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*Original Article*

# Microenvironmental interactions and expression of molecular markers associated with epithelial-to-mesenchymal transition in colorectal carcinoma

Sun-Jae Lee[1], Chun-Seok Yang[2], Dae-Dong Kim[2], Yu-Na Kang[3], Sang Gyu Kwak[4], Jae-Bok Park[1], Chang-Ho Cho[1], Kwan-Kyu Park[1]

*Departments of [1]Pathology, [2]Surgery, [4]Medical Statistics, College of Medicine, Catholic University of Daegu, Republic of Korea; [3]Department of Pathology, Keimyung University School of Medicine, Republic of Korea*

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**Abstract:** The tumor microenvironment is known to play a critical role in tumor progression, invasion and metastasis. The epithelial-to-mesenchymal transition (EMT) is understood as a process of tumor invasion and metastasis. Therefore, we investigated the relation between the EMT and the microenvironment of colorectal carcinoma (CRC). The histological features and expression of EMT markers in tumor cells and surrounded stromal cells were obtained from the surgically resected tissues of 39 patients using microscopic review and immunohistochemistry. The loss of expression of E-cadherin was more prominent in the invasive front of tumor than the surface, where α-smooth muscle actin-positive carcinoma-associated fibroblasts (CAFs) are accumulated. The signaling molecules of the Wnt and TGF-β1-Smad pathway were expressed more frequently in the tumor cells and/or CAFs of the invasive margin than those of the tumor surface. The expressions of related transcription factors, such as SNAIL and ZEB1, were increased in the tumor cells and CAFs. The process of EMT may be activated in the tumor margin of CRC under the control of CAFs. Related signaling molecules and transcription factors might be induced by paracrine effects of the surrounding CAFs.

**Keywords:** Colorectal carcinoma, epithelial-to-mesenchymal transition, microenvironment, carcinoma-associated fibroblasts

## Introduction

Colorectal carcinoma (CRC) is one of the most frequent causes of death from cancer worldwide [1]. Disparate factors increase a person's risk of developing the tumor, such as age, inflammatory bowel disease, personal and/or family history of colorectal tumors, and environmental factors [2, 3]. The molecular genetic alterations along the process leading to colon cancer are one of the best characterized processes in cancer progression [4].

As with many other solid tumors, metastatic disease, especially that in the lymph nodes, is the most important prognostic factor in patients with CRCs [5]. Cancer cells can invade as single or collective cell migrations that retain epithelial characteristics [6]. The epithelial-to-mesenchymal transition (EMT), a cellular process during which epithelial cells lose their polarized organization and cell-cell junctions, undergo changes in cell shape and in cytoskeletal organization and acquire mesenchymal characteristics, such as fibroblast-like cell morphology [7], is thought to enhance motility, invasion, and apoptosis resistance [8].

There are some major signal transduction pathways known to be important for the EMT of CRCs [9]. The downregulation of E-cadherin expression is the most crucial characteristic of EMT development. ZEB1 and SNAIL are the major transcription inhibitors of E-cadherin [10]. Many studies have shown that the loss of E-cadherin expression can lead to a loss of contact inhibition, infinite proliferation, dedifferentiation, and loose intercellular connec-

tions which enhanced invasion and migration feature of cancer cells [11-13]. EMT may be transient, as observed in CRCs, where cells at the invasive front lose E-cadherin expression and exhibit nuclear β-catenin localization, while the cells of liver metastasis have redifferentiated into epithelial cells and morphologically resemble primary tumor cells [14]. Hence, the actual occurrence of the EMT in human cancers is still controversial.

In fully formed neoplastic glands (as well as in normal colonic crypt epithelium) β-catenin together with E-cadherin reside at the cell membrane to form junctional complexes, but β-catenin released to the cytoplasm is taken up by the so-called destruction complex that consists of the adenomatous polyposis coli (APC) protein and glycogen synthase kinase-3 (GSK-3) [9]. In Wnt-signaling, the binding of the evolutionarily conserved Wnt-growth factors (Wnts) to the frizzled receptor induces a release of β-catenin from the destruction complex [9]. Cytosolic β-catenin then translocates to the nucleus to activate transcription factors cooperating to induce the EMT [9].

TGF-β1 enhances cell invasion, migration and evasion of immune surveillance in the advanced stages of carcinogenesis [15]. TGF-β1 and its receptor are expressed in the majority of CRCs [16] and it is particularly frequent in CRCs with prominent tumor budding [17], which is a phenotype of tumor invasion and regarded as a process of the EMT. The EMT in response to TGF-β1 is mediated mainly by Smad-dependent mechanisms [18]. SNAIL in cell cultures of CRC has been shown to repress E-cadherin [19] and the loss of E-cadherin by CRC cells occurs as an early event in the EMT [20]. Thus, induction of snail and slug and their effect on E-cadherin link the outside-in signals set by the microenvironment to the tumor invasion [9]. Similarly, ZEB1 (also known as Smad-interacting protein-1; Sip-1) forms repressive complexes with Smad proteins and binds to the E-cadherin gene promoter to suppress it [7]. ZEB proteins promote the EMT, cell migration and cell invasion [21, 22]. ZEB1 is a critical regulator of EMT by its transcriptional repression of E-cadherin and other epithelial marker genes, which directly affect the epithelial versus mesenchymal status of tumor cells, and thus EMT, cell migration invasion and metastasis [7].

EMT has been recognized as crucial phenomenon when the tumor cell invades the surrounding stroma [23]. In this tumor-associated stroma, the extracellular matrix, growth factors, cytokines, and a variety of nonepithelial cell types, including vascular space-related cells (endothelial cells, pericytes, and smooth-muscle cells), inflammatory response cells (lymphocytes, macrophages, and mast cells), and fibroblasts, come together [24]. In this complex microenvironment, a variety of interactions take place between its various components and determine a series of events, such as tumor cell proliferation, metastatic potential, and location of metastases [25, 26]. Recently, numerous studies reported that these complex processes are associated with the EMT and constitute an important mechanism in the development of tumor invasiveness [27].

Fibroblasts of the tumor stroma have received various names: tumor-associated fibroblasts, carcinoma-associated fibroblasts (CAFs), or myofibroblasts [28]. They are similar to those found in the wound-healing process, although CAFs are constantly activated [28]. CAFs are involved in various tumor mechanisms, such as extracellular matrix remodeling, immune suppression, secretion of the growth factors and cytokines that extensively affect tumor cell growth, invasion, differentiation, angiogenesis, chronic inflammation, regulation of tumor cell motility, and the specialization of tumor metabolism or tumor cell implantation [29, 30]. CAFs' activated phenotype is characterized by the expression of different proteins, such as α-smooth muscle actin (α-SMA) [31]. Notably, emerging data have revealed that CAFs are the most important component of tumor stroma with respect to their effect on tumor progression and metastasis [32]. However, there are a few concerns about the interaction between the tumor and the microenvironment [33]. Despite the above mentioned findings, CAFs from primary colon cancer have not been fully studied yet.

In this study, we identified the EMT process that occurs in CRCs and investigated the relationship between the density of α-SMA-positive myofibroblasts and cancer cell EMT with the expression of EMT related signaling molecules and transcription factors by immunohistochemical studies. We established experiments to

**Table 1.** The clinical parameters and histological assessments of case distribution

| Variable | | N (%) or mean ± SD |
|---|---|---|
| Age (years) | | 62.795±12.872 |
| Gender | M | 16 (41.0) |
| | F | 23 (59.0) |
| Size (cm) | | 5.046±2.659 |
| Location | Proximal | 13 (33.3) |
| | Distal | 17 (43.6) |
| | Rectal | 9 (23.1) |
| pT stage | 0 | 3 (7.7) |
| | 1, 2 | 5 (12.8) |
| | 3, 4 | 31 (79.5) |
| pN stage | 0 | 26 (66.7) |
| | 1 | 11 (28.2) |
| | 2 | 2 (5.1) |
| M stage | 0 | 28 (75.7) |
| | 1 | 9 (24.3) |
| Stage (TNM) | Early (0, 1, 2) | 23 (59) |
| | Advanced (3, 4) | 16 (41) |
| Gross type | Fungating | 9 (23.1) |
| | Ulcerative | 30 (76.9) |
| Histologic type | Tubular | 38 (97.4) |
| | Mucinous | 1 (2.6) |
| Differentiation | Well | 8 (20.5) |
| | Moderate | 25 (64.1) |
| | Poorly | 6 (15.4) |
| Perineural invasion | Absent | 26 (66.7) |
| | Present | 13 (33.3) |
| Lymphovascular invasion | Absent | 24 (61.5) |
| | Present | 15 (38.5) |

test the capability of primary colon CAFs to promote the tumorigenesis of colon cancer cells and found that CAFs work in this direction to augment cancer cell invasion. Therefore, we hypothesized that the roles of α-SMA-positive CAFs in CRCs are associated with EMT and invasion. Finally, we correlated these findings with clinicopathologic features statistically to define the significance clinically.

## Materials and methods

### *Case selection*

From January to June 2012, 39 patients who underwent surgical resection of CRC were enrolled in this study. Carcinomas arising from the colorectal mucosa were selected in this study. Tumors which were located in the serosa or the subserosal space of the intestinal wall without mucosal involvement were considered as secondary carcinomas metastasized to the intestine, and were excluded. A tumor with mucosal involvement, regardless of the serosal extension, was characterized as a primary colorectal lesion. The histologic features of all specimens were reviewed. The patients' biological data and demographic information (age, gender, and diagnoses of metastatic lesions, M stage) were collected through the review of medical records. Histologic data were obtained from pathologic reports and microscopic review. The tumor's size, location, and growth pattern were collected from the patients' pathologic reports. Microscopic features, including differentiation of tumors, invasion depth (pT stage), lymph nodal metastasis (pN stage), and the invasion status of nerve fibers or lymphovascular tracts were obtained from the microscopic review of hematoxylin and eosin (H&E) stained slides. The clinical parameters and histological assessments of the respective cases are summarized in **Table 1**.

### *Histologic examination*

To characterize the tumor microenvironment, the status of desmoplasia, peritumoral inflammatory infiltrates and tumor budding were obtained from the microscopic review of H&E slides. Desmoplasia was defined as a basophilic myxoid stroma around the tumor regardless of the cellularity of the stromal cells. The status of tumor-associated inflammation was categorized using a three point scoring system; no inflammatory infiltration was given a score of 0, infiltration of any type of inflammatory cell was scored as 1, and inflammatory aggregation with abscess formation was scored as 2. Tumor budding was a histologic finding in the invasion front of CRC tumor cells, singly or in small aggregates, that is detached from the neoplastic glands. Tumor buds were defined as comprising five or fewer tumor cells [9].

### *Immunohistochemistry*

The formalin-fixed paraffin embedded tissues were cut into 4 μm sections and deparaffinized. Immunohistochemical staining using the BOND-

**Table 2.** Clinicopathologic features compared by histologic features associated with EMT and invasion

| Variable | | Tumor budding, n (%) | | Desmoplasia, n (%) | | CAF, n (%) | | Inflammation, n (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Absent (n=31) | Present (n=8) | Absent (n=5) | Present (n=34) | Absent (n=3) | Present (n=36) | 0 (n=26) | 1 (n=7) | 2 (n=6) |
| pT stage[b,c] | 0 | 3 (9.7) | 0 (0) | 2 (40.0) | 1 (2.9) | 2 (66.7) | 1 (2.8) | 2 (7.7) | 0 (0) | 1 (16.7) |
| | 1, 2 | 4 (12.9) | 1 (12.5) | 1 (20.0) | 4 (11.8) | 1 (33.3) | 4 (11.1) | 2 (7.7) | 2 (28.6) | 1 (16.7) |
| | 3, 4 | 24 (77.4) | 7 (87.5) | 2 (40.0) | 29 (85.3) | 0 (0) | 31 (86.1) | 22 (84.6) | 5 (71.4) | 4 (66.7) |
| TNM stage[b,c] | Early (0, 1, 2) | 18 (58.1) | 5 (62.5) | 5 (100) | 18 (52.9) | 3 (100) | 20 (55.6) | 13 (50.0) | 7 (100) | 3 (50.0) |
| | Advanced (3, 4) | 13 (41.9) | 3 (37.5) | 0 (0) | 16 (47.1) | 0 (0) | 16 (44.4) | 13 (50.0) | 0 (0) | 3 (50.0) |
| Gross type[a,c] | Fungating | 9 (29.0) | 0 (0) | 2 (40.0) | 7 (20.6) | 3 (100) | 6 (16.7) | 5 (19.2) | 2 (28.6) | 2 (33.3) |
| | Ulcerative | 22 (71.0) | 8 (100) | 3 (60.0) | 27 (79.4) | 0 (0) | 30 (83.3) | 21 (80.8) | 5 (71.4) | 4 (66.7) |
| Differentiation[c] | Well | 8 (25.8) | 0 (0) | 2 (40.0) | 6 (17.6) | 3 (100) | 5 (13.9) | 5 (19.2) | 2 (28.6) | 1 (16.7) |
| | Moderately | 20 (64.5) | 5 (62.5) | 1 (20.0) | 24 (70.6) | 0 (0) | 25 (69.4) | 16 (61.5) | 5 (71.4) | 4 (66.7) |
| | Poorly | 3 (9.7) | 3 (37.5) | 2 (40.0) | 4 (11.8) | 0 (0) | 6 (16.7) | 5 (19.2) | 0 (0) | 1 (16.7) |
| Lymphovascular invasion | Absent | 19 (61.3) | 5 (62.5) | 5 (100) | 19 (55.9) | 3 (100) | 21 (58.3) | 14 (53.8) | 5 (71.4) | 5 (83.3) |
| | Present | 12 (38.7) | 3 (37.5) | 0 (0) | 15 (44.1) | 0 (0) | 15 (41.7) | 12 (46.2) | 2 (28.6) | 1 (16.7) |
| Perineural invasion[d] | Absent | 20 (64.5) | 6 (75) | 4 (80.0) | 22 (64.7) | 3 (100) | 23 (63.9) | 14 (53.8) | 7 (100) | 5 (83.3) |
| | Present | 11 (35.5) | 2 (25) | 1 (20.0) | 12 (35.3) | 0 (0) | 13 (36.1) | 12 (46.2) | 0 (0) | 1 (16.7) |

[a]Statistically significant with $P<0.05$ by tumor budding; [b]Statistically significant with $P<0.05$ by desmoplasia; [c]Statistically significant with $P<0.05$ by CAF; [d]Statistically significant with $P<0.05$ by inflammation.

MAX slide stainer (Leica Biosystems) was carried out in accordance with the manufacturer's instructions. Anti-E-cadherin (1:3000, BD Biosciences), cytokeratin (CK, 1:3000, SIGNET), α-SMA (1:500, Dako-patts), β-catenin (1:200, Leica Biosystems), Wnt5b (1:100, ThermoFisher Scientific, Inc.), TGF-β1 (1:100, Santa Cruz Biotechnology, Inc.), p-Smad2 (1:200, Novus Biologicals), Smad4 (1:100, Santa Cruz Biotechnology, Inc.), ZEB1 (1:100, Bethyl Laboratories, Inc.) and anti-SNAIL (1:100, Abcam) were applied to the sections. Simple immunohistochemical stains of β-catenin, Wnt5b, TGF-β1, p-Smad2, Smad4, ZEB1 and anti-SNAIL were done using a Bond polymer refine detection kit (DAB, 3,3'-diaminobenzidine tetrahydrochloride, Leica Biosystems). Two sets of double immunohistochemical stains were performed: E-cadherin - CK and E-cadherin - α-SMA. The double immunohistochemical stains were performed by a combination of the Bond polymer refine detection kit and the Bond primer Ap Red detection kit (Fast Red, Leica Biosystems).

The stained sections were interpreted without any knowledge of the clinical data of the patient cohort. Diffuse cytoplasmic staining was interpreted as positive for CK, α-SMA, Wnt5b, and TGF-β1. Only nuclear staining was assessed as positive reactivity for Smad2, Smad4, ZEB1 and SNAIL. Membranous staining of E-cadherin and b-Catenin was interpreted as normal, and nuclear staining of β-catenin was considered to be translocational expression. Any other patterns of staining were regarded as false positive. Negative staining in the internal controls was regarded as false negative staining. The immunoreactivity on the tumor surface and tumor margin (invasion front) were analyzed separately. α-SMA-positive fibroblasts of the periphery of the tumor cells were interpreted as activated CAFs [31].

*Statistical analysis*

Statistical analyses were calculated with SPSS Amos 19.0 (IBM corp., Armonk, NY, USA). The two sample t-test, Pearson Chi-square test, and logistic regression were used to compare differences in qualitative data. The data were expressed as mean ± standard deviation for continuous variables and a $P$-value < 0.05 was regarded as statistically significant.

## Results

**Table 2** analyzes variables stratified histologic features associated with the EMT and invasion, such as desmoplasia, CAFs, tumor budding and peritumoral inflammation. Thirty-four cases (87.2%) revealed desmoplasic reactions and 36 cases (92.3%) showed α-SMA-positive CAFs accumulation. Tumor budding was found in eight cases (20.5%). Of the aggressive histopathologic factors analyzed, the patients with desmoplasia were correlated with pT ($P$=0.032) and TNM stage ($P$=0.035). The accumulation of CAFs was related with ulceration ($P$=0.001)

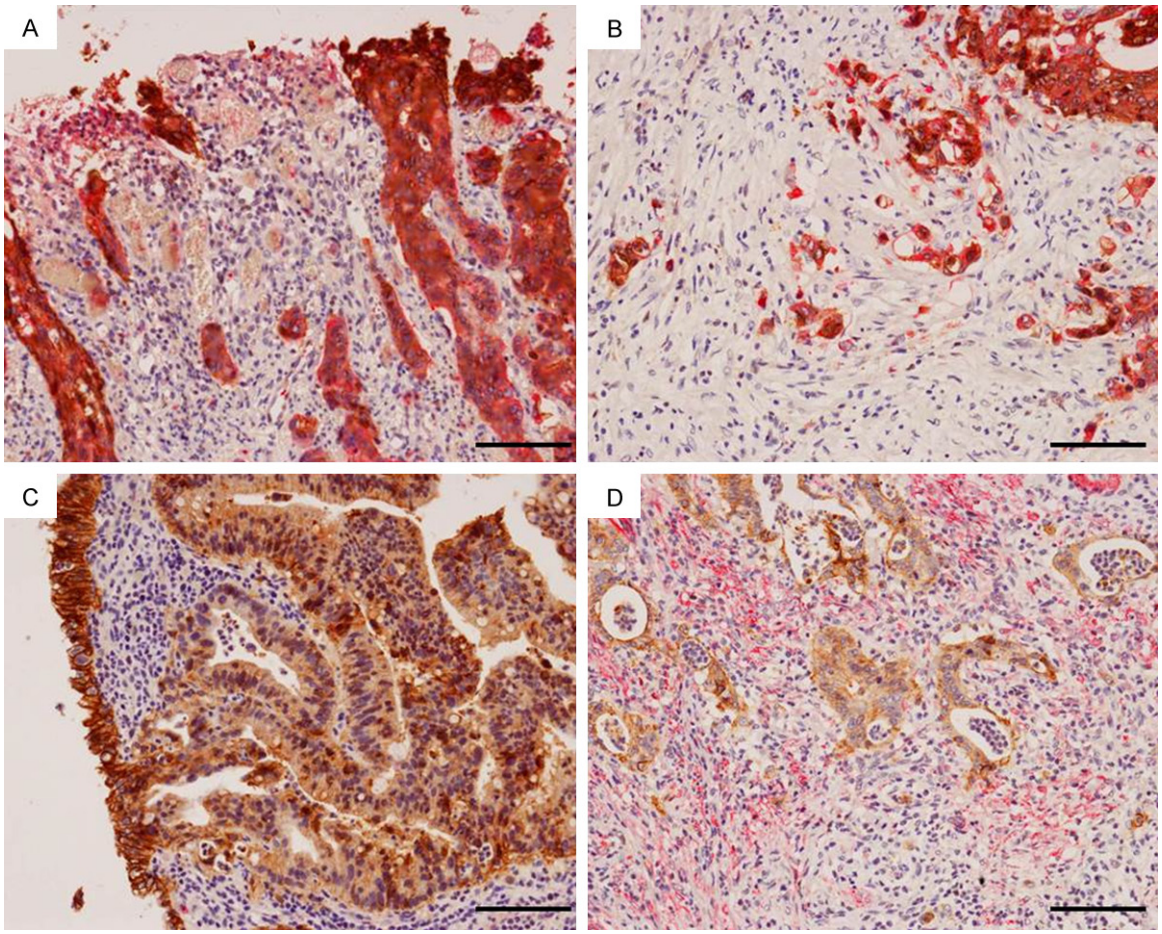


**Figure 1.** Expression of E-cadherin compared to CK (A and B) and α-SMA (C and D) via double immunohistochemistry. The loss of expression of E-cadherin in CK-positive tumor cells is more prominent in the invasive front of tumor than at the surface, where α-SMA-positive myofibroblasts (CAFs) are accumulated. (A and C) Cancer surface, (B and D) Invasive front (×200, scale bar: 100 μm).

and differentiation ($P$=0.002) as well as pT ($P$=0.000) and TNM stage ($P$=0.001). The cases with budding margins of the tumor were correlated with the ulcerative gross type of tumor ($P$=0.002) associated with the inflammatory lesion of the surrounding stroma. As shown in **Table 2**, 26 cases of CRC showed few inflammatory infiltrates (score 0, 66.7%). Diffuse abscess formation was observed in six cases (score 2, 15.4%), and seven cases showed focal inflammatory infiltration (score 1, 17.9%). The status of peritumoral inflammation was correlated with perineural invasion ($P$=0.046).

On the surface of the tumor, all the samples showed diffuse and strong expression of E-cadherin on the CK-positive tumor cell membrane (**Figure 1A**). The expression of E-cadherin was less strongly and focally on the cell membrane at the margin of the tumor than on the tumor surface (**Figure 1B**). The loss of E-cadherin expression was more prominent in the infiltrating tumor margins of scattered singly or clustered with a few tumor cells, so called tumor budding, than expanding tumor margins. However, α-SMA expression was rarely detected (**Figure 1C**) on the tumor surface. The diffuse α-SMA expressed cells were observed in the 36 cases around the tumor cells at the invasion front (**Figure 1D**).

The expression of Wnt5b was observed in both tumor cells and CAFs, on the tumor surface and at the invasive margin (**Figure 2A** and **2B**). In the normal colorectal mucosa, β-catenin expression was observed at the basolateral surface of mucosal epithelial cells. In contrast, increased expression and nuclear translocation of β-catenin (**Figure 2C** and **2D**) were

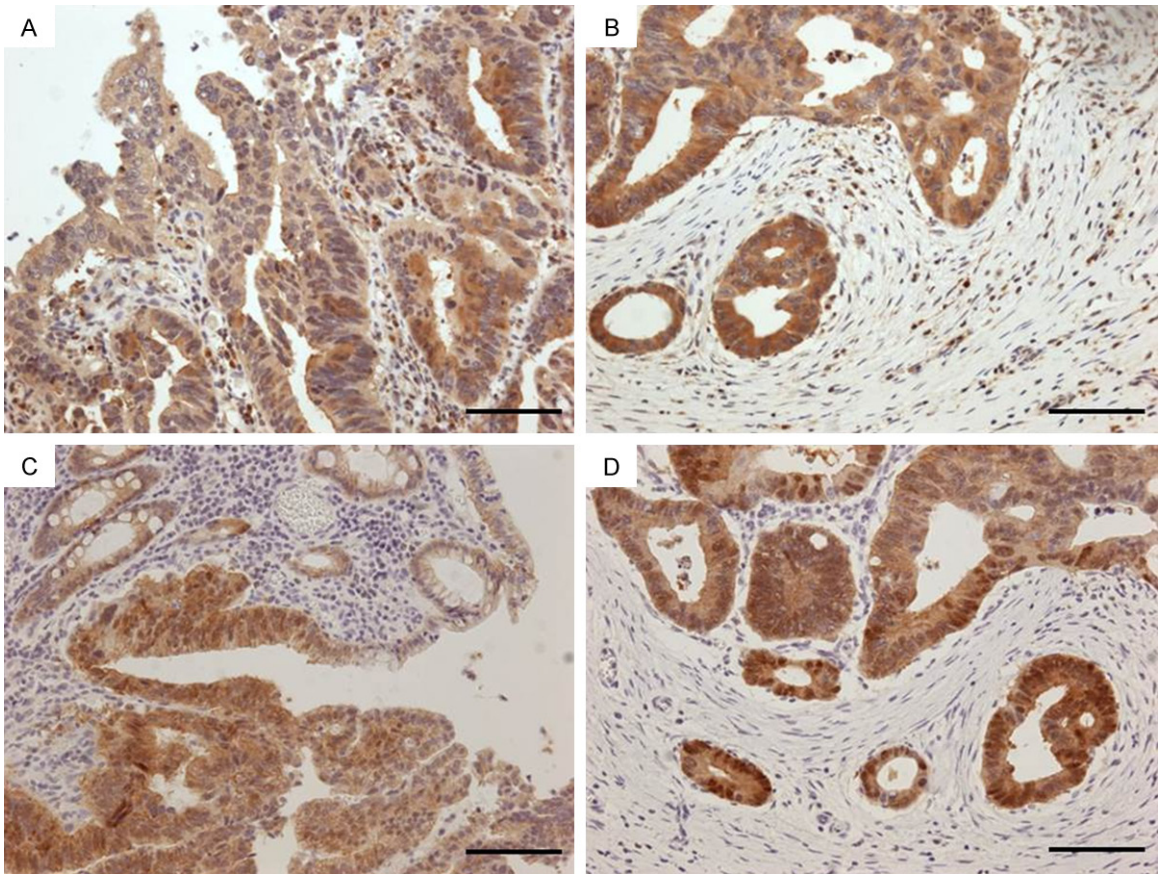


**Figure 2.** Expressions of signaling molecules of Wnt pathway, Wnt5b (A and B) and β-catenin (C and D). The expression of Wnt5b is observed in both tumor cells and CAFs, at the tumor surface (A) and the invasive margin (B). The increased expression and nuclear translocation of β-catenin is observed more frequently in the tumor cells of the invasive margin (D) than those of the tumor surface (C). (A and C) Cancer surface, (B and D) Invasive front (×200, scale bar: 100 μm).

observed in some of the CRCs. Ten cases of CRCs showed complete membranous staining of β-catenin (25.6%). Diffuse and marked staining of nuclear translocation was observed in 11 cases (28.2%), and 18 cases were focally stained in the nuclei (46.2%). The nuclear translocation of β-catenin expression was more frequently found in the tumor cells of the invasive margin (**Figure 2D**) than those of the tumor surface in the focally expressed cases (**Figure 2C**). This finding was prominent in the tumor budding site.

TGF-β1 staining was seen in the cytoplasm of the tumor cells and CAFs (**Figure 3A** and **3B**) in the invasive tumor margin. Similarly, nuclear p-Smad2 staining was seen in the tumor cells and CAFs of the invasive margin (**Figure 3C** and **3D**). Diffuse Smad4 expression was observed in CAFs of the tumor margin in all experimental cases (**Figure 3E** and **3F**). On the other hand, in the tumor cells, five cases showed Smad4-negative findings (**Figure 3E**).

**Table 3** analyzes variables stratified between Smad4-positive and Smad4-negative tumors. However, there was no significant association between the Smad4 expression of tumor cells and the clinicopathological features of the CRCs, such as, tumor location, stage, gross type, differentiation, and perineural and/or lymphovascular invasion. It was not correlated with any characteristics of the tumor microenvironment, such as, desmoplasia, accumulation of CAFs, inflammation and tumor budding, either. Analysis by the expression status of β-catenin shows that there was a significant ($P$=0.027) increase of lymphovascular invasion from CRCs (**Table 3**). Most diffuse expression cases were found in the grossly ulcerative type

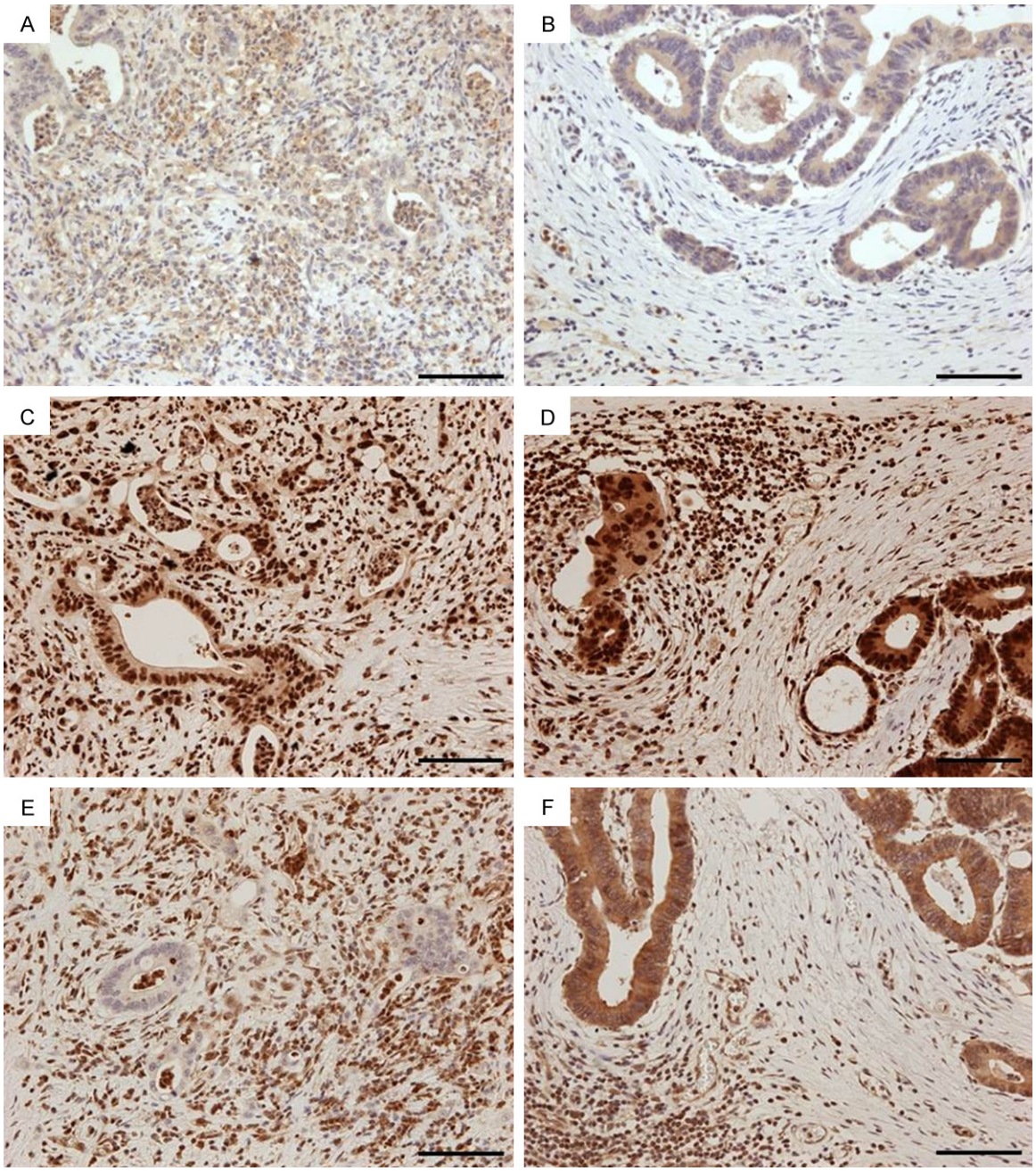


**Figure 3**. Expressions of signaling molecules of TGF-β1-Smad pathway. The immunohistochemical stains of TGF-β1 (A and B), p-smad2 (C and D), and smad4 (E and F) are diffusely positive in the CAFs of the invasive front of tumor in both of Smad4-negative (A, C and E) and Smad4-positive CRCs (B, D and F). (×200, scale bar: 100 μm).

(n=8, 72.7%) though the gross type was not significantly associated with β-catenin expression (*P*=0.205).

Immunohistochemical staining of SNAIL was detected at the tumor surface and tumor margin in all (100%) cases. However, some of the positive expression of SNAIL was limited only to the cytoplasm, and was absent from the nuclei.

The nuclear expression of SNAIL was observed in the tumor cells and CAFs (**Figure 4A**). The staining patterns in the nuclei were focal and weakly stained in some cases. Nuclear accumulation of snail1 was mainly found in tumor budding cells displaying the EMT-like phenotypic changes described above. The nuclear staining of ZEB1 was observed in all (100%) cases. The cells with positive ZEB1 staining were spin-

**Table 3.** Comparison of clinicopathologic features by the expression of Smad4 and β-catenin

| Variable | | Smad4, n (%) | | β-catenin, n (%) | | |
|---|---|---|---|---|---|---|
| | | Negative (n=5) | Positive (n=34) | Negative (n=10) | Focal (n=18) | Diffuse (n=11) |
| Location | Proximal | 1 (20.0) | 12 (35.3) | 6 (60.0) | 4 (22.2) | 3 (27.3) |
| | Distal | 2 (40.0) | 15 (44.1) | 2 (20.0) | 9 (50) | 6 (54.5) |
| | Rectal | 2 (40.0) | 7 (20.6) | 2 (20.0) | 5 (27.8) | 2 (18.2) |
| pT stage | 0 | 0 (0) | 3 (8.8) | 2 (20.0) | 0 (0) | 1 (9.1) |
| | 1, 2 | 1 (20.0) | 4 (11.8) | 1 (10.0) | 2 (11.1) | 2 (18.2) |
| | 3, 4 | 4 (80.0) | 27 (79.4) | 7 (70.0) | 16 (88.9) | 8 (72.7) |
| pN stage | 0 | 3 (60.0) | 23 (67.6) | 9 (90.0) | 9 (50) | 8 (72.7) |
| | 1, 2 | 2 (40.0) | 9 (26.5) | 1 (10.0) | 7 (38.9) | 3 (27.3) |
| | 3, 4 | 0 (0) | 2 (5.9) | 0 (0) | 2 (11.1) | 0 (0) |
| pM stage | 0 | 4 (80.0) | 24 (75.0) | 9 (90.0) | 11 (68.8) | 8 (72.7) |
| | 1, 2 | 1 (20.0) | 8 (25.0) | 1 (10.0) | 5 (31.3) | 3 (27.3) |
| Stage (pTNM) | Early (0, 1, 2) | 3 (60.0) | 20 (58.8) | 9 (90.0) | 8 (44.4) | 6 (54.5) |
| | Advanced (3, 4) | 2 (40.0) | 14 (41.2) | 1 (10.0) | 10 (55.6) | 5 (45.5) |
| Gross type | Fungating | 0 (0) | 9 (26.5) | 4 (40.0) | 2 (11.1) | 3 (27.3) |
| | Ulcerative | 5 (100) | 25 (73.5) | 6 (60.0) | 16 (88.9) | 8 (72.7) |
| Histologic type | Tubular | 5 (100) | 33 (97.1) | 10 (100) | 17 (94.4) | 11 (100) |
| | Mucinous | 0 (0) | 1 (2.9) | 0 (0) | 1 (5.6) | 0 (0) |
| Differentiation | Well | 1 (20.0) | 7 (20.6) | 3 (30.0) | 1 (5.6) | 4 (36.4) |
| | Moderate | 4 (80.0) | 21 (61.8) | 5 (50.0) | 14 (77.8) | 6 (54.5) |
| | Poorly | 0 (0) | 6 (17.6) | 2 (20.0) | 3 (16.7) | 1 (9.1) |
| Perineural invasion | Absent | 3 (60.0) | 23 (67.6) | 8 (80.0) | 9 (50) | 9 (81.8) |
| | Present | 2 (40.0) | 11 (32.4) | 2 (20.0) | 9 (50) | 2 (18.2) |
| Lymphovascular invasion[a] | Absent | 2 (40.0) | 22 (64.7) | 8 (80.0) | 7 (38.9) | 9 (81.8) |
| | Present | 3 (60.0) | 12 (35.3) | 2 (20.0) | 11 (61.1) | 2 (18.2) |
| Desmoplasia | Absent | 0 (0) | 5 (14.7) | 3 (30.0) | 1 (5.6) | 1 (9.1) |
| | Present | 5 (100) | 29 (85.3) | 7 (70.0) | 17 (94.4) | 10 (90.9) |
| CAF | Absent | 0 (0) | 3 (8.8) | 2 (20.0) | 0 (0) | 1 (9.1) |
| | Present | 5 (100) | 31 (91.2) | 8 (80.0) | 18 (100) | 10 (90.9) |
| Inflammation | 0 | 3 (60.0) | 23 (67.6) | 7 (70.0) | 12 (66.7) | 7 (63.6) |
| | 1 | 2 (40.0) | 5 (14.7) | 2 (20.0) | 3 (16.7) | 2 (18.2) |
| | 2 | 0 (0) | 6 (17.6) | 1 (10.0) | 3 (16.7) | 2 (18.2) |
| Budding | Absent | 3 (60.0) | 28 (82.4) | 7 (70.0) | 15 (83.3) | 9 (81.8) |
| | Present | 2 (40.0) | 6 (17.6) | 3 (30.0) | 3 (16.7) | 2 (18.2) |

[a]Statistically significant with $P<0.05$ by β-catenin.

dle-shaped stromal elements which overlapped with the distribution of α-SMA-positive CAFs (**Figure 4B**).

## Discussion

The EMT is originally known as a process during embryonic development in which cells acquire the mesenchymal phenotype and lose the epithelial phenotype [23]. The EMT was first recognized as a distinct cell differentiation process in the late 70 s and, over the years, it has received increasing attention, since it not only occurs in embryonic development but also contributes to various pathological conditions, including wound healing, tissue-regeneration, inflammation, and fibrosis as well as in the conversion of differentiated epithelial cancer cells into migratory mesenchymal cancer cells which may lead to cancer invasion and metastasis [34]. As the EMT progresses, the tumor cells acquire a motile and invasive phenotype [8,

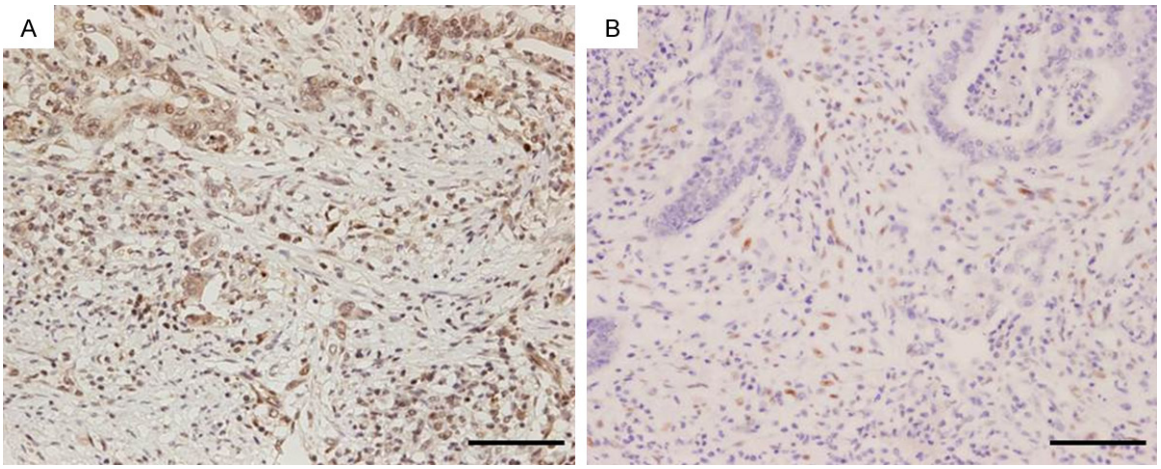


**Figure 4.** Expressions of SNAIL (A) and ZEB1 (B) in the invasive portion of CRCs. The nuclear expression of SNAIL is observed in tumor cells and CAFs especially at the tumor budding site (A). The nuclear staining of ZEB1 is observed in CAFs (B). (×200, scale bar: 100 μm).

34]. The EMT involves the loss of epithelial markers, such as the tight junction proteins claudins and occludins, the adherens junction proteins E-cadherin, β-catenin, and CKs [7]. Concomitantly, a number of mesenchymal markers are increased in their expression, including N-cadherin, vimentin, fibronectin, matrix metalloproteinase, and α-SMA [35].

Nabeshima *et al*. [36] reported that large aggregates of CRC cells (much larger than tumor buds) induced matrix degradation and moved as large, coherent clusters. They initiate and sustain the remodeling of the adjacent extracellular matrix [36] but, in contrast to tumor budding, they retain cell-cell contacts to remain in large aggregates. In our study, tumor buddings were noted in eight cases (20.5%) and were significantly related with the presence of surface ulceration. These findings suggest that the EMT is increased in the presence of tumor surface ulceration, which is related with inflammation. Actually, peritumoral inflammation is significantly associated with perineural invasion, suggesting a relationship between the presence of inflammation and tumor cell invasiveness. Further studies for the presence of inflammation related to the EMT are needed.

Tumor progression and metastasis are influenced by tumor-associated stroma as well as the tumor cell itself [37]. The tumor-associated stroma is composed of the extracellular matrix and many different cells such as inflammatory cells, macrophages, endothelial cells, and fibroblasts [38]. Tumor epithelial cells within a tumor coexist with a complex microenvironment [31]. Recently, numerous studies reported that these complex processes are associated with the EMT and it constitutes an important mechanism in the development of tumor invasiveness [5, 27, 32]. Vered *et al*. [32] reported that EMT markers are commonly expressed in both primary and metastatic oral cancers. Cancer cells with decreased E-cadherin expression are primarily located at the tumor periphery and directly contact CAFs, revealing that the EMT may be modulated by CAFs [32].

As the most abundant component of tumor microenvironment, CAFs are widely known to be co-conspirators in tumor initiation, progression and metastasis [5, 32]. CAFs acquire a phenotype similar to myofibroblasts, which are activated in wound healing and fibrosis and possess a different morphology and function from normal fibroblasts [29]. Unlike the myofibroblasts removed by apoptosis in normal wound healing, fibroblasts of the tumor stroma, CAFs, are constantly activated [28] and promote tumor growth and tumor progression, favoring a variety of tumor-specific mechanisms [39], including extracellular matrix remodeling, immune suppression, and secretion of the growth factors and cytokines that extensively affect tumor cell growth, invasion, differentiation, angiogenesis, and chronic inflammation [29, 30]. Some clinical researchers have reported that CAFs have a significant correlation with the regional lymphatic metastasis and progno-

sis in mobile tongue squamous cells carcinoma, ovarian cancer, and gastric cancer [40-42].

In our study, desmoplasia was found more frequently in the advanced stage of CRCs. The number of α-SMA-positive CAFs is increased further in the advanced pT stage, the presence of surface ulceration, and in poorly differentiated cancer. It is suggested that tumor invasiveness and prognosis are affected by the presence of CAF. Furthermore, it should be noted that the increasing number of CAFs is associated with direct stimulation by the surface ulceration of the tumor. In addition, we observed the characteristic findings of the EMT; the decreased expression of E-cadherin and increased expression of SMA. The loss of expression of E-cadherin is more prominent in the invasive front of the tumor than the surface, where α-SMA-positive myofibroblasts myofibroblasts (CAFs) accumulated. The process of the EMT may be more activated in the deep invasive portion of the CRC under the control of CAFs.

In CRCs, Wnt disruption is expected to be common [43]. As direct evidence of Wnt dysregulation, β-catenin immunohistochemistry in CRCs revealed a striking pattern [44]. In our study, the nuclear translocation of β-catenin expression was observed more frequently in the tumor cells of the invasive margin than those of the tumor surface. These findings were especially prominent in the tumor budding and overlapped to the EMT site as shown by the double immunohistochemistry of E-cadherin and CK, which is consistent with the previous studies [44]. The status of nuclear translocated β-catenin was significantly correlated with lymphatic invasion. The expression of Wnt5b was observed in the cytoplasm of tumor cells and CAFs. These findings suggest that the expression of Wnt5b in tumor cells and CAFs controls nuclear translocated expression of β-cateninin tumor cells of CRC.

In this study, we observed similar findings in the expression of TGF-β1-Smad signaling molecules in tumor cells and CAFs in the tumor invasive margin with the expression of Wnt signaling molecules. TGF-β1 and the TGF-β receptor are expressed in the majority of CRCs [16] and their expression is notably frequent in CRCs with prominent tumor budding [17]. TGF-β1 binding to TGF-β receptors (TβRI and TβRII) initiates a Smad2/3 complex formation to phosphorylate Smad2 or Smad3. These form heterodimers with Smad4 and this heterodimer translocates to the nucleus to regulate the transcription of target genes [9]. Various different effects of the TGF-β1-Smad signaling pathway have been observed *in vitro*, including the regulation of cell migration, increased synthesis of extracellular matrix, and changes of the epithelial cells paralleling EMT [45, 46]. In this study, there was no significant relation between the Smad4 expression of tumor cells and the clinicopathologic factors and histologic features of tissue invasion and the tumor microenvironment. However, we found that the immunohistochemical staining of signaling molecules of the TGF-β1-Smad pathway are diffusely positive in the CAFs of the invasive front of tumor, regardless the positivity of smad4 expression in tumor cells. These findings suggest that the TGF-β1-Smad pathway in CAFs might affect the EMT of cancer cells to invade the stroma.

The clinical importance of the expression of transcription factors, such as SNAIL and ZEB1, is also known to contribute to a higher tumor grade and a poorer prognosis in various carcinomas [8, 47, 48]. The precise molecular mechanisms behind the altered expression of ZEB1 in CRC are unclear. Recent studies found that ZEB1 also inhibited the expression of E-cadherin, and thus plays an important role in the carcinogenesis, progression, invasion and metastasis of various tumors [49-51]. Putzke *et al*. [51] reported that the overexpression of ZEB1 in prostate cancer cells inhibited the expression of E-cadherin protein, thereby promoting the invasion and metastasis of prostate cancer cells. The overexpression of ZEB1 was also found to promote the invasion and metastasis of lung cancer cells [48]. Using immunohistochemical study, Mang *et al*. reported that the nuclear expression of ZEB1 and SNAIL & SLUG was observed in the tumor center and invasive margin of human lung adenocarcinoma [23]. Although the nuclear staining of SNAIL & SLUG was more frequently observed in the margin of the tumor, the expression of transcription factors within the tumor was unexpectedly homogenous between both areas analyzed. However, those studies focused on the expression of the transcription factors in tumor cells, *in vivo* as well as *in vitro*. In this study, the nuclear expression of ZEB1 was

observed in all cases, both at the tumor surface and the periphery, mostly in CAFs, but also rarely in tumor cells. The ZEB1-positive CAFs were markedly richer in the tumor invasive margin than at the tumor surface. The expression of SNAIL was detected in tumor margin in tumor cells and CAFs. These transcription factors, which are known as driving factors of the EMT, were produced by the CAFs and might act with a paracrine effect on the neighboring tumor cells, resulting in the EMT.

In summary, we found that the EMT process occurred in CRCs, especially in the deep invasive portion with a marked accumulation of CAFs. The TGF-β1-Smad and Wnt pathways were associated with the control system of the interaction between cancer cells and CAFs during the EMT process. In addition, the expressions of related transcription factors, such as SNAIL and ZEB1, were increased in cancer cells and the surrounding CAFs. In particular, these expressions were markedly increased in the deep invasive portions of the cancer. These signaling molecules and transcription factors which were produced by the CAFs might act in a paracrine manner from the CAFs to the neighboring tumor cells, which then induces the EMT process. This study may be the first to investigate the relationship between the EMT of CRC and CAFs in a tumor microenvironment by using immunohistochemical staining of human CRC tissue. However, these results still have some limitations, such as the small number of the study cases. Further studies should be conducted with a focus on the detailed pathogenesis of these EMT processes. This would allow novel approaches for the treatment of CRCs in and advanced stage through a deeper understanding of the interaction between the EMT process and the surrounding microenvironment.

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## Disclosure of conflict of interest

None.

**Address correspondence to:** Drs. Chang-Ho Cho and Kwan-Kyu Park, Department of Pathology, College of Medicine, Catholic University of Daegu, 3056-6 Daemyung 4-Dong, Nam-Gu 705-718, Daegu, Republic of Korea. Tel: +82-53-650-4160; Fax: +82-53-650-4834; E-mail: chcho@cu.ac.kr (CHC); Tel: +82-53-650-4149; Fax: +82-53-650-4834; E-mail: kkpark@cu.ac.kr (KKP)

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