Increased expression of connective tissue growth factor, epithelial membrane antigen, and fibroblast activation protein in hepatocellular carcinoma with aggressive behavior

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Directed by Professor Young Nyun Park

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Gi Jeong Kim

<TABLE OF CONTENTS>

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

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Tumor behavior is affected by not only malignant tumor cells themselves, but also by stromal cells in the tumor microenvironment, including cancer-associated fibroblasts (CAFs). Hepatocellular carcinoma (HCC) with "stemness" reportedly exhibits fibrous stroma and aggressive behavior suggestive of tumor-stroma interaction. However, the activation mechanism thereof remains unclear.

In the present study, the expressions of connective tissue growth factor (CTGF), epithelial membrane antigen (EMA), fibroblast activation protein (FAP), and keratin 19 (K19) were studied in specimens taken from 314 cases of HCC (cohort 1), 42 cases of HCC with fibrous stroma (cohort 2), and 36 cases of chronic hepatitis/cirrhosis by immunohistochemistry. Clinicopathological parameters in the HCC specimens were analyzed according to the expression status of CTGF, EMA, and FAP, respectively. The topographic expression patterns thereof were further assessed in the HCC specimens with fibrous

stroma of cohort 2.

CTGF and EMA expressions were detected in 15.3% (48/314) and 17.2% (54/314) of the HCCs of cohort 1, respectively. The expressions of CTGF and EMA were well correlated with each other (P = 0.001), and were significantly higher in HCCs with fibrous stroma, compared to those without (P = 0.028 and P = 0.003, respectively). K19 expression rate was significantly higher in HCCs with CTGF expression (17/48, P = 0.018). In HCCs with fibrous stroma, the expressions of CTGF, EMA, and FAP were noted in 40.5% (17/42), 40.5% (17/42), and 66.7% (28/42), respectively, and EMA expression was positively correlated with expressions of CTGF, K19, and FAP (P = 0.046, P = 0.026, and P = 0.020, respectively). EMA expression was found at the periphery of tumor nests in contact with fibrous stroma in 3 of 6 cases that exhibited large tumor nests, whereas it was rather diffuse in all 11 HCCs with small nests or that showed a trabecular pattern. CTGF expression rate was higher in large HCCs (≥ 5 cm), and FAP expression rate was higher in HCCs with vascular invasion (P = 0.030). Analysis of disease-free survival indicated CTGF expression as a worse prognostic factor in both cohort 1 (P = 0.005) and cohort 2 (P = 0.023), and EMA as a worse prognostic factor in HCCs with fibrous stroma (P = 0.048).

Thus, we discerned that the expressions of CTGF, EMA, and FAP are important to the activation of CAFs and gain of “stemness” in HCC, giving rise to aggressive behavior. Frequent coexpression of EMA and FAP and their

topographic expression patterns suggest possible cross-talk between epithelial cells and stromal cells in the tumor microenvironment.

Key words : hepatocellular carcinoma, connective tissue growth factor,

mucin core protein 1, epithelial membrane antigen, fibroblast activation protein, fibrous stroma, tumor microenvironment

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I. INTRODUCTION

The biological behavior of tumors is reportedly affected by not only malignant tumor cells themselves, but also by the tumor microenvironment including tumor stroma[1-3]. The tumor stroma is a complicated system that consists of signaling molecules, extracellular matrix proteins, proteolytic enzymes, blood vessels, and a variety of cellular components, such as fibroblasts and immune cells[4,5].

Cancer-associated fibroblasts (CAFs) in tumor stroma are histologically categorized as myofibroblasts or activated fibroblasts, and they have been reported to be associated with aggressive biological behavior, poor prognosis, and resistance to chemotherapy and radiation therapy in breast cancer, pancreatic cancer, and colon cancer[6-10]. Therefore, CAFs could influence the

characteristics of tumor cells through tumor-stroma cross-talk. However, the activation mechanism thereof has not yet been fully explored.

Hepatocellular carcinoma (HCC) is the seventh most common malignancy worldwide and the third greatest cause of cancer related mortality, especially in Asia and sub-Saharan Africa[11]. Most HCCs contain no or only little amounts of fibrous stroma; nevertheless, some HCCs without history of preoperative treatment exhibit various amounts of fibrous stroma between tumor nests. In a previous study, we showed that HCC specimens with abundant fibrous stroma, scirrhous HCC, exhibits an aggressive biological behavior and the expression of "stemness"-related markers with activation of TGF-β signature and epithelial-mesenchymal transition (EMT)-related genes[12].

Connective tissue growth factor (CTGF), a fibrogenic cytokine, is involved in virtually all fibrotic pathologies, both benign and malignant[13]. Recently, CTGF expression was reported to be impeded by TGF-β receptor inhibition, resulting in a decrease of the stromal components in HCC[14]. Additionally, the expression levels of CTGF mRNA and TGF-β mRNA were markedly increased in the surrounding tumor stroma of HCCs, compared to normal control specimens and engendering migration, invasion, and progressed tumor specimens[15].

Epithelial membrane antigen (EMA) is a member of a family of transmembrane mucin glycoproteins, with a high carbohydrate content and extensive O-linked glycosylation of its extracellular domain[16]. Recent clinical studies have reported a relationship between EMA expression and poor

prognosis in various malignant tumors, including lung cancer, gastric cancer, gallbladder cancer, and HCC[17-20]. Furthermore, EMA mRNA was reported to be up-regulated in a co-culture study of hepatoma cells and activated hepatic stellate cells (HSCs), compared to stromal cells cultured alone[21].

Fibroblast activation protein (FAP), a member of the serine protease family, has been reported to increase stromal cell proliferation and invasiveness, as well as reduce cell apoptosis[22]. FAP is also recognized as a useful marker of CAFs, selectively expressed in fibroblasts of several epithelial cancers, and is reported to be related to worse prognosis of pancreatic adenocarcinoma and colon cancer[22-25].

Concerning the tumor microenvironment of HCCs, the molecular mechanism involved in the formation of fibrous stroma and tumor-stroma cross-talk remains unclear. Thus, we attempted to evaluate the expressions of CTGF, EMA, and FAP and their correlation with clinicopathological features of HCCs. As well, their topographic expression patterns were further examined in HCCs with fibrous stroma.

II. MATERIALS AND METHODS

1. Patients and clinicopathological analysis

The HCC specimens included in this study were morphologically typical of HCC, and cases that could be classified as combined hepatocellular-cholangiocarcinoma or with a history of preoperative treatment were excluded. Formalin-fixed, paraffin-embedded HCC specimens were obtained from the archives of the Department of Pathology, Severance Hospital, Yonsei University College of Medicine.

This study was performed in specimens from two cohorts of patients with HCC. Cohort 1 consisted of 314 cases of HCC from January 2007 to March 2011; there were 254 males and 60 females, ranging in age from 28 to 81 years (55.6 ± 10.1, mean ± SD). To investigate the relationship between epithelial cells and stromal cells, cohort 2 included 42 cases of HCC with fibrous stroma from September 2001 to December 2010. Among these, there were 29 males and 13 females, whose ages ranged from 27 to 71 years (range, 53.7 ± 8.3, mean ± SD). Twenty-five cases were included in both of cohort 1 and 2. As a control group, 36 non-tumor tissues of chronic hepatitis/cirrhosis were studied in comparison to those of cohort 2.

Histopathologic analysis was performed for both cohorts on whole sections

of representative tissue blocks. For each case, tumor size, differentiation according to Edmondson-Steiner grade, tumor capsule formation, lymphovascular invasion, multiplicity of tumors, presence of fibrous stroma, and non-neoplastic liver disease were recorded. Fibrous stroma was defined as fibrotic areas that occupied more than 5% of the tumor area.

Clinical data from each patient were obtained from a careful review of their medical records, including hepatitis B virus surface antigen status, hepatitis C virus antibody, and tumor-node-metastasis (TNM) classification according to the 7th American Joint Committee on Cancer/International Union against Cancer (AJCC/UICC) staging system. This study was approved by the ethics committees of Severance Hospital (Seoul, Korea).

2. Tissue microarray construction

A representative formalin-fixed, paraffin-embedded block containing HCC was selected for each of the 314 available cases of cohort 1. The arrays were constructed in triplicate using a 3-mm punch on a tissue-arraying instrument (Beecher Instruments, Silver Springs, FL, USA). The cases were reviewed on conventional hematoxylin and eosin-slides, and representative areas were marked on each slide. Using a marker pen, the corresponding region was circled on the "donor" paraffin block. The samples were then arrayed on to a "recipient" block.

3. Immunohistochemistry

Immunohistochemical stain was performed using tissue microarray in cohort 1 and paraffin-embedded whole tissue sections for topographic assessment in cohort 2. To compare the phenotypical characteristics between tumor fibrous stroma and benign fibrous stroma, 36 cases of chronic hepatitis or cirrhosis, which included at least 10 portal tracts, were also immunostained.

The primary antibodies used were anti-CTGF (1:300, Abcam, Cambridge, UK), anti-EMA (1:100, Dako, Glostrup, Denmark), anti-FAP (D8, 1:100, Vitatex, Stony Brook, NY, USA), and Keratin 19 (K19) (1:100, Dako). Briefly, 4-㎛-thick sections were taken with a microtome, transferred to adhesive slides, and dried at 62℃ for 30 min. After incubation with primary antibodies, immunodetection was performed with biotinylated anti-mouse immunoglobulin, followed by horseradish peroxidase-labeled streptavidin using a labeled streptavidin biotin kit with 3, 3'-diaminobenzidine chromogen substrate. All slides were counterstained with Harris hematoxylin for 7 min. All immunohistochemical markers were assessed by a light microscope. The immunohistochemical staining results were interpreted in a semiquantitative way and given a staining score, from 0 to 3, as follows: 0, staining in <5% of tumor cells; 1, weak staining in ≥ 5%; 2, moderate staining in ≥ 5%; and 3, strong staining in ≥ 5% of the tumor cells. Positive staining was defined as a

staining score of 2 or 3, whereas scores of 0 and 1 were regarded as negative. Two pathologists (G. J. Kim and Y. N. Park) assessed the staining results without awareness of the clinicopathological data for each case.

4. Statistical analysis

Statistical analyses were performed using SPSS software version 21.0 for Windows (SPSS Inc., Chicago, IL, USA). Categorical variables were analyzed by the chi-square test or Fisher's exact test. On survival analysis, clinicopathologic variables were dichotomized and analyzed according to their effect on prognosis. Disease-free survival (DFS) and overall survival (OS) analysis was performed using the Kaplan–Meier method, and differences between the groups were assessed using the log-rank test. Univariate and multivariate survival analyses were carried out using Cox proportional hazard regression models. Only variables significant in the univariate analysis of factors affecting survival were used in the stepwise multivariate analysis. Estimated relative risks of death were expressed as adjusted hazard ratios (HR) and corresponding 95% confidence intervals (CI). Statistical significance was assumed for *P*-values < 0.05.

III. RESULTS

1. CTGF, EMA, and FAP expression and the clinicopathological characteristics of HCC (Cohort 1, *n* = 314)

The expressions of CTGF, EMA, K19, and FAP were evaluated by immunohistochemistry using tissue microarray in specimens from 314 cases of HCC. Positive expression rates of CTGF, EMA, K19, and FAP were 15.3% (48/314), 17.2% (54/314), 22.3% (70/314) and 6.7% (21/314), respectively. Expressions of CTGF and EMA were significantly correlated with each other (*P* = 0.001). Among 48 cases that exhibited CTGF expression, 16 cases (33.3%, 16/48) were positive for EMA. In the CTGF negative cases, however, the expression rate of EMA was relatively low (14.3%, 38/266). The expressions of CTGF and FAP showed no significant correlation with each other (*P* = 0.752); 2 cases (4.2%, 2/48) were positive for FAP in the CTGF positive group and 19 cases (7.1%, 19/266) were positive for FAP in the CTGF negative group. As well, the expressions of EMA and FAP also showed no significant correlation with each other (*P* = 0.406); 5 cases (9.3%, 5/54) were positive for FAP in the EMA positive group and 16 cases (6.2%, 16/260) were positive for FAP in the EMA negative group.

CTGF, EMA, and K19 expression was noted in tumor epithelial cells, but not in tumor stromal cells. CTGF was diffusely expressed throughout the tumor cells upon cytoplasmic staining (Figure 1. A-B). Expression of EMA, on the contrary, was either patchy or diffuse in the tumor cells upon membranous and/or cytoplasmic staining (Figure 1C-D). K19 was focally expressed in the tumor cells upon cytoplasmic and/or membranous staining (Figure 1E-F).

The expressions of CTGF and EMA were evaluated according to clinicopathologic parameters of HCC (Table 1). CTGF expression was significantly related to background cirrhosis ($P = 0.035$), absence of tumor capsule ($P = 0.049$), and presence of fibrous stroma in the tumors ($P = 0.028$). EMA expression demonstrated a significant association with presence of tumor fibrous stroma only ($P = 0.003$). Expression of CTGF was significantly correlated with K19 immunoreactivity ($P = 0.018$), whereas EMA expression was not.

FAP was expressed in the cytoplasm of tumor stromal cells, but not in tumor epithelial cells (Figure 1G-H). FAP expression was observed in 21 of 314 (6.7%) specimens, and was significantly correlated with solitary tumor mass ($P = 0.031$) (Table 1). FAP expression was not significantly correlated with any other clinicopathologic parameter ($P > 0.05$).

Table 1. Clinicopathological characteristics of HCCs according to CTGF, EMA, and FAP expression in cohort 1 (n = 314)

	CTGF			EMA			FAP		
	Positive (%)	**Negative (%)**	***P***	**Positive (%)**	**Negative (%)**	***P***	**Positive (%)**	**Negative (%)**	***P***
Frequency	48 (15.3)	266 (84.7)		54 (17.2)	260 (82.8)		21 (6.7)	293 (93.3)	
Sex			0.259			0.795			0.254
Female	12 (25.0)	48 (18.0)		11 (20.4)	49 (18.8)		6 (28.6)	54 (18.4)	
Male	36 (75.0)	218 (82.0)		43 (79.6)	211 (81.2)		15 (71.4)	239 (81.6)	
Age (years)			0.328			0.494			0.207
<55	18 (37.5)	120 (45.1)		26 (48.1)	112 (43.1)		12 (57.1)	126 (43.0)	
≥55	30 (62.5)	146 (54.9)		28 (51.9)	148 (56.9)		9 (42.9)	167 (57.0)	
Etiology			0.697			0.112			0.089
Non-viral	5 (10.4)	33 (12.4)		10 (18.5)	28 (10.8)		5 (23.8)	33 (11.3)	
HBV	39 (81.3)	220 (82.7)		41 (75.9)	218 (83.8)		16 (76.2)	243 (82.9)	
HCV	4 (8.3)	13 (4.9)		3 (5.6)	14 (5.4)		0 (0.0)	17 (5.8)	
Cirrhosis			0.035			0.169			0.495
Absent	15 (31.2)	127 (47.7)		29 (53.7)	113 (43.5)		11 (52.4)	131 (44.7)	
Present	33 (68.8)	139 (52.3)		25 (46.3)	147 (56.5)		10 (47.6)	162 (55.3)	
Tumor size (cm)			0.388			0.138			0.586
<5	36 (75.0)	214 (80.5)		39 (72.2)	211 (81.2)		18 (85.7)	232 (79.2)	
≥5	12 (25.0)	52 (19.5)		15 (27.8)	49 (18.8)		3 (14.3)	61 (20.8)	
Edmondson grade			0.141			0.844			0.128
I/II	19 (39.6)	136 (51.1)		26 (48.1)	129 (49.6)		7 (33.3)	148 (50.5)	
III/IV	29 (60.4)	130 (48.9)		28 (51.9)	131 (50.4)		14 (66.7)	145 (49.5)	
Capsule formation			0.049			0.141			0.406
Absent	13 (27.1)	41 (15.4)		13 (24.1)	41 (15.8)		5 (23.8)	49 (16.7)	
Present	35 (72.9)	225 (84.6)		41 (75.9)	219 (84.2)		16 (76.2)	244 (83.3)	
Vascular invasion			0.604			0.400			0.990
Absent	19 (39.6)	116 (43.6)		26 (48.1)	109 (41.9)		9 (42.9)	126 (43.0)	
Present	29 (60.4)	150 (56.4)		28 (51.9)	151 (58.1)		12 (57.1)	167 (57.0)	
Multiplicity			0.198			0.705			0.031
Single	37 (77.1)	225 (84.6)		46 (85.2)	216 (83.1)		21 (100.0)	241 (82.3)	
Multiple	11 (22.9)	41 (15.4)		8 (14.8)	44 (16.9)		0 (0.0)	52 (17.7)	
Stage (by AJCC)			0.096			0.747			0.615
I-II	43 (89.6)	254 (95.5)		52 (96.3)	245 (94.2)		21 (100.0)	276 (94.2)	
III-IV	5 (10.4)	12 (4.5)		2 (3.7)	15 (5.8)		0 (0.0)	17 (5.8)	
Fibrous stroma			0.028			0.003			0.219
Absent	36 (75.0)	232 (87.2)		39 (72.2)	229 (88.1)		16 (76.2)	252 (86.0)	
Present	12 (25.0)	34 (12.8)		15 (27.8)	31 (11.9)		5 (23.8)	41 (14.0)	
K19 expression			0.018			0.730			0.863
Negative	31 (64.6)	213 (80.1)		41 (75.9)	203 (78.1)		16 (76.2)	228 (77.8)	
Positive	17 (35.4)	53 (19.9)		13 (24.1)	57 (21.9)		5 (23.8)	65 (22.2)	

HCC, Hepatocellular carcinoma; CTGF, Connective tissue growth factor; EMA, Epithelial membrane antigen; FAP, Fibroblast activation protein

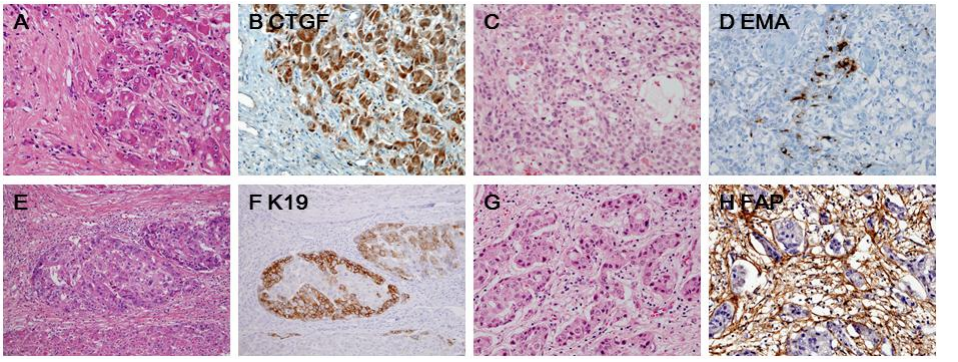

Figure 1. Connective tissue growth factor (CTGF), epithelial membrane antigen (EMA), fibroblast activation protein (FAP), and keratin 19 (K19) expression in hepatocellular carcinomas (HCCs) of cohort 1. (A) Small nests of tumor cells are surrounded by fibrous stroma. (B) Immunohistochemistry for CTGF exhibits diffuse cytoplasmic staining in most HCC cases. (C-D) Immunohistochemistry for EMA reveals patchy or focal expression patterns in most HCC cases. (E-F) K19 is focally positive in the periphery of tumor nests. (G-H) FAP is expressed in the cytoplasm of tumor stromal cells.

2. CTGF, EMA, and FAP expression and the clinicopathological characteristics of HCCs with fibrous stroma (Cohort 2, $n = 42$)

In paraffin-embedded whole tissue sections, the expression rates of CTGF and EMA were significantly higher in HCCs with fibrous stroma than in those without, and the expression patterns thereof were further investigated in HCCs with fibrous stroma. Whole tumor sections from representative paraffin-embedded tissue blocks were used to assess the topographic expression patterns CTGF, EMA, K19, and FAP in HCCs with fibrous stroma. Therein, the positive expression rates of CTGF, EMA, K19, and FAP were 40.5%, 40.5% (17/42), 33.3% (14/42), and 66.7% (28/42), respectively. The EMA expression rate was significantly higher in HCCs with CTGF expression compared to those without ($P = 0.046$). Ten cases (58.8%, 10/17) were positive for EMA among CTGF positive specimens, while EMA was expressed in only 7 (28.0%, 7/25) CTGF negative specimens. Moreover, the EMA expression rate was also significantly higher in HCCs with FAP expression compared to those without ($P = 0.020$). Fifteen cases (53.6%, 15/28) were positive for EMA among FAP positive specimens, while EMA was expressed in only 2 (14.3%, 2/14) FAP negative specimens. The expressions of CTGF and FAP showed no significant correlation with each other ($P = 0.331$); 13 cases (76.5%, 13/17) were positive for FAP among CTGF positive specimens and 15 cases (60.0%, 15/25) were positive for FAP among CTGF negative specimens.

The clinicopathological features of HCC according to the expressions of CTGF, EMA, and FAP in HCCs with fibrous stroma are summarized in Table 2. CTGF was diffusely expressed in tumor cells in a non-specific pattern (Figure 2A-C). In HCCs with fibrous stroma, CTGF expression rate was significantly higher in large tumors (≥ 5 cm), compared to small ones (< 5 cm) (P = 0.023); no other clinicopathological features were significantly different according to CTGF expression.

EMA exhibited focal or diffuse expression in the cytoplasm and/or membranes of tumor epithelial cells. The topographical expression pattern thereof was further assessed in 17 cases positive for EMA expression, including 6 cases of a large tumor nest pattern and 11 cases of a small tumor nest/trabecular pattern. Interestingly, EMA expression was noted at the periphery of tumor nests in contact with the tumor stroma in 50% (3/6) of cases of a large tumor nest pattern (Figure 2D-F). Meanwhile, all 11 cases of a small nest/trabecular pattern showed diffuse expression of EMA, where tumor cells were closely surrounded by tumor stroma (Figure 2G-I). This difference in EMA expression pattern between HCCs with small nests/trabeculae and those with large nests was statistically significant (P = 0.029). Furthermore, EMA expression revealed a significant association with K19 expression (P = 0.026).

FAP expression was detected in tumor stromal cells that surrounded tumor epithelial cells (Figure 2). Among HCCs with fibrous stroma, FAP expression rate was significantly higher in tumors with vascular invasion compared to

those without (P = 0.030) (Table 2).

In comparison of HCC and non-neoplastic liver specimens, the expressions of CTGF, EMA and FAP were investigated in 36 cases of chronic hepatitis/cirrhosis (Figure 3). CTGF and EMA were not detected in either non-tumor hepatocytes or stromal cells. As well, FAP expression was not found in benign hepatocytes, and it was only focally detected in stromal cells from 3 cases (3/36, 8.3%) of chronic hepatitis/cirrhosis. FAP expression was, however, significant different between the benign fibrous stroma of chronic hepatitis/cirrhosis specimens and the tumor fibrous stroma of HCC specimens (P < 0.001).

Table 2. Clinicopathological characteristics of HCCs with fibrous stroma according to CTGF, EMA, and FAP expression in cohort 2 (n = 42)

	CTGF			EMA			FAP		
	Positive (%)	**Negative (%)**	***P***	**Positive (%)**	**Negative (%)**	***P***	**Positive (%)**	**Negative (%)**	***P***
Frequency	17 (40.5)	25 (59.5)		17 (40.5)	25 (59.5)		28 (66.7)	14 (33.3)	
Sex			0.859			0.505			0.238
Female	5 (29.4)	8 (32.0)		4 (23.5)	9 (36.0)		7 (25.0)	6 (42.9)	
Male	12 (70.6)	17 (68.0)		13 (76.5)	16 (64.0)		21 (75.0)	8 (57.1)	
Age (years)			0.753			0.346			1.000
<55	8 (47.1)	13 (52.0)		10 (58.8)	11 (44.0)		14 (50.0)	7 (50.0)	
≥55	9 (52.9)	12 (48.0)		7 (41.2)	14 (56.0)		14 (50.0)	7 (50.0)	
Etiology			1.000			1.000			1.000
Non-viral	3 (17.6)	5 (20.0)		3 (17.6)	5 (20.0)		5 (17.9)	3 (21.4)	
HBV	13 (76.5)	20 (80.0)		14 (82.4)	19 (76.0)		22 (78.6)	11 (78.6)	
HCV	1 (5.9)	0 (0.0)		0 (0.0)	1 (4.0)		1 (3.5)	0 (0.0)	
Cirrhosis			0.542			0.963			1.000
Absent	10 (58.8)	17 (68.0)		11 (64.7)	16 (64.0)		18 (64.3)	9 (64.3)	
Present	7 (41.2)	8 (32.0)		6 (35.3)	9 (36.0)		10 (35.7)	5 (35.7)	
Tumor size (cm)			0.023			0.324			0.653
<5	7 (41.2)	19 (76.0)		9 (52.9)	17 (68.0)		18 (64.3)	8 (57.1)	
≥5	10 (58.8)	6 (24.0)		8 (47.1)	8 (32.0)		10 (35.7)	6 (42.9)	
Edmondson grade			0.346			0.116			0.100
I/II	7 (41.2)	14 (56.0)		6 (35.3)	15 (60.0)		11 (39.3)	10 (71.4)	
III/IV	10 (58.8)	11 (44.0)		11 (64.7)	10 (40.0)		17 (60.7)	4 (28.6)	
Capsule formation			0.158			1.000			0.545
Absent	2 (11.8)	0 (0.0)		1 (5.9)	1 (4.0)		2 (7.1)	0 (0.0)	
Present	15 (88.2)	25 (100.0)		16 (94.1)	24 (96.0)		26 (92.9)	14 (100.0)	
Vascular invasion			0.731			0.731			0.030
Absent	4 (23.5)	8 (32.0)		4 (23.5)	8 (32.0)		5 (17.9)	7 (50.0)	
Present	13 (76.5)	17 (68.0)		13 (76.5)	17 (68.0)		23 (82.1)	7 (50.0)	
Multiplicity			0.286			1.000			0.590
Single	14 (82.4)	24 (96.0)		15 (88.2)	23 (92.0)		26 (92.9)	12 (85.7)	
Multiple	3 (17.6)	1 (4.0)		2 (11.8)	2 (8.0)		2 (7.1)	2 (14.3)	
Stage (by AJCC)			0.556			1.000			0.254
I-II	15 (88.2)	24 (96.0)		16 (94.1)	23 (92.0)		27 (96.4)	12 (85.7)	
III-IV	2 (11.8)	1 (4.0)		1 (5.9)	2 (8.0)		1 (3.6)	2 (14.3)	
K19 expression			0.824			0.026			0.313
Negative	11 (64.7)	17 (68.0)		8 (47.1)	20 (80.0)		17 (60.7)	11 (78.6)	
Positive	6 (35.3)	8 (32.0)		9 (52.9)	5 (20.0)		11 (39.3)	3 (21.4)	

HCC, Hepatocellular carcinoma; CTGF, Connective tissue growth factor; EMA, Epithelial membrane antigen; FAP, Fibroblast activation protein

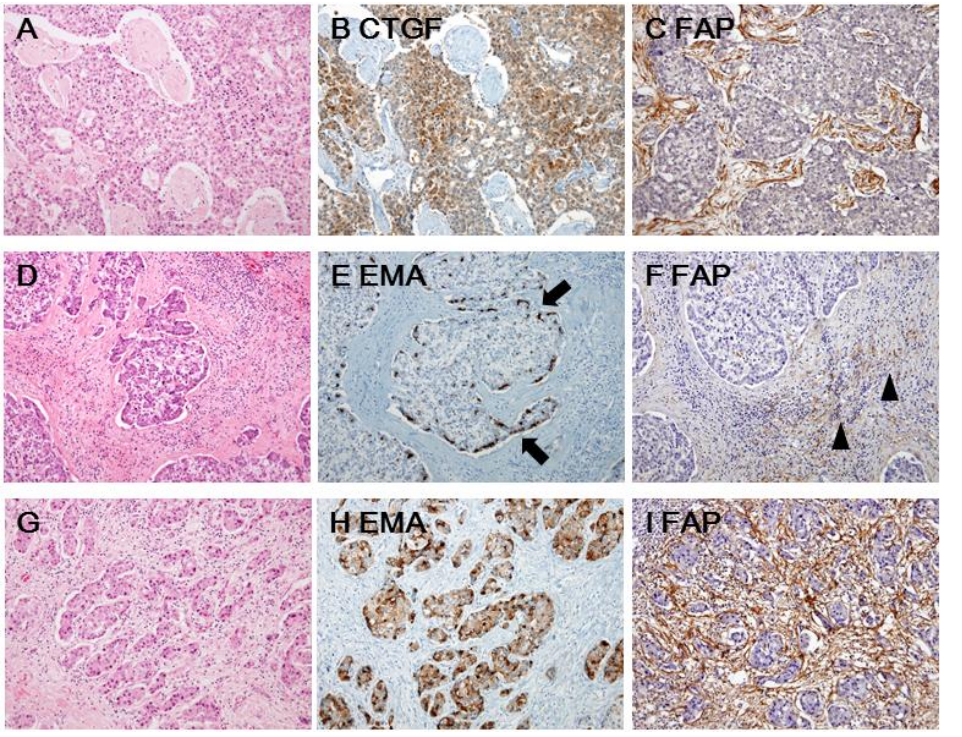

Figure 2. Connective tissue growth factor (CTGF), epithelial membrane antigen (EMA), and fibroblast activation protein (FAP) expression in hepatocellular carcinomas (HCCs) with fibrous stroma of cohort 2. A-C) CTGF (B) is diffusely expressed in the nests of tumor epithelial cells, and the tumor stromal cells between the tumor nests exhibit strong FAP expression (C). D-F) EMA is mainly expressed in the periphery (E, arrow) of large tumor nests in contact with fibrous stroma, which is also positive for FAP (F, arrowhead). G-I) HCCs with small nests or a trabecular pattern show diffuse expression for EMA in the tumor epithelial cells (H), which are closely admixed with FAP-positive tumor stromal cells (I).

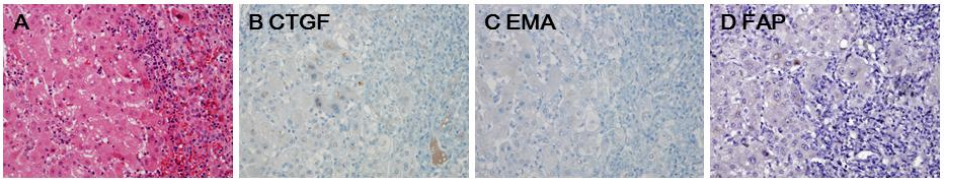

Figure 3. Connective tissue growth factor (CTGF), epithelial membrane antigen (EMA), and fibroblast activation protein (FAP) expression in chronic hepatitis/cirrhosis.

Fibrotic area with inflammatory cells in chronic hepatitis/cirrhosis. CTGF (B), EMA (C), and FAP (D) expression is negative

3. Prognostic significance of CTGF, EMA, and FAP expression

All HCC patients underwent curative resection, and the mean follow-up times were 31.2 months (range, 0-76) in cohort 1 and 44.6 months (range, 8-138) in cohort 2.

Among the 314 HCC patients of cohort 1, DFS rate was significantly lower in HCC patients expressing CTGF compared to those that did not (P = 0.005, Figure 4A); EMA and FAP expression was not associated with patient outcomes in this cohort (P = 0.362 and P = 0.287, respectively, Figure 4. B-C). Univariable analysis revealed background cirrhosis (P = 0.002), tumor size of more than 5 cm (P = 0.001), high Edmondson grade (P = 0.007), vascular invasion (P < 0.001), multiple tumors (P < 0.001), tumor stage III/IV (P < 0.001), and CTGF expression (P = 0.005) to be adverse prognostic factors for DFS after surgery. Subsequent multivariable analysis indicated background cirrhosis (HR = 1.815, P = 0.004) and vascular invasion (HR = 1.764, P = 0.015) as independent prognostic factors for DFS after surgery; CTGF expression was not significant in multivariate analysis (HR = 1.561, P = 0.056, Table 3). Additionally, OS rate was not significant different according to expression of these markers (P > 0.05).

In cohort 2, consisting of 42 HCCs with fibrous stroma, DFS rates were significantly lower for both CTGF-positive and EMA-positive specimens, compared to negative specimens (P = 0.023 and P = 0.048, respectively, Figure

4. D-E). Nevertheless, there were no differences in OS rates according to CTGF and EMA expression (P = 0.484 and 0.230, respectively). As well, expression of FAP showed no correlation with DFS and OS (P = 0.283 and P = 0.820, respectively, Figure 4F).

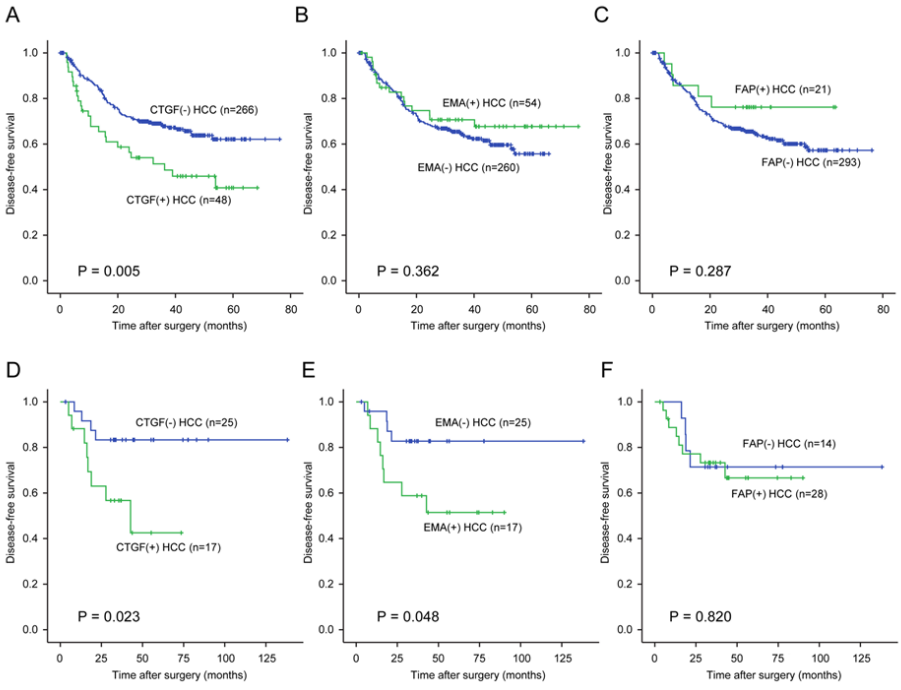

Figure 4. Disease-free survival (DFS) analysis of hepatocellular carcinoma (HCC) patients according to the expressions of connective tissue growth factor (CTGF), epithelial membrane antigen (EMA), and fibroblast activation protein (FAP). A-C) DFS analysis of HCC patients in cohort 1. HCC patients with positive expression of CTGF (A) exhibited a significantly worse DFS curve compared to those without (P = 0.005). There was no difference in DFS rate according expression of EMA (B) or FAP (C). D-F) DFS analysis of HCC patients with fibrous stroma in cohort 2. CTGF (D) and EMA (E) expression significantly influenced DFS rates among the HCC patients with fibrous stroma (P = 0.023 and P = 0.048, respectively), whereas there was no difference in DFS rate according to FAP expression (F).

Table 3. Univariate and stepwise multivariate analysis of disease-free survival rate for HCC in cohort 1

	Univariate			Multivariate		
	HR	**95% CI**	***P***	**HR**	**95% CI**	***P***
Sex						
Female	1					
Male	0.989	0.620-1.579	0.965			
Age (years)						
<55	1					
≥55	1.200	0.820-1.755	0.349			
Etiology						
Non-viral	1					
Viral	0.939	0.536-1.645	0.825			
Cirrhosis						
Absent	1			1		
Present	1.859	1.251-2.761	0.002	1.815	1.208-2.729	0.004
Tumor size (cm)						
<5	1			1		
≥5	1.977	1.309-2.986	0.001	1.533	0.952-2.468	0.079
Edmondson grade						
I/II	1			1		
III/IV	1.685	1.150-2.469	0.007	1.186	0.779-1.804	0.427
Vascular invasion						
Absent	1			1		
Present	2.179	1.437-3.302	< 0.001	1.764	1.118-2.784	0.015
Multiplicity						
Single	1			1		
Multiple	2.250	1.470-3.445	< 0.001	1.399	0.787-2.488	0.253
Stage (by AJCC)						
I-II	1			1		
III-IV	4.652	2.636-8.211	< 0.001	2.214	0.921-4.898	0.077
Fibrous stroma						
Not abundant	1					
Abundant	0.931	0.548-1.581	0.790			
K19 expression						
Negative	1					
Positive	0.952	0.609-1.487	0.828			
CTGF expression						
Negative	1			1		
Positive	1.886	1.207-2.948	0.005	1.561	0.989-2.465	0.056
EMA expression						
Negative	1					
Positive	0.782	0.460-1.329	0.363			
FAP expression						
Negative	1					
Positive	0.617	0.252-1.515	0.292			

HCC, Hepatocellular carcinoma; CTGF, Connective tissue growth factor; EMA, Epithelial membrane antigen; FAP, Fibroblast activation protein

IV. DISCUSSION

Solid tumors comprise a number of components, such as cancer cells, surrounding inflammatory cells, endothelial cells, and activated fibroblasts or myofibroblasts[2,4,5]. Although CAFs in tumor stroma are recognized as being related to the aggressive biological behavior of several cancers[6,7,9,10], their activation mechanisms remain poorly understood. Activated HSCs, which were previously considered as the source of hepatic fibrosis in chronic hepatitis[26,27], are now thought to also be responsible for the formation of fibrous stroma in primary hepatic carcinoma, including HCC and cholangiocarcinoma[28,29]. Interestingly, our previous study revealed that HCCs expressing "stemness"-related markers, such as K19, exhibit greater formation of fibrous stroma and more aggressive clinical outcomes upon activation of EMT-related genes[30].

FAP was initially identified as being expressed in reactive fibroblasts for embryonic development or in chronic inflammation[31,32]. More importantly, FAP is recognized as a marker of CAFs, and is reported to increase stromal cell proliferation and invasiveness, reduce cell apoptosis, and to be associated with worse prognosis in colon cancer and pancreas cancer[22-25]. Consistent with previous reports, the present study found that FAP is expressed predominantly in the activated fibroblasts in tumor stroma and is significantly correlated with frequent vascular invasion in HCCs with fibrous stroma. In contrast, FAP

expression was rarely found in fibrotic tissue of chronic hepatitis/cirrhosis. These findings suggest that FAP is involved in the activation of CAFs in tumor stroma, which differ from benign fibroblasts in the fibrous tissue of chronic hepatitis/cirrhosis.

Herein, we observed more frequent expression of CTGF in HCCs with fibrous stroma. CTGF is a fibrogenic cytokine that mediates almost all fibrotic processes[13]. Overexpression of CTGF in fibroblasts produces large amounts of extracellular matrix and enhances benign fibrotic changes in the pancreas, kidney, lung, and liver[33-36]. In addition to benign fibrotic processes, CTGF overexpression is also known to be responsible for pathologic fibrosis, including desmoplastic reaction in cancer[37]. This study revealed that CTGF expression was more frequently observed in HCCs with fibrous stroma. Inhibition of TGF-β, which is typically activated in HCCs with fibrous stroma[12], was reported to downregulate CTGF and block tumor-stroma cross-talk and tumor progression in HCC[14]. Two prior studies assessing the prognostic effects of CTGF expression in HCCs disclosed that the expression levels of intra tumoral CTGF were significantly higher in HCCs with bone metastasis[38,39]. Moreover, the expression levels of CTGF mRNA and TGF-β mRNA were markedly increased in tumor stroma of HCCs, facilitating migration, invasion, and progression of the tumor[15]. In accordance with these studies, we revealed that CTGF expression is related to worse DFS. Taken together, CTGF expression may be involved in the formation of fibrous stroma, which is related

to aggressive biological behavior of HCC.

Interestingly, CTGF expression was well correlated with K19 expression in the HCC specimens of this study. We recently reported that the expression rate of Yes-associated protein (YAP), a transcriptional coactivator, was significantly higher in HCCs with "stemness"-related marker expression, compared to those without[40]. Actually, CTGF expression is known to be dependent on the activity of YAP[41]. Taken together, YAP activation may provoke the expression of CTGF in tumor cells, which might be involved in the activation CAFs of tumor stroma and gain of "stemness" via tumor-stroma cross-talk, potentially explaining the aggressive behavior of HCCs with fibrous stroma.

In pancreatic cancer, EMA reportedly enhances invasiveness and metastasis by EMT[42]. An *in vitro* co-culture model study of human hepatoma cells and activated HSCs demonstrated increases in EMA mRNA when those cells were cultured together, compared to culture of stromal cells alone[21]. Our study revealed significantly higher rates of EMA expression in HCCs with fibrous stroma compared to those without, and this was related to DFS rate in HCC patients with fibrous stroma. These findings were consistent with previous reports that EMA was a poor prognostic factor in HCC[17,43].

In HCCs with large tumor nests, EMA expression was higher at the peripheral portions of the tumor nests where tumor cells were in contact with fibrous stroma. On the contrary, in HCCs with small clusters or trabeculae patterns, EMA expression was rather diffuse and lacked a specific pattern; the

tumor cells closely intermingled with stromal cells expressing FAP. This topographic expression pattern was similar to that of K19 expression in HCCs with fibrous stroma reported in our previous study[12]. Furthermore, the frequency of EMA expression was shown to be significantly correlated with that of FAP expression in HCCs with fibrous stroma. Taken together, we discerned that EMA and FAP may be important to tumor-stroma cross-talk for activation of CAFs, which might be involved in "stemness" of HCC. To our knowledge, this is the first study to verify topographically the expression patterns of EMA in human HCC tissues with activated CAFs.

V. CONCLUSION

The expressions of CTGF, EMA, and FAP may be involved in the activation of CAFs and gain of “stemness” in HCC, giving rise to aggressive behavior. Frequent coexpression of EMA and FAP and their topographic expression patterns suggest possible cross-talk between epithelial cells and stromal cells in the tumor microenvironment.

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ABSTRACT (IN KOREAN)

공격적 특성을 가진 간세포암에서의 결합조직 성장 인자, 상피막 항원, 섬유아세포 활성화 단백질의 발현 증가

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김기정

종양의 특성은 종양 세포뿐만 아니라 종양간질세포를 비롯한 종양미세환경에 의해서도 영향을 받는다. 줄기세포 성질을 가진 간세포암은 종양-간질 상호작용에 의하여 섬유성 간질이 풍부하고 공격적 특성을 가지는 것으로 알려져 있으나 그 활성화 기전에 대해서는 이해가 부족한 실정이다.

본 연구에서는 314명의 간세포암 환자군 (환자군 1)과 42명의 간질세포가 풍부한 간세포암 환자군 (환자군 2), 36명의 만성간염/간경변 환자 각각에 대하여 결합조직성장인자, 상피막항원, 섬유아세포활성화단백질, keratin 19에 대한 면역조직화학염색을 시행하였고, 상기 표지자의 발현 유무에 따른 임상병리학적 특성 차이를 분석하였다. 42명 환자군 2

조직에 대하여 추가적으로 면역조직화학염색의 발현 패턴을 분석하였다.

환자군 1의 간세포암에서 결합조직성장인자, 상피막항원은 각각 15.3% (48/314), 17.2% (54/314)에서 발현이 확인되었다. 결합조직성장인자와 상피막항원의 발현은 상호 연관성을 보였고 (P = 0.001), 섬유성 간질이 풍부한 간세포암에서 모두 발현 빈도가 증가하였다 (P = 0.028 and P = 0.003). Keratin 19의 발현 빈도는 결합조직성장인자 양성인 간세포암에서 유의하게 증가하였다 (17/48, P = 0.018). 섬유성 간질이 풍부한 간세포암에서는 결합조직성장인자, 상피막항원, 섬유아세포활성화단백질 발현이 각각 40.5% (17/42), 40.5% (17/42), 66.7% (28/42)에서 보고되었고, 상피막항원의 발현은 결합조직성장인자 (P = 0.046), keratin 19 (P = 0.026), 섬유아세포활성화단백질 (P = 0.020) 발현과 상관성을 보였다. 상피막항원의 발현은 큰 종양군집을 만드는 6증례 중 3증례에서 섬유성 간질과 맞닿아 있는 종양군집 변연부에서 나타났다. 그에 반하여 작은 종양군집 또는 소주양상을 보이는 간세포암 11증례에서는 모두 미만성 발현 양상을 나타냈다.

결합조직성장인자 발현은 5 cm 이상의 간세포암에서, 섬유아세포활성화단백질 발현은 혈관 침범이 있는 간세포암세에서 빈도가 증가하였다. 결합조직성장인자는 환자군 1 (P = 0.005)과 환자군 2 (P = 0.023)에서, 상피막항원은 환자군 2 (P = 0.048)에서 환자의 무병 생존률을 유의하게 저하시키는 요인임을 확인하였다.

결합조직성장인자, 상피막항원, 섬유아세포활성화단백질의 발현은 종양간질세포의 활성화와 간세포암의 줄기세포성 획득에 중요하고, 종양에 공격적 특성을 부여한다. 상피막항원과 섬유아세포활성화단백질의 빈번한 동시 발현 및 특징적인 발현 패턴은 종양미세환경에서 종양-간질상호작용의 가능성을 시사한다.

핵심되는 말 : 간세포암, 결합조직성장인자, 상피막항원, 섬유아세포활성화단백질, 섬유성 간질, 종양미세환경