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Comparisons of cancer-associated fibroblasts in the intratumoral stroma and invasive front in colorectal cancer

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Abstract

The aim of this study was to evaluate the cytomorphologic maturity and molecular activation of cancer-associated fibroblasts (CAFs) in the intratumoral stroma and invasive front in colorectal cancer and understand how they affect cancer invasion and long-term oncological outcomes.

The cytomorphologic maturity of and α-smooth muscle actin (α-SMA), fibroblast activation protein α (FAPα), and fibroblast-specific protein 1 (FSP-1) expression in CAFs in the intratumoral stroma (CAF^{IT}) and the invasive front (CAF^{IF}) of colorectal cancer tissues were compared (n = 147). The correlations between CAF maturation, molecular activity markers, and cancer invasion were evaluated by network analysis. Overall survival and systemic recurrence were analyzed to assess the oncological effects of CAF properties.

The cytomorphologic maturation rate was comparable between CAF^{IT} and CAF^{IF}. The presence of mature CAFs was related to epidermal growth factor receptor overexpression in cancer cells. Expression rates of α-SMA (96.6%–98.0%) and FAPα (18.6%–22.9%) were similar between CAF^{IT} and CAF^{IF}. FSP-1 expression was more frequent in CAF^{IT} than in CAF^{IF} (66.4% vs 58.2%, $P = .038$). There was a significant decrease in FSP-1 expression in CAF^{IT} and CAF^{IF} in higher stages. The infiltrating growth pattern of the tumor was more frequent in the immature CAF^{IT}. In colorectal cancer with perineural invasion and lymph node metastasis, FSP-1 expression in CAF^{IF} was significantly lower. On multivariate analysis using the Cox proportional hazards model, immature CAF^{IF} was found to be an independent prognostic factor of overall survival. In non-metastatic (stage I-III) colorectal cancer patients, CAF maturity was not a prognostic factor for systemic recurrence.

Cytomorphologic maturity and molecular activation markers were similar between CAFs in the intratumoral stroma and invasive front of colorectal cancer.

Abbreviations: α-SMA = α-smooth muscle actin, BMI = body mass index, CAF = cancer-associated fibroblast, CAF^{IF} = cancer-associated fibroblast invasive front, CAF^{IT} = cancer-associated fibroblast intratumoral stroma, CEA = carcinoembryonic antigen, CI = confidence interval, EGFR = epidermal growth factor receptor, EMT = epithelial-mesenchymal transition, FAPα = fibroblast activation protein α, FSP-1 = fibroblast-specific protein 1, HE = hematoxylin and eosin, HPFs = high power fields, IHC = immunohistochemistry, LOX = lysyl oxidase, PDGFR = platelet-derived growth factor receptor, TGF-β = transforming growth factor-β.

Keywords: α-SMA, cancer invasion, cancer-associated fibroblasts, colorectal cancer, FAPα, FSP-1, maturity

1. Introduction

Colorectal cancer is one of the most common gastrointestinal cancers worldwide. Early diagnosis and advancements in treatment options have improved survival, but almost half of colorectal cancer patients still have metastasis to the liver or lung.[1] Cancer invasion and metastasis are affected by various stromal cells in the tumor microenvironment, which are manifested by various pathological reactions.[2] Desmoplasia represents a histological structure similar to wound healing. Mature fibrosis with dense collagen fibers and spindle-shaped fibroblasts are expected to inhibit the spread of cancer cells and be a favorable prognostic factor of colorectal cancer.[3,4] Immature desmoplasia with large, plump myofibroblast-like cells is more similar to keloid scars and could promote cancer infiltration, leading to undesirable prognosis.[5,6]

Fibroblasts around cancer cells have been considered cancer-associated fibroblasts (CAFs). Activated CAFs can express several molecular markers, such as α-smooth muscle actin (α-SMA), fibroblast activation protein α (FAPα), and fibroblast specific protein-1 (FSP-1). α-SMA expressing myofibroblasts are representative of activated CAFs, as well as wound healing.[7] FAPα is a type II integral membrane protein belonging to the

Editor: Martin S. Staege.

This study was supported by Research Institute for Convergence of biomedical science and technology Grant (30–2017–008), Pusan National University Yangsan Hospital.

The authors have no conflicts of interests to disclose.

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Medicine (2019) 98:18(e15164)

Received: 27 May 2018 / Received in final form: 27 November 2018 / Accepted: 18 March 2019

http://dx.doi.org/10.1097/MD.0000000000015164

family of plasma membrane-bound serine proteases.[8] FAPα is expressed in reactive stroma and areas of tissue remodeling or wound healing. Normal, healthy adult tissues have no detectable FAPα expression.[9] FSP-1, also called S100A4, is a member of the S100 family of small, calcium binding proteins. FSP-1–positive fibroblasts can also be derived from local epithelial-mesenchymal transition (EMT), indicating that non-transformed epithelial cells may be an additional source of CAFs by undergoing EMT in response to stimuli from surrounding cells.[10]

CAFs can not only produce type I collagen fibers, but also release lysyl oxidase (LOX) for the cross-linking of fibers to form mature fibrosis that induces matrix stiffness and cancer invasion.[11] CAFs can be regulated by various growth factors from cancer cells and support cancer proliferation, metabolism, invasion, and metastasis.[12–16]

The diverse results of the cancer-promoting and -inhibitory effects by desmoplasia and CAFs are not understood clearly. Although fibroblasts account for a high proportion of tumor stroma (30%–60%), the differences of CAFs in the intratumoral stroma and invasive front are not well known. In particular, the correlation between cytomorphology and molecular properties of CAFs in colorectal cancer invasion is still insufficient.

The aim of this study was to evaluate the cytomorphologic maturity and molecular activation of CAFs in the intratumoral stroma and invasive front, and how they affect cancer invasion and the long-term oncologic outcomes in colorectal cancer patients.

2. Materials and methods

2.1. Patients

Of 217 patients with colorectal cancer, 66 were excluded because of incomplete paraffin blocks or unidentifiable tumor lesions on hematoxylin and eosin (HE) staining. Four patients with no α-SMA, FAPα, or FSP-1 expression were also excluded. We finally analyzed the CAF properties of 147 patients with colorectal cancer who underwent a follow-up of more than 5 years. Patients with complete preservation of paraffin blocks and identifiable tumor stromal lesion were included for pathologic evaluations. This study was conducted after receiving the approval of the Institutional Review Board (IRB No. 05–2016–097) of the Pusan National University Yangsan Hospital. Written informed consent was obtained from all patients. Clinicopathologic characteristics were obtained from a prospectively-recorded patient database.

All patients underwent postoperative surveillance using abdominal and thoracic computed tomography and serum carcinoembryonic antigen (CEA) testing every 6 months. Local recurrence was defined as recurrence at the site of the primary tumor. Metastasis to other organs, such as the liver and lung, and intra-abdominal disseminated metastasis were considered systemic metastasis.

2.2. Cytomorphologic evaluation

Pathological samples were sliced into sections of 4-μm thickness, deparaffinized, and treated with HE. Cytomorphologic evaluation was performed in the intratumoral stroma and the invasive front to classify fibroblast maturation; stromal maturity classifies predominant tissue types according to the Ueno classification.[3] Thin, wavy, spindle-like fibroblasts surrounded with elongated collagen fibers were classified as mature fibroblasts, and large fibroblasts with plump nuclei enclosed by keloid-like collagen bundles were counted as immature fibroblasts.

2.3. Immunohistochemistry (IHC)

Paraffin tissues were cut into 4-μm thick sections and fixed with xylene and ethanol. For antigen retrieval, slides were placed in a microwave oven for 10 minutes at 5-minute intervals in 10 mmol/L citrate buffer, pH 6.0, and cooled to room temperature for 1 hour, after which endogenous peroxidases were blocked with 3% H_2O_2. The cells were then blocked with 1% bovine serum albumin (Thermo Scientific, Rockford, USA) for 30 minutes. Slides were incubated with primary antibodies for CEA (mouse monoclonal antibody, clone: II-7, DAKO, Glostrup, Denmark), epidermal growth factor receptor (EGFR; mouse monoclonal antibody, clone: E30, M7239, DAKO), p53 (mouse monoclonal antibody, clone: DO-7, NCL-L-p53-DO7, Novocastra), α-SMA (Dako), FAPα (Novusbio, San Diego, CA), and FSP-1 (S100A4, Novusbio) at 4°C for 24 hours. Slides were washed and incubated with the appropriate secondary antibody for 1 hour. Slides were treated with a solution of diaminobenzidine tetrahydrochloride (Dako), stained with hematoxylin, and sealed through dehydration. Nuclear accumulation of p53 was considered positive when it was recognized in more than 10% of tumor cells. Cytoplasmic CEA accumulation was considered positive in cases where more than 10% of tumor cells showed cytoplasmic staining. EGFR expression was considered positive when it was recognized in more than 10% of cancer cells. IHC grades of α-SMA, FAPα, and FSP-1 in fibroblasts were measured using intensity and percentage of staining, as follows[17,18]: grade 1, weak staining in <50% or moderate staining in <20% of stromal cells; grade 2, weak staining in ≥50%, moderate staining in 20% to 49%, or strong staining in <20%; and grade 3, moderate staining in ≥50% or strong staining in ≥20%. IHC grades 1 to 2 were considered negative and grade 3 was counted as positive. In this study, we defined cells as CAFs in the intratumoral area (CAF^{IT}) or invasive front (CAF^{IF}) when one or more molecular activation markers, such as α-SMA, FAPα, and FSP-1, were positive.

2.4. Cancer invasiveness

To evaluate the invasiveness of cancer cells, T status, N status, lymphatic invasion, venous invasion, perineural invasion, tumor budding, and tumor growth pattern (expanding or infiltrating type) were examined. Cancer stage was classified according to the 7th edition of the American Joint Committee on Cancer.[19] Lymphatic invasion was identified by the presence of tumor cell clusters in endothelial-lined spaces without a muscular coat. Venous invasion was defined as tumor cell clusters in spaces with muscular coats. Perineural invasion was defined as cancerous cells spreading to the space surrounding nerves. Tumor budding was defined as a single cell or a cluster of fewer than 5 cells in the stroma around the tumor. Tumor budding was evaluated in 10 high power fields (HPFs) at high magnification (×40) and stratified as grade 1 (0–4/10 HPF), grade 2 (5–9/10 HPF), grade 3 (10–19/10 HPF), and grade 4 (≥ 20/10 HPF). Tumor budding of grades 3–4 (≥10/10 HPF) was considered positive.[20] Tumor growth patterns were classified into the expanding type, which grows by pushing the surrounding stroma, and the infiltrating type, which grows by penetrating the stroma. Two pathologists reviewed the HE slides independently. When they had different

views, they reevaluated the slides together. All pathological evaluations were performed by 2 pathologists who had no prior knowledge of clinical information.

2.5. Statistics

The primary outcome of this study was overall survival according to CAF maturation, which has been previously reported as approximately 85% and 65%, respectively, for colorectal cancer patients with mature and immature CAFs.[3] Therefore, a total of 217 patients were enrolled with a significance level of 5%, a power of 90%, and a dropout rate of 30%. Chi square tests and Pearson correlation tests were used to evaluate the correlation between CAF properties and cancer invasiveness. The McNemar test was used to compare the incidence of positive rates of CAF maturation and marker expressions according to tumor region. The Kaplan–Meier method and log-rank test were used to analyze overall survival and systemic recurrence for long-term oncologic outcomes. A Cox proportional hazards model was used for multivariate analysis. The covariance input criterion was less than 0.1 and the elimination criterion was less than 0.05. SPSS 24.0 (Statistical Package for Social Science Version 24.0, IBM SPSS, Armonk, NY) was used for statistical analysis, and the significance level was $P < .05$. We also performed a network analysis between clinicopathologic factors, CAF properties, and cancer invasiveness. Network analysis was based on robust Spearman correlation (rho) measures for all collected parameters. The depth of each node indicates the number of edges. Cut-off values of rho for positive and negative associations were ±0.2. Network visualization was conducted on the Gephi platform.[21]

3. Results

A comparison of clinicopathologic characteristics and CAF maturity is shown in Table 1. EGFR expression was significantly lower in both immature CAF^{IT} and CAF^{IF}.

As the intensity of staining increased, the staining area became wider, but staining patterns were varied, so positive expression was identified using a grading system based on intensity and area (Fig. 1). In CAF^{IT} and CAF^{IF}, the expression rates of α-SMA (96.6 vs 98.0%, $P = .68$) and FAPα (22.9 vs 18.6%, $P = .33$) were not different. FSP-1 expression was more frequent in CAF^{IT} than CAF^{IF} (66.4% vs 58.2%, $P = .03$).

Cytomorphologic maturation rates were comparable between CAF^{IT} and CAF^{IF} (57.1% vs 61.2%, $P = .21$). α-SMA expression was higher in immature CAF^{IT} (94.0% vs 100%, $P = .04$), but FAPα and FSP-1 expression in CAF^{IT} and CAF^{IF} were not different (Fig. 2).

The incidences of tumor budding and lymphatic, venous, and perineural invasion increased with stage progression (Fig. 3). However, infiltrating tumor growth was dominant even in the early stage and maintained in advanced stages. The frequency of immature CAF did not significantly differ according to stage. α-SMA and FAPα were expressed consistently in CAF^{IT} and CAF^{IF} from early stages. Interestingly, there was a significant decrease in FSP-1 expression in CAF^{IT} and CAF^{IF} as stage increased.

In network analysis, cancer invasiveness factors were analyzed as the critical node-forming major network island to indicate a more aggressive phenotype. FAPα and FSP-1 expression in CAFs were linked to each other and were associated with aggressive behavior in the inhibitory direction. Another small island was formed by the association of EGFR expression and mature CAFs connecting to lymphatic invasion.

Correlations of CAF properties and cancer invasiveness are shown in Table 2. The infiltrating growth pattern of the tumor was significantly higher in the immature CAF^{IT}. In colorectal cancer with the perineural invasion and lymph node metastasis, FSP-1 expression of CAF^{IF} was significantly lower. The α-SMA and FAPα expression in CAFs were not related with cancer invasiveness. FSP-1 expression in CAF^{IT}

Table 1

Clinicopathologic characteristics (n = 147).

	CAF^{IT}			CAF^{IF}		
	Mature (n = 84)	Immature (n = 63)	*P* value	Mature (n = 90)	Immature (n = 57)	*P* value
Clinical factors						
Age (≥70 yr)	28 (33.3)	14 (22.2)	.14	29 (32.2)	13 (22.8)	.21
Male	46 (54.8)	42 (66.7)	.14	52 (57.8)	36 (63.2)	.51
BMI (≥25 kg/m^2)	27 (32.1)	17 (27.0)	.49	29 (32.2)	15 (26.3)	.44
VSerum CEA (>5 mg/dL)	32 (38.1)	25 (39.7)	.84	35 (38.9)	22 (38.6)	.97
Colon obstruction	32 (38.1)	24 (38.1)	1.00	33 (36.7)	23 (40.4)	.65
Tumor size (>5 cm)	38 (45.2)	32 (50.8)	.50	39 (43.3)	31 (54.4)	.19
T status 1–2	23 (27.4)	15 (23.8)	.62	28 (31.1)	10 (17.5)	.06
3–4	61 (72.6)	48 (76.2)		62 (68.9)	47 (82.5)	
N status 0	42 (50.0)	30 (47.6)	.77	43 (47.8)	29 (50.9)	.71
1–2	42 (50.0)	33 (52.4)		47 (52.2)	28 (49.1)	
Pathologic stage I	17 (20.2)	9 (14.3)	.13	19 (21.1)	7 (12.3)	.25
II	25 (29.8)	21 (33.3)		24 (26.7)	22 (38.6)	
III	28 (33.3)	29 (46.0)		34 (37.8)	23 (40.4)	
IV	14 (16.7)	4 (6.3)		13 (14.4)	5 (8.8)	
Differentiation well	5 (6.0)	4 (6.3)	.92	7 (7.8)	2 (3.5)	.29
Molecular factors						
Cytoplasmic CEA	63 (75.0)	50 (79.4)	.53	66 (73.3)	47 (82.5)	.20
p53 accumulation	54 (64.3)	34 (54.0)	.20	55 (61.1)	33 (57.9)	.69
EGFR overexpression	27 (39.7)	12 (22.2)	.04	29 (40.3)	10 (20.0)	.01

CAF = Cancer-associated fibroblast, CAF^{IT} = CAFs in the intratumoral stroma, CAF^{IF} = CAFs in the invasive front, BMI = Body mass index, CEA = carcinoembryonic antigen, EGFR = Epidermal growth factor receptor.

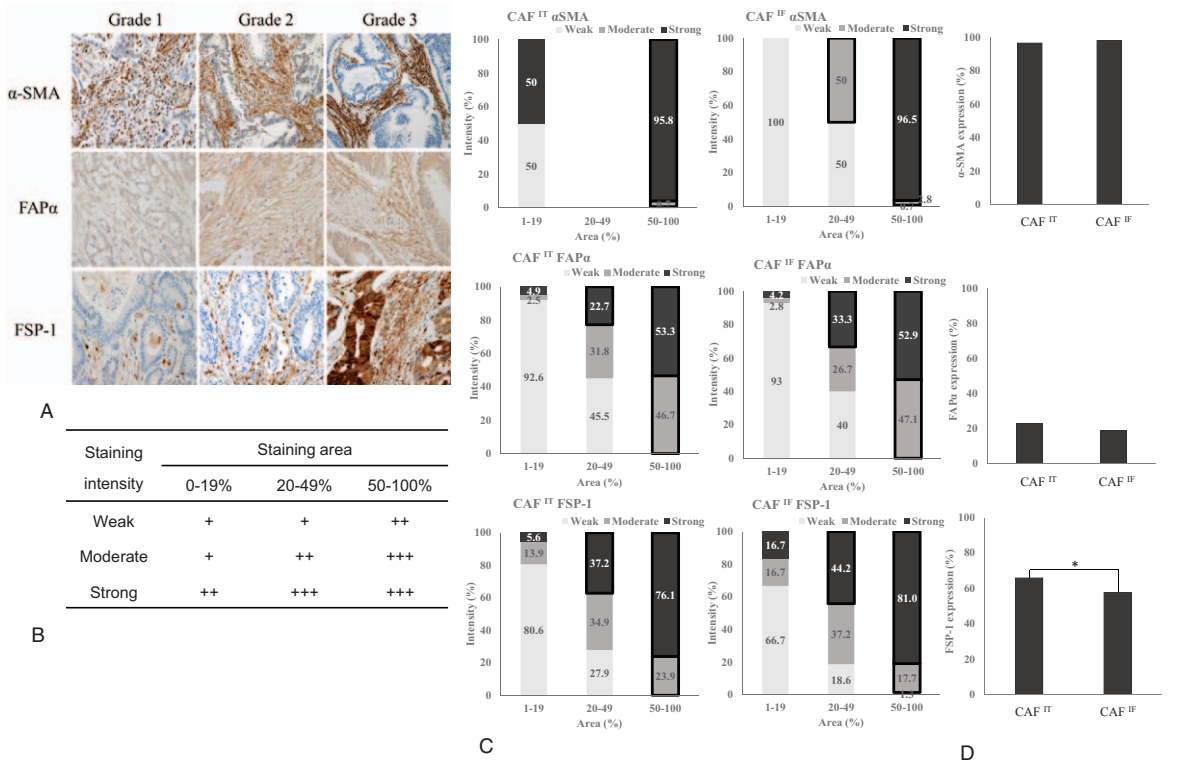

Figure 1. Expression of molecular activation markers in CAFs. (A) immunohistochemical (IHC) staining of α-SMA, FAPα, and FSP-1 on CAFs, (B) IHC grading system, (C) IHC staining intensity according to tumor staining area, (D) IHC strong positivity according to tumor location of CAF (intratumoral stroma vs invasive front). CAF = Cancer-associated fibroblast, CAF^{IT} = CAFs in the intratumoral stroma, CAF^{IF} = CAFs in the invasive front, α-SMA = α-smooth muscle actin, FAPα = fibroblast activation protein α, FSP-1 = fibroblast specific protein-1. * $P < .05$.

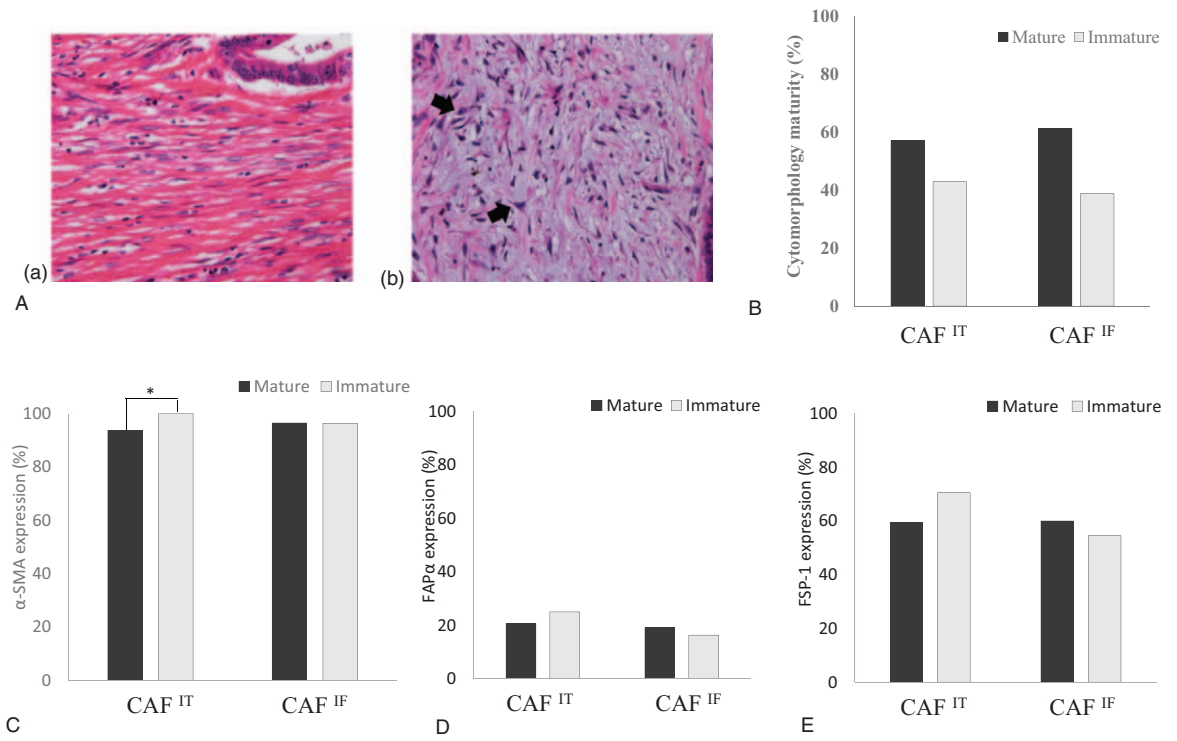

Figure 2. Expression of molecular activation markers according to maturity of CAFs. (A) cytomorphologic maturity of CAFs was defined as spindle-shaped fibroblasts indicating mature CAFs (a) and the large, plump myofibroblast-like cells indicating immature CAFs (b). (B) The proportions of mature CAF^{IT} and CAF^{IF} and (C) α-SMA expression, (D) FAPα expression, and (E) FSP-1 expression in CAF^{IT} and CAF^{IF} according CAF maturity were shown in the bar charts. CAF = Cancer-associated fibroblast, CAF^{IT} = CAFs in the intratumoral stroma, CAF^{IF} = CAFs in the invasive front, α-SMA = α-smooth muscle actin, FAPα = fibroblast activation protein α, FSP-1 = fibroblast specific protein-1.

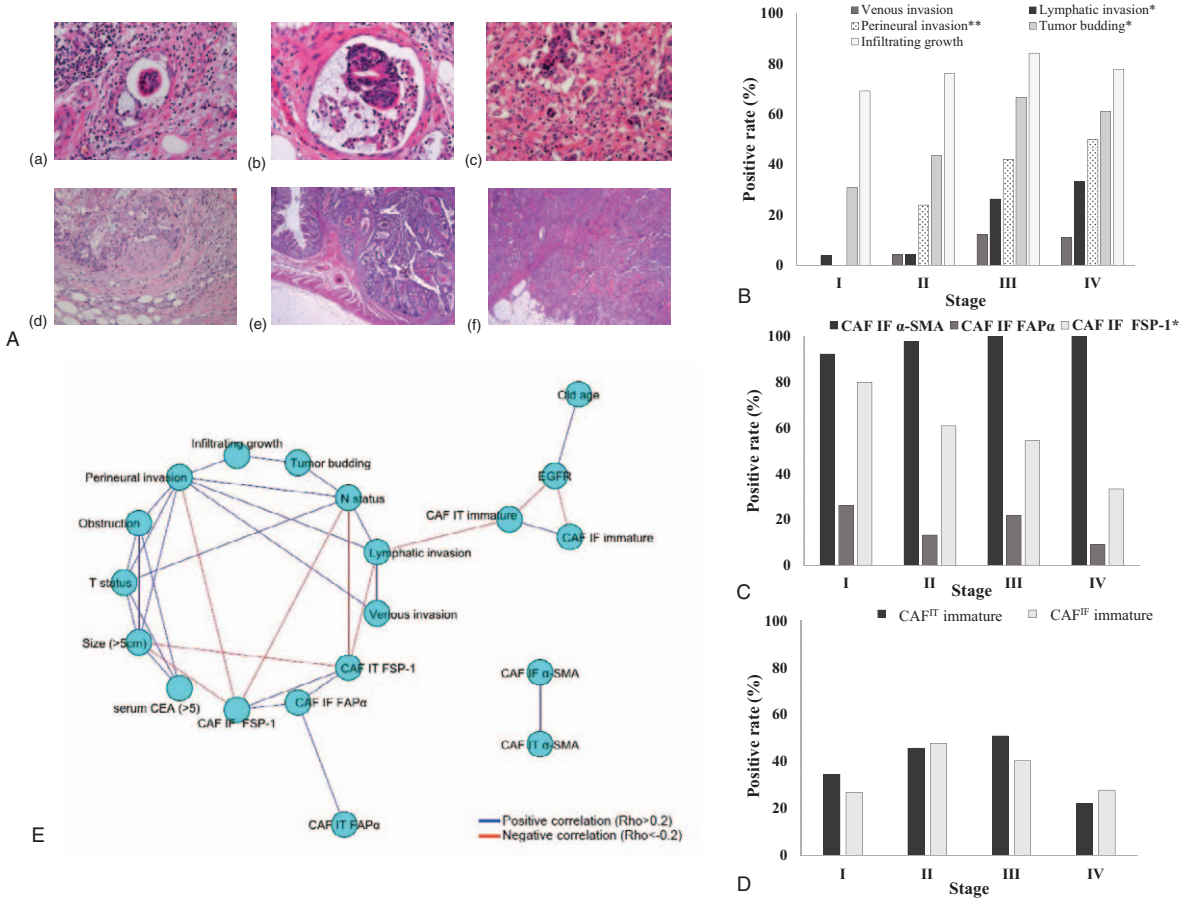

Figure 3. Changes in the incidence of cancer invasiveness factors and CAF properties by cancer stage. (A) Tumor invasiveness properties, including lymphatic invasion (a), venous invasion (b), tumor budding (c), perineural invasion (d), expanding growth (e), and infiltrating growth (f), were evaluated on the Hematoxylin and Eosin staining. (B) The incidence changes of cancer invasiveness factors, (C) α-SMA, FAPα, and FSP-1 expression of CAF^{IF}, (D) proportions of immature CAF^{IT} and CAF^{IF} by cancer stage were expressed in the bar charts. (E) Network analysis shows complex correlation link between clinicopathologic factors, CAF properties, and cancer invasiveness. All edge lines in the network represent a significant correlation ($P < .05$), and the color indicates whether the correlation is positive (blue) or negative (red). CAF = cancer-associated fibroblast, CAF^{IT} = CAFs in the intratumoral stroma, CAF^{IF} = CAFs in the invasive front, α-SMA = α-smooth muscle actin, FAPα = fibroblast activation protein α, FSP-1 = fibroblast specific protein-1. * $P < .05$, ** $P < .01$.

The presence of mature CAF^{IF} was an unfavorable prognostic factor for overall survival in stage I-IV colorectal cancer patients (n = 147). In stage I-III colorectal cancer patients with curative resection (n = 129), CAF maturity was not prognostic factor for systemic recurrence (Fig. 4). On multivariate analysis using Cox proportional hazards model, immature CAF^{IF} were an independent prognostic factor of overall survival (Table 3). In non-metastatic (stage I-III) colorectal cancer patients, CAF properties were not prognostic factors for systemic recurrence on univariate and multivariate analysis (Table 4).

4. Discussion

Dvorak declared that cancer is a non-healing wound; activated fibroblasts are key players in wound healing and expected to cause cancer proliferation, invasion, and metastasis.[22] However, targeted therapy to FAPα-positive CAFs showed survival deterioration in colorectal cancer patients, because this treatment had presumably simultaneous removal of both cancer-promoting and -suppressive CAFs.[23] To date, no specific marker has been identified that can differentiate cancer-promoting CAFs from cancer-suppressive CAFs.

In previous pathological studies, large, plump myofibroblast-like cells, termed immature CAFs, were found in 17.3% to 26.2% of tumor samples, and were considered an unfavorable prognostic factor.[3–6] Immature desmoplasia and CAFs are associated with aggressive cancer invasion. In a multicenter retrospective study, immature desmoplasia in colorectal cancer was significantly associated with higher incidences of tumor budding, lymphatic invasion, venous invasion, advanced T status, and lymph node metastasis.[3]

In the present study, immature CAFs were found in 38.8% of invasive fronts, which is slightly higher than the results of previous studies. In particular, immature CAF^{IF} were determined to be a favorable prognostic factor of overall survival. This difference might be because of patient selection and the classification method of CAF maturity. Ueno's study included stage II and III colorectal cancer patients and classified 3 categories of CAFs: mature, intermediate, and immature.[3] We enrolled patients with stage I to IV colorectal cancer and classified them into 2 groups, without an intermediate CAF group, so the proportion of immature CAFs may be increased. In particular, the proportion of immature CAFs increased to 60% in stage III, but decreased abruptly to less than 30% in

Table 2

Correlations of cytomorphologic and molecular properties of CAFs and cancer invasiveness in colorectal cancer patients.

	Lymphatic invasion n (%)	Venous invasion n (%)	Perineural invasion n (%)	Tumor budding n (%)	Infiltrating tumor growth n (%)	T status (3–4) n (%)	N status (1–2) n (%)
CAFIT							
Mature (n = 84)	18 (21.4)	8 (9.5)	26 (31.0)	47 (56.0)	60 (71.4)*	61 (72.6)	42 (50.0)
Immature (n = 63)	6 (9.5)	3 (4.8)	18 (28.6)	30 (47.6)	55 (87.3)	48 (76.2)	33 (52.4)
α-SMA							
Positive (n = 142)	23 (16.2)	11 (7.7)	42 (29.6)	75 (52.8)	112 (78.9)	106 (74.6)	73 (51.4)
Negative (n = 5)	1 (20.0)	0 (0.0)	2 (40.0)	2 (40.0)	3 (60.0)	3 (60.0)	2 (40.0)
FAP α							
Positive (n = 27)	6 (22.2)	3 (11.1)	10 (37.0)	15 (55.6)	21 (77.8)	22 (81.5)	16 (59.3)
Negative (n = 91)	13 (14.3)	6 (6.6)	23 (25.3)	50 (54.9)	71 (78.0)	65 (71.4)	41 (45.1)
FSP-1							
Positive (n = 97)	12 (12.4)	8 (8.2)	26 (26.8)	55 (56.7)	76 (78.4)	69 (71.1)	44 (45.4)*
Negative (n = 49)	12 (24.5)	3 (6.1)	18 (36.7)	22 (44.9)	39 (79.6)	40 (81.6)	31 (63.3)
CAF^{IF}							
Mature (n = 90)	17 (18.9)	7 (7.8)	27 (30.0)	52 (57.8)	67 (74.4)	62 (68.9)	47 (52.2)
Immature (n = 57)	7 (12.3)	4 (7.0)	17 (29.8)	25 (43.9)	48 (84.2)	47 (82.5)	28 (49.1)
α-SMA							
Positive (n = 144)	24 (16.7)	11 (7.6)	44 (30.6)	76 (52.8)	114 (79.2)	108 (75.0)	75 (52.1)
Negative (n = 3)	0 (0.0)	0 (0.0)	0 (0.0)	1 (33.3)	1 (33.3)	1 (33.3)	0 (0.0)
FAP α							
Positive (n = 22)	5 (22.7)	3 (13.6)	7 (31.8)	14 (63.6)	17 (77.3)	16 (72.7)	11 (50.0)
Negative (n = 96)	14 (14.6)	6 (6.3)	26 (27.1)	51 (53.1)	75 (78.1)	71 (74.0)	46 (47.9)
FSP-1							
Positive (n = 61)	13 (15.3)	8 (9.4)	17 (20.0) **	47 (55.3)	65 (76.5)	59 (69.4)	37 (43.5) *
Negative (n = 85)	11 (18.0)	3 (4.9)	27 (44.3)	30 (49.2)	50 (82.0)	50 (82.0)	38 (62.3)

CAF = Cancer-associated fibroblast, CAF^{IT} = CAFs in the intratumoral stroma, CAF^{IF} = CAFs in the invasive front, α-SMA = α-smooth muscle actin, FAPα = fibroblast activation protein α, FSP-1 = fibroblast specific protein-1. *$P < .05$, ** $P < .01$.

stage IV, which could affect the inverse survival results of immature CAF^{IF}. Further analysis showed that the overall survival of non-metastatic (stage I-III) colorectal cancer patients with immature CAF^{IF} was not significantly different from that of those with mature CAF^{IF} (92.3% vs 84.2%, $P = .21$).

When we compared CAF maturity to aggressive invasion properties, infiltrating tumor growth and advanced T status were common in patients with immature CAFs. However, lymphatic invasion was related to mature CAFs. Both mature and immature CAFs can promote cancer invasion in different ways. These results suggest that the cytomorphologic changes of CAFs and

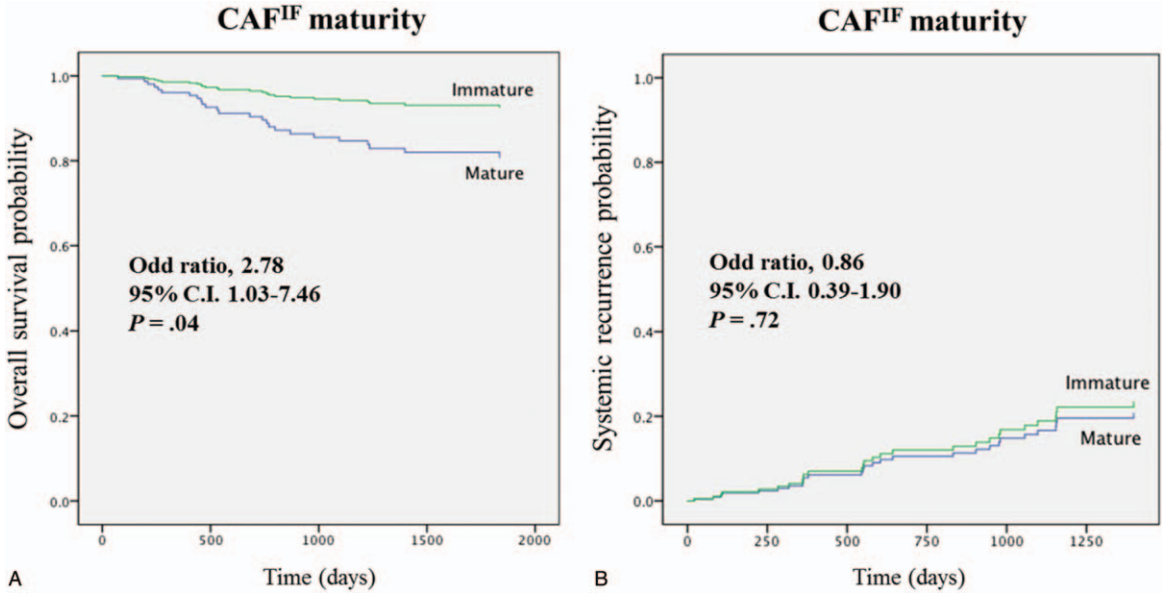

Figure 4. Effects of CAF^{IF} maturity on the long-term oncologic outcomes using Cox's proportional hazard model. (A) 5-year overall survival of patients with colorectal cancer (n = 147) and (B) systemic recurrence in patients with non-metastatic (stage I-III) colorectal cancer (n = 129) were represented by the Kaplan Meier curve. CAF^{IF} = cancer-associated fibroblast in the invasive front, CI = confidence interval.

Table 3

Univariate and multivariate analysis of 5-year overall survival using Cox regression model in colorectal cancer patients (n = 147).

	Univariate analysis			Multivariate analysis		
Overall survival	**Odd ratio**	**95% C.I.**	***P* value**	**Odd ratio**	**95% C.I.**	***P* value**
Age (≥70 years)	2.45	1.18–5.08	.01	2.32	1.05–5.12	.03
Serum CEA (>5 mg/dL)	3.03	1.43–6.43	.002	2.07	0.92–4.65	.07
Colon obstruction	2.54	1.21–5.32	.01			
T status (3–4)	2.37	0.82–6.81	.09			
N status (1–2)	3.29	1.40–7.72	.004	5.42	1.60–18.29	.006
Lymphatic invasion	1.77	0.75–4.15	.18			
Venous invasion	0.91	0.21–3.86	.90			
Perineural invasion	1.27	0.59–2.74	.53			
Tumor budding	2.52	1.14–5.82	.01			
Infiltrating growth	1.40	0.53–3.68	.48			
EGFR overexpression	2.60	1.18–5.71	.01			
CAF ^{IT}						
Immature	0.39	0.16–0.92	.02			
α-SMA	1.01	0.13–7.43	.99			
FAP α	0.70	0.23–2.07	.52			
FSP-1	0.47	0.22–0.97	.03			
CAF ^{IF}						
Immature	0.38	0.15–0.95	.03	2.78	1.03–7.46	.04
α-SMA	20.79	–	.41			
FAP α	0.92	0.31–2.72	.88			
FSP-1	0.52	0.25–1.08	.07			

CEA = carcinoembryonic antigen, EGFR = epidermal growth factor receptor, CAF = cancer-associated fibroblast, CAF^{IT} = CAFs in the intratumoral stroma, CAF^{IF} = CAFs in the invasive front, α-SMA = α-smooth muscle actin, FAPα = fibroblast activation protein α, FSP-1 = fibroblast specific protein-1, C.I. = confidence interval.

desmoplasia might be understood in a sequential manner. Dvorak speculated that "granulation tissue" with highly venous connective tissue would be replaced by vascular resorption and collagen synthesis, resulting in the formation of dense fibrous connective tissue, called "desmoplasia," in cancer.[24] Loose stroma could be favorable for cancer cell migration, infiltrating growth, and tumor budding, but dense fibrous tissue may also influence the mechanism of cancer invasion in different ways. Tissue stiffness has been experimentally proven to promote cancer invasion. When fibrosis forms a dense structure around

Table 4

Univariate and multivariate analysis of 5-year systemic recurrence using Cox regression model in non-metastatic colorectal cancer patients (n = 129).

	Univariate analysis			Multivariate analysis		
Systemic recurrence	**Odd ratio**	**95% C.I.**	***P* value**	**Odd ratio**	**95% C.I.**	***P* value**
Age (≥70 yr)	3.25	1.60–6.60	.001	2.82	1.30–6.10	.009
Serum CEA (>5 mg/dL)	1.33	0.64–2.74	.43			
Colon obstruction	1.98	0.98–4.01	.05			
T status (3–4)	3.15	1.10–9.01	.02	3.58	1.03–12.40	.04
N status (1–2)	2.13	1.03–4.39	.03			
Lymphatic invasion	2.69	1.20–6.03	.01	2.62	1.10–6.19	.02
Venous invasion	2.41	0.84–6.90	.09			
Perineural invasion	1.80	0.87–3.72	.10			
Tumor budding	1.02	0.50–2.07	.94			
Infiltrating growth	1.24	0.51–3.04	.62			
EGFR overexpression	3.21	1.52–6.74	.001	3.24	1.49–7.04	.003
CAF ^{IT}						
Immature	0.99	0.49–2.01	.98			
α-SMA	1.13	0.15–8.30	.90			
FAP α	1.36	0.59–3.11	.45			
FSP-1	1.37	0.59–3.19	.45			
CAF ^{IF}						
Immature	1.12	0.54–2.28	.75			
α-SMA	0.83	0.11–6.10	.85			
FAP α	1.08	0.43–2.69	.85			
FSP-1	1.24	0.58–2.63	.57			

CEA = carcinoembryonic antigen, EGFR = epidermal growth factor receptor, CAF = cancer-associated fibroblast, CAF^{IT} = CAFs in the intratumoral stroma, CAF^{IF} = CAFs in the invasive front, α-SMA = α-smooth muscle actin, FAPα = fibroblast activation protein α, FSP-1 = fibroblast specific protein-1, C.I. = confidence interval.

cancer cells, the elevated internal pressure causes rupture of cell clusters, allowing the rapid spread of cancer cells.[25] In addition, elaborately aligned fibers form a track to promote the movement of cancer cells, thereby promoting invasion and metastasis.[4] Expression of LOX, a target of transforming growth factor-β (TGF-β) involved in collagen arrangement, may form mature desmoplasia and affect oncologic outcomes.[11]

Expression of α-SMA, FAPα, FSP-1, and platelet-derived growth factor receptor (PDGFR), which are considered markers of activated CAFs, were expected to have an effect on cancer proliferation and invasion.[26,27] In esophageal cancer patients, the expression of α-SMA, FAPα, and FSP-1 tended to be higher in immature CAFs.[17] In oral squamous cell cancer patients, α-SMA was expressed in all immature CAFs and 75% of mature CAFs, and FSP-1 was expressed in 80% of CAFs.[26] Until now, the molecular activation status and CAF maturation have not been fully evaluated in colorectal cancer patients.

In the present study, α-SMA was expressed in all immature CAF^{IT}, but 94% of mature CAF^{IT} also expressed α-SMA strongly. α-SMA–positive myofibroblast-like cells could be a representative form of activated CAFs, but most spindle-shaped fibroblasts in the tumor stroma also expressed α-SMA, a marker of activated CAFs. Interestingly, the survival rate of patients with α-SMA–negative CAFs (both immature and mature) was 100%, but it was not statistically significant due to the small number of patients.

The reason for different positivity of IHC markers might be due to different grading systems, cut-off values for positivity, and organ-specific properties. In particular, the positive rate of FAPα in our study was 18.6%, which was different from the 80% positivity reported in previous studies in which grade 2 to 3 was counted as FAPα positivity, even though the frequency of strong staining was similar in both studies.[26]

When CAF properties were compared between the intratumoral stroma and invasive front, FSP-1 expression was more frequent on CAF^{IT}, but cytomorphologic maturation and α-SMA and FAPα expression did not differ according to CAF location. There was no difference in α-SMA, FAPα, and FSP-1 expression according to the cytomorphologic maturities of CAF^{IT} and CAF^{IF}. Therefore, the activation status of CAFs cannot be determined only by cytomorphologic properties, but the expression of various molecular activation markers should also be considered.

In a laboratory study, α-SMA, FAPα, or FSP-1-positive fibroblasts contributed to cancer cell invasion. In colorectal cancer patients, α-SMA expression was reported to have associations with advanced tumor stage, infiltrating tumor growth, and tumor budding. FSP-1 was related to lymphatic invasion and tumor budding.[16]

Although not statistically significant, α-SMA-positive $CAFs^{IF}$ were more frequent in patients with infiltrating growth and lymph node metastasis in the present study. However, FAPα expression in CAFs was not related to cancer invasiveness factors. Rather, low FSP-1 expression was related to lymphatic invasion, perineural invasion, and lymph node metastasis. The FSP-1 expression decreased with stage progression, but the incidence of cancer invasiveness increased. It is possible that, as the stage progresses, CAFs could be introduced from diverse origins, differentiated for various functions, and accumulate as senescent fibroblasts, resulting in varied molecular marker expression in the advanced stages.[28,29] Therefore, it is assumed that FSP-1 expression is relatively decreased in advanced cancer stages.

In the network analysis, "old age" did not count as an invasiveness factor. However, we conducted an unbiased analysis without excluding any single factor or parameter. The Spearman's correlation coefficient (rho) used in the network analysis was analyzed to include positive correlations greater than 0.2 or negative correlations less than −0.2 that also had significant *P* values, as observed from the bivariate analysis. All edge link lines in the network represent a significant positive or negative correlation ($P < .05$). As shown in the network analysis, the CAF activation marker expression formed a main complex island associated with cancer invasiveness, but CAF maturity formed a different small island with cancer EGFR expression.

This study has several limitations. First, clinical data from a small number of patients in a single institution will have statistically low power and require careful interpretation, and a larger scale study is therefore needed. Second, we considered that the heterogeneity of patients with stage I-IV may be a critical bias for survival analysis, which may be different from previous studies. CAF maturity was analyzed as an independent factor reflecting survival in all patients (n = 147), including stage I-IV patients. Systemic recurrence was analyzed in patients with non-metastatic (stage I-III) colorectal cancer patients who underwent curative resection (n = 129). Patients who were at stage IV at the time of colorectal cancer diagnosis (n = 18) were excluded from the systemic recurrence analysis. Generally, recurrence rates can be analyzed in stage IV patients after curative resection, including primary tumor resection and metastasectomy. However, in this study, stage IV patients were excluded from the analysis of systemic recurrence because palliative resection of primary tumors was performed in stage IV patients. Therefore, further analysis might be needed only for stage II to III colorectal cancer patients to eliminate selection bias. Third, differences in agents and procedures of IHC staining and subjective readings by pathologists may have resulted in other findings.[30] The IHC grading system used in this study was adopted for an objective evaluation of CAF activation markers FAP, FSP, and SMA.[17] The same scoring system was used for CAF IHC in colon cancer tissues.[18] This scoring system can be evaluated at 3 levels—weak, moderate, and strong expression—by combining IHC staining area and staining intensity for more objective and quantitative analysis. However, the results may still be different if the cut-off differs. In particular, the FAP expression rate was only 20% in this study. However, as shown in previous studies where positive staining rates were as high as 80%, the difference in the FAP expression rates is likely due to the differences in the cut-off values. If moderate and strong staining were all considered positive FAP staining in our study, an 80% positive staining rate, similar to the previous study, could be obtained. IHC is a relatively simple method that can be widely used for various pathological studies. However, even in the same laboratory, different results may be obtained depending on the staining procedure, such as antibody selection, dilution, and incubation time. In addition, objective and quantitative assessment can be difficult as the interpretation is based on subjective evaluation by the pathologist.[30] Therefore, it is expected that large-scale studies should be continued for elucidation of the role and clinical application of cytomorphologic maturity and molecular activation of CAF in colorectal cancer.

In conclusion, cytomorphologic maturity and molecular activation marker expression on CAFs were not different between the intratumoral stroma and invasive front of colorectal cancer. FSP-1 expression and immature CAF^{IF} were favorable

prognostic factors of survival, but further evaluation using specific molecular markers of CAFs is needed to define novel targets for cancer treatment.

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