgery for 6 months to 1 year.

Clinical response and histopathological tumor regression. The clinical response after pre-operative CRT was evaluated by barium enema, endoscopy and magnetic resonance imaging, and was then graded as complete response (CR), partial response (PR), no change (NC), or progressive disease (PD). The degree of histopathological tumor regression based on the Guidelines for the Clinical and Pathological Studies on Carcinoma of the Colorectum was classified into five categories: Grade 0, no necrosis or regressive changes; grade 1a, more than two-thirds vital residual tumor cells (VRTCs); grade 1b, approximately one-third to two-thirds VRTCs; grade 2, fewer than one-third VRTCs; and grade 3, no VRTCs (20). We defined non-responders as histopathological tumor regression grade 0 to 1b, and responders as grade 2 and 3.

Microdissection of FFPE specimens. FFPE specimens cut in 10-μm sections were stained with nuclear fast red and subsequently manually microdissected to collect stromal cells separated from residual cancer cells with reference to hematoxylin and eosin slides.

RNA extraction from FFPE specimens. Microdissected samples were digested with proteinase K in lysis buffer containing Tris-HCl, ethylenediaminetetraacetic acid, and sodium dodecyl sulfate, as previously described, with minor modifications (21). RNA was purified by phenol-chloroform extraction.

cDNA synthesis. cDNA was synthesized with random hexamer primers and SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (RT-PCR). Quantitative RT-PCR analysis was performed with the SYBR-Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) using the Applied Biosystems 7500 Real-Time PCR System according to the manufacturer's instructions. Primers for FAP-α, SDF-1 and β-actin were designed with Primer3 software (Biology Workbench Version 3.2, San Diego Supercomputer Center, University of California, San Diego, CA). The following primer sequences were used: FAP-α-specific primers (sense, CACCTGATCGGCAATTTGTA; antisense, TTGGACGAGGAAGCTTT), SDF-1-specific primers (sense, ATGAACGCCAAGGTCGTG; antisense, ACATGGCTTTCGAAGAATCG), and β-actin (sense, ACAGAGCCTCGCCTTTGC; antisense, GCGGCGATATCA TCATCC). PCR was performed in a final volume of 25 μl with SYBR-Green PCR Master Mix, using 1 μl cDNA and 400 nM of each primer for the respective genes. Cycling conditions were 50˚C for 2 min and 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min each.

Relative expression levels of FAP-α and SDF-1. Relative gene expression levels were determined by the standard curve method. Standard curves and line equations were generated using 5-fold serially diluted solutions of cDNA from qPCR Human Reference Total RNA (Clontech, Mountain View, CA, USA) for FAP-α and SDF-1. All standard curves were linear in the analyzed range with an acceptable correlation co-efficient. The amount of target gene expression was calculated from the standard curve followed by quantitative normalization of cDNA in each sample using β-actin gene expression as the internal control. Target gene mRNA levels were described as ratios to β-actin mRNA levels. RT-PCR assays were performed in duplicate for each sample. Cases in which the β-actin mRNA level was unstable (not reliable) or not available were excluded from this study.

Serum FAP-1a and SDF-1 levels. We investigated whether FAP-1α and SDF-1 mRNA expression levels in cancer stroma influenced serum FAP-1α and SDF-1 protein levels by enzyme-linked immunosorbent assay (ELISA). FAP-1α and SDF-1 concentrations were measured using ELISA kits for human FAP (catalog no: DY3715, R&D Systems, Minneapolis, MN, USA) and human CXCL12/SDF-1α (catalog no: DSA00, SSA00 and PDSA00, R&D Systems), respectively, using the

sandwich method. The lower limit of detection for serum FAP-1α and SDF-1 concentration was 0.01 pg/ml.

Immunohistochemistry for FAP-α and SDF-1. FFPE specimens were sliced into 2-3-µm sections. After deparaffinization and dehydration, specimens were brought to a boil in 10 mM sodium citrate buffer for antigen unmasking. Specimens were then blocked and incubated with primary antibody overnight at 4˚C. The antibody was detected using Envision reagents (Envision kit/HRP, Dako Cytomation, Denmark). Primary monoclonal anti-human CXCL12/SDF-1 antibody (clone 79018, R&D Systems) and rabbit polyclonal FAP-α antibody (ab53066; Abcam, Cambridge, MA, USA), were used at a dilution of 1:100, for implementation of the labeled streptavidin-biotin method (LASB2 kit/HRP, Dako Cytomation), and 3,3'-diaminobenzidine (Dako Cytomation). All sections were counterstained with hematoxylin, and were dehydrated and mounted. Negative controls were also run simultaneously.

Statistical analysis. All statistical analyses were done using Stat View 5.0 for Windows (SAS Institute Inc., Cary, NC, USA). The contingency tables were analyzed using the Chi-square test with Yates' correction. The correlations among mRNA levels and serum concentration were assessed with the Spearman rank correlation co-efficient. A non-parametric receiver operating characteristic (ROC) analysis was performed to calculate the best cut-off value for each serum level that would be predictive of distant recurrence and survival, using Medcalc 7.2 for Windows (Mariakerke, Belgium). Disease-free and overall survival probabilities were calculated using the Kaplan-Meier product limit method and intergroup differences were determined using the log-rank test. The influence of distant recurrence and survival predictors identified by univariate analysis was assessed by multivariate analysis using Cox's proportional hazards model. Two-sided P-values of <0.05 were considered to be statisticaly signficant.

Table I. Patient and tumor characteristics.

Variables	Number (%)
Gender	
Male	42 (81)
Female	10 (19)
Age (mean; 64.5 years)	
<65	26 (50)
≥65	26 (50)
T classification	
1/2	17 (33)
3/4	35 (67)
N classification	
Absent	35 (67)
Present	17 (33)
Post-operative stage	
I/II	33 (64)
III	19 (36)
Lymphatic invasion	
Absent	13 (25)
Present	39 (75)
Vascular invasion	
Absent	22 (42)
Present	30 (58)
Histology	
Well/moderate	44 (85)
Poorly/signet/mucinous	8 (15)
Pathological response	
Non-responder, grade 0/1a/1b	36 (69)
Responder, grade 2/3	16 (31)
Distant recurrence	
Absent	44 (85)
Present	8 (15)

Results

Patient and tumor characteristics. Fifty-two patients were included in this study. Their median age was 64 years (range, 37 to 78 years) and the male-to-female ratio was 4.2:1. Post-CRT pathological T stages were pT1 (n=5), pT2 (n=13), pT3 (n=32) and pT4 (n=2). Seventeen patients (33%) had pathological lymph node metastases. Thirty-three patients (64%) had post-operative stage I and II. Forty-four tumors (85%) showed well- or moderately-differentiated adenocarcinoma histology. No patients had local failure. Patterns of distant recurrence included liver and lung metastases (n=2), lung metastasis alone (n=5) and peritoneal metastasis (n=1). Histopathological tumor regression grades included grade 0 (n=0), grade 1a (n=11), grade 1b (n=25) and grade 2 (n=16) (Table I). The median follow-up period was 43 months (range, 14 to 105 months).

Stromal FAP-α and SDF-1 gene expression. Quantitative RT-PCR revealed that of the 52 patients, 7 (15.6%) and 14 (36.8%) showed detectable FAP-α and SDF-1 mRNA expression, respectively, in residual rectal cancer stroma after CRT, whereas the remaining patients had no detectable expression despite positive gene expression of β-actin. FAP-α and SDF-1 mRNA levels were 0.118±0.108 (range, 0-5.633) and 0.2975±0.2665 (range, 0-13.86), respectively. Patients were divided into two groups according to the positivity of each gene expression.

Serum FAP-α and SDF-1 concentration in rectal cancer after CRT. Serum FAP-α and SDF-1 concentration in a total of 40 serum samples matched the microdissected specimens were measured by enzyme-linked immunosorbent assay. Serum concentrations of FAP-α and SDF-1 were 4.424±0.299 ng/ml (range, 0.362-6.477) and 2.580±0.067 ng/ml (range, 1.763-3.404), respectively.

Association between FAP-α and SDF-1 expression levels and clinicopathological variables. Table II shows the association between FAP-α and SDF-1 gene expression levels and clinicopathological variables. Positive gene expressions of FAP-α and SDF-1 were significantly correlated with distant recurrence (FAP-α, P=0.030; and SDF-1, P=0.014, respectively). No significant correlation was noted between the positivity of these genes and other clinicopathological variables. In serum FAP-1α and SDF-1 levels, no associations between the serum

Table II. Association of FAP-α and SDF-1 gene expression with clinicopathological variables.

Variables	FAP-α gene expression			SDF-1 gene expression		
	Negative	Positive	P-value	Negative	Positive	P-value
Gender						
Male	37	5	0.500	30	12	0.583
Female	8	2		8	2	
Age (mean; 62 years)						
<65	23	3	0.685	19	7	>0.9999
≥65	22	4		19	7	
T classification						
1/2	17	1	0.224	13	5	0.920
3/4	28	6		25	9	
N classification						
Absent	30	5	0.803	28	7	0.106
Present	15	2		10	7	
Post-operative stage						
I/II	28	5	0.638	26	7	0.221
III	27	2		12	7	
Lymphatic invasion						
Absent	11	2	0.815	11	2	0.279
Present	34	5		27	12	
Vascular invasion						
Absent	20	2	0.429	16	6	0.961
Present	25	5		22	8	
Histology						
Well/moderate	38	6	0.931	31	13	0.317
Poorly/signet/mucinous	7	1		7	1	
Pathological response						
Non-responder, grade 0/1a/1b	30	6	0.310	25	11	0.376
Responder, grade 2/3	15	1		13	3	
Distant recurrence						
Absent	40	4	0.030	35	3	0.014
Present	5	3		9	5	

Table III. Correlation among stromal mRNA level and serum concentration.

	Spearman's ϱ	P-value
Stroma FAP-α mRNA/stroma SDF-1 mRNA	0.760	<0.001
Stroma FAP-α mRNA/serum FAP-α concentration	0.080	0.628
Stroma SDF-1 mRNA/serum SDF-1 concentration	0.337	0.036
Serum FAP-α concentration/serum SDF-1 concentration	0.127	0.440

levels of these proteins and other clinicopathological variables were observed (data not shown).

Correlation between FAP-α and SDF-1 expression. A significant positive correlation was observed between FAP-α and SDF-1 gene expression levels in residual cancer stroma (Spearman's ϱ=0.760, P<0.0001). A significant correlation was also observed between the stromal SDF-1 mRNA level and serum SDF-1 concentration (Spearman's ϱ=0.337, P=0.036), but not between the FAP-α stromal mRNA level and serum FAP-α protein level (Table III).

Predictive value of FAP-α and SDF-1 expression levels for distant recurrence and survival. On the basis of these results, ROC analysis was used to identify each cut-off value of FAP-α and SDF-1 serum concentrations that was predictive of distant recurrence and survival. A non-parametric ROC analysis determined that the optimal cut-off values of FAP-α and

Table IV. Uni- and multivariate analyses for predictors of distant recurrence and survival.

A, Predictors of distant recurrence

Variables	Hazard ratio	95% CI	P-value
Univariate analysis			
T classification (T1/2 vs. T3/4)	0.244	0.134-3.322	0.622
N classification (absent vs. present)	3.678	0.059-1.032	0.055
Post-operative stage (I/II vs. III)	2.854	0.069-1.219	0.091
FAP-α gene expression (negative vs. positive)	6.223	0.030-0.658	0.013
SDF-1 gene expression (negative vs. positive)	7.478	0.018-0.517	0.006
Serum FAP-α (≥cut-off vs. <cut-off)	0.222	0.275-8.249	0.637
Serum SDF-1 (≥cut-off vs. <cut-off)	0.099	0.169-11.718	0.753
Multivariate analysis			
FAP-α gene expression (negative vs. positive)	0.429	0.087-2.830	0.429
SDF-1 gene expression (negative vs. positive)	4.159	0.020-0.925	0.041

B, Predictors of survival

Variables	Hazard ratio	95% CI	P-value
Univariate analysis			
T classification (T1/2 vs. T3/4)	0.035	0.233-3.328	0.852
N classification (absent vs. present)	2.913	0.108-1.166	0.088
Post-operative stage (I/II vs. III)	6.022	0.050-0.715	0.014
Distant recurrence (absent vs. present)	5.412	0.066-0.799	0.020
FAP-α gene expression (negative vs. positive)	3.960	0.059-0.979	0.047
SDF-1 gene expression (negative vs. positive)	8.181	0.044-0.558	0.004
Serum FAP-α (≥cut-off vs. <cut-off)	1.300	0.511-12.648	0.254
Serum SDF-1(≥cut-off vs <cut-off)	3.053	0.019-1.256	0.081
Multivariate analysis			
Post-operative stage (I/II vs. III)	6.165	0.033-0.668	0.013
Distant recurrence (absent vs. present)	0.040	0.159-4.472	0.841
FAP-α gene expression (negative vs. positive)	0.141	0.197-11.198	0.707
SDF-1 gene expression (negative vs. positive)	5.589	0.015-0.675	0.018

CI, confidence interval.

SDF-1 were 3.271 and 3.007 ng/ml for distant recurrence, and 5.448 and 2.397 ng/ml for survival. Cox's univariate proportional hazards analysis showed that positive FAP-α and SDF-1 gene expression in residual cancer stroma was significantly associated with a higher rate of developing distant recurrence after pre-operative CRT compared to negative cases (P=0.013 and P=0.006, respectively). In multivariate analysis, positive SDF-1 gene expression independently predicted distant recurrence after CRT followed by surgery (P=0.041) (Table IVA). Regarding survival, Cox's univariate proportional hazard analysis showed that post-operative stage, distant recurrence, positive stromal SDF-1 and FAP-α gene expression were significantly associated with poor survival (P=0.014, P=0.020, P=0.004 and P=0.047, respectively). Cox's multivariate hazard model analysis showed that post-operative stage and positive stromal SDF-1 gene expression independently predicted poor survival (P=0.013 and P=0.018, respectively) (Table IVB).

As shown in Fig. 1A and B, patients with positive FAP-α and SDF-1 gene expression in cancer stroma tissue experienced significantly worse recurrence-free survival (FAP-α, P=0.0038; SDF-1, P=0.0008). Moreover, the positive expression of both of these genes was significantly associated with poor overall survival (FAP-α, P=0.0310; SDF-1, P=0.0012). Fig. 1C and D show the association of each serum level with recurrence-free and overall survival. An elevated serum SDF-1 level was associated with poor overall survival (P=0.0442).

Immunohistochemical analysis of FAP-α and SDF-1 expression. As shown in Fig. 2, FAP-α and SDF-1 staining were detected not only in stromal tissue but also in residual cancer cells. FAP-α staining was present in the cytoplasm of residual cancer cells, stromal fibroblasts, endothelial cells and intravascular granules. SDF-1 expression was observed in the cytoplasm of residual cancer cells, as well as in stromal fibroblasts, endothelial cells and neural cells. FAP-α and SDF-1 expression was also observed in activated fibroblasts. In these fibroblasts, FAP-α expression was observed in the nuclei and cytoplasm, while SDF-1 was expressed in the cytoplasm.

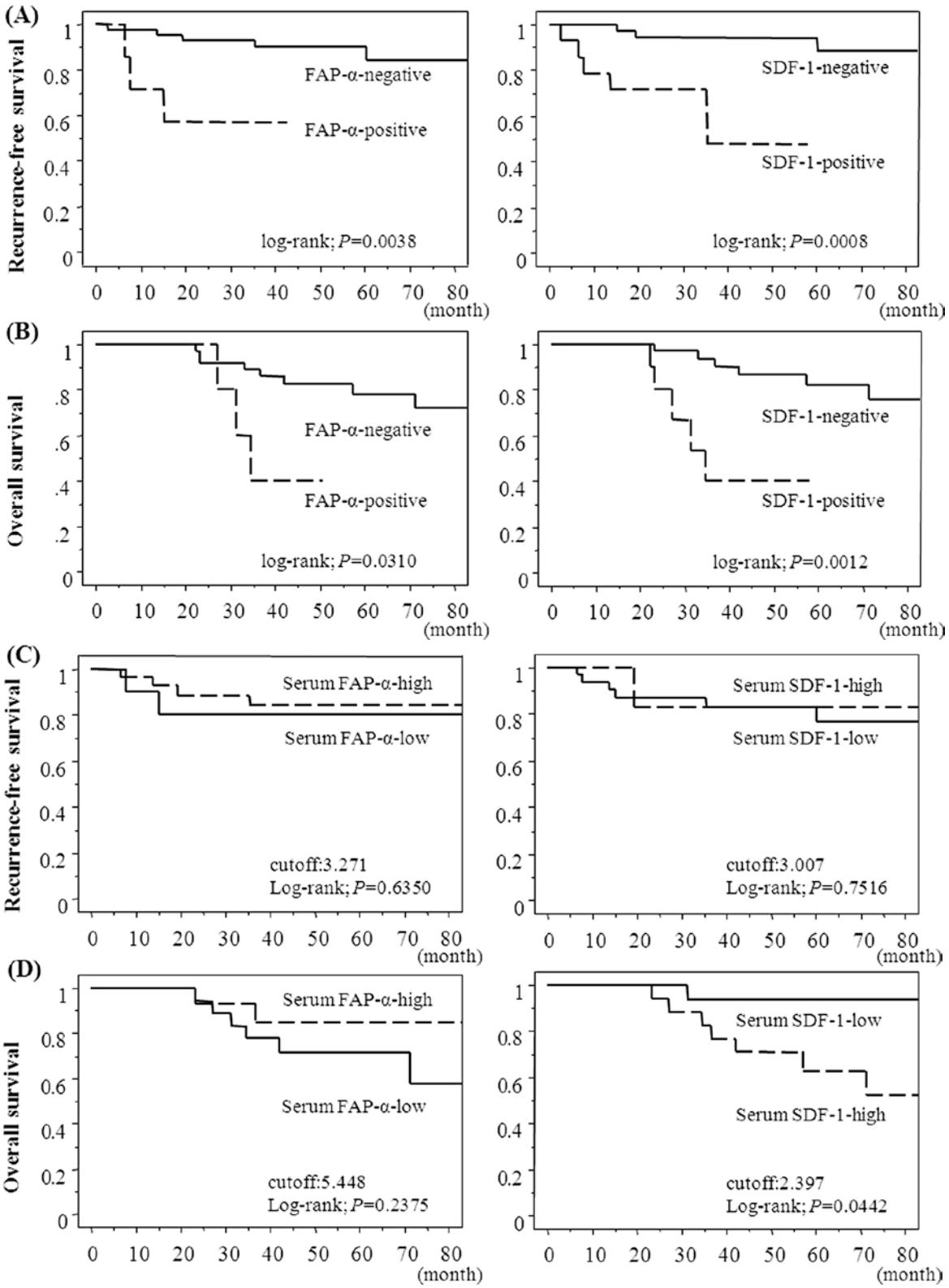

Figure 1. Recurrence-free survival (A) and overall survival (B) curves according to the positivity of FAP-α and SDF-1 mRNA expression in stromal tissue after CRT. Recurrence-free survival (C) and overall survival (D) curves in patients subdivided on the basis of serum FAP-α and SDF-1 levels using individual cut-off values.

Discussion

Over the years, the interaction between cancer and stromal cells in tumor growth, invasion and metastasis has become increasingly clear (4-6). Cancer tissue is composed not only of cancer cells but also of cancer-associated stromal cells, including extracellular matrix molecules, endothelial cells, fibroblasts and immune cells. CAFs have been reported to release potentially oncogenic signals such as the transforming growth factor-β (TGF-β) and vascular endothelial growth

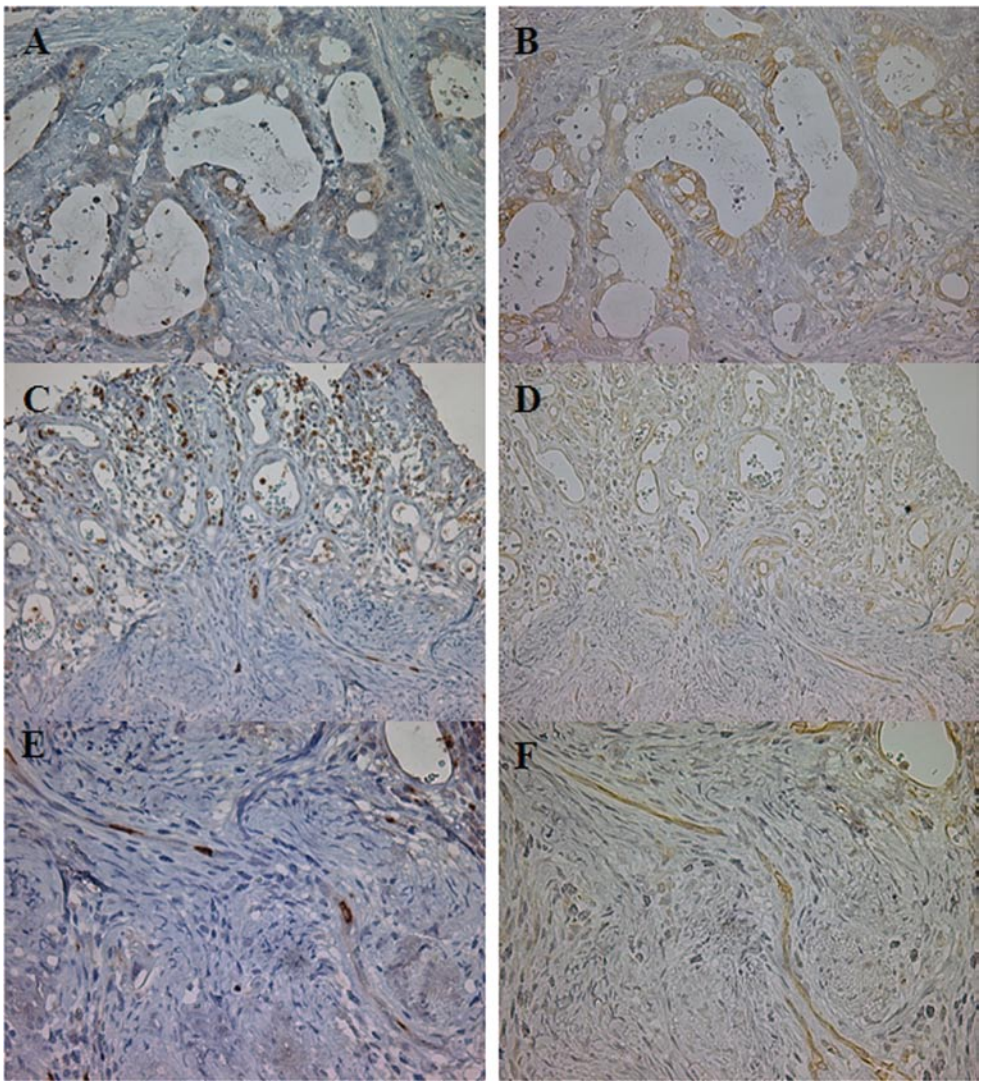

Figure 2. FAP-α (A) and SDF-1 (B) expression in residual cancer cells. Both FAP-α (C) and SDF-1 (D) are expressed in endothelial cells. FAP-α (E) and SDF-1 (F) expression in activated fibroblasts (E and F). Original magnification, x200 (A-D), x400 (E and F).

factor (22). Additionally, Karnoub *et al* showed that the co-injection of breast cancer cells and bone marrow-derived mesenchymal stem cells promoted their metastatic activity via signaling through the chemokine CCL5 in an *in vivo* study (23). This result suggests a direct pro-metastatic effect of CAFs. Therefore, CAFs play an important role in both tumor progression and regulation of the tumor microenvironment.

Certain studies have suggested that in colorectal cancer, CAFs are associated with poor prognosis and distant metastasis (4,5,24). However, the evaluation of FAP-α and SDF-1 expression as CAF-associated markers and the association of their expression with clinicopathological variables in rectal cancer after CRT, have not been previously reported. In the present study, we found that the expression of FAP-α and SDF-1 in residual rectal cancer stroma after CRT was associated with distant recurrence and poor prognosis, although the rate of detection of their gene expressions was very low. Furthermore, we found that FAP-α and SDF-1 expression levels exhibited a significant positive correlation to each other in residual cancer stroma after CRT. Immunohistochemical results demonstrated that these proteins were expressed in activated fibroblasts. These results suggest that CAFs also play an important role in distant recurrence in rectal cancer after CRT. Hwang *et al* demonstrated that CAFs can influence chemo- and radio-sensitivity by using pancreatic cancer cells co-cultured in CAF-conditioned medium (25). However, no significant association between FAP-α and SDF-1 gene expression and histopathological response after CRT were observed in the present study.

Residual cancer cells were separated from stromal tissue using microdissection to enable accurate evaluation of FAP-α and SDF-1 gene expression in stromal tissue. We speculated that mRNA levels in stromal tissue might affect serum protein levels if these gene products are host-derived factors that promote re-growth and distant metastasis after CRT. While a correlation was observed between the SDF-1 mRNA level in stromal tissue and serum SDF-1 levels, no such correlation was found for FAP-α. Additionally, patients with elevated serum SDF-1 concentration had equally poor overall survival as those with positive stromal SDF-1 gene expression. One possible reason for these different results in FAP-α and SDF-1 is that SDF-1 is one of many chemokines, while FAP-α is a

cell-surface serine protease that acts only locally. Our results suggest that serum SDF-1 levels can reflect the local microenvironment surrounding residual cancer cells.

Many studies have reported that FAP-α expression in stromal tissue is correlated with tumor invasion and metastasis in colorectal cancer based on immunohistochemical analysis (26,27). Our results also suggest that FAP-α expression can be used to predict poor prognosis in rectal cancer patients who have undergone pre-operative CRT.

The origin of CAFs has been suggested to be local fibroblasts or bone marrow-derived cells that are recruited into the developing tumor and adopt a CAF phenotype. These cells originate from epithelial or endothelial cells (4). Epithelial-mesenchymal transition (EMT) is known as one potential mechanism of migration, invasion and metastasis of cancer cells. EMT facilitates cell migration and metastasis by the conversion of epithelial-derived cancer cells to a more mesenchymal-like state (5,22,28). Zeisberg *et al* suggested that endothelial-mesenchymal transition should be categorized as a specialized form of EMT as a source for CAFs (29). In this study, FAP-α and SDF-1 were commonly expressed in residual cancer cells, endothelial cells and activated fibroblasts in the stroma, although a small number of fibroblasts displayed both FAP-α and SDF-1 staining in cancer stroma tissue compared to other fibroblasts. Our immunohistochemical findings suggest that CAFs could be derived from endothelial cells. As certain reports have suggested the occurrence of radiation-induced EMT (30), we believe that CAFs could be associated with tumor re-growth and distant recurrence in rectal cancer after CRT. In *in vitro* studies, Onoue *et al* demonstrated that the SDF-1/CXCR4 axis is involved in EMT in oral squamous cell carcinoma (31). We believe that SDF-1 also strongly contributes to EMT.

FAP-α antibody immunotherapy as well as the inhibition of the platelet-derived growth factor receptor and TGF-β have been suggested as therapeutic strategies that target CAF recruitment or CAF-derived tumorigenic signals (32-34). In the near future, such CAF-targeted therapies could be established.

In rectal cancer, pre-operative CRT and TME result in significantly decreased local recurrence and significantly increased survival rates. However, further improvement in prognosis cannot be achieved in the absence of control of distant recurrence after pre-operative CRT. The 'seed and soil' hypothesis has been suggested as one explanation for distant metastasis (35). To control distant recurrence, both the 'seed' and the 'soil' should therefore be targeted. Controlling the condition of the cancer stroma, including CAFs, could be a useful strategy for preventing distant recurrence after CRT in rectal cancer. We plan to investigate the expression of various genes in the stromal tissue of metastatic sites, such as the liver and lung.

The data reported in this study should be interpreted with some caution. Major limitations included the small number of patients (n=52), particularly those patients with distant recurrence (n=8), as well as the retrospective nature of the study. We believe that a larger study population and long-term follow-up will allow us to validate the conclusions presented here.

In conclusion, FAP-α and SDF-1 expression was involved in tumor re-growth and recurrence, and the control of CAFs could be effective for preventing distant recurrence in rectal cancer patients treated with pre-operative CRT.

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