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Expression of E-cadherin and β-catenin is Altered at Tumor Budding Sites, Whose Number is Associated with the Progression of Colorectal Carcinoma

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Background : Tumor budding is present in the stroma at the invasive margin of colorectal carcinomas (CRC). The disintegration of cell adhesion molecules is closely related to this process. This study investigated the role of tumor budding in the progression of CRC, and compared the expression of β-catenin and E-cadherin between tumor budding and tumor center to determine whether epithelial-to-mesenchymal transitions (EMTs) occur in tumor budding. **Methods :** The number of tumor budding (NTB) instances was determined in 58 cases of CRC, and immunoreactivities of E-cadherin and β-catenin were compared at the tumor center and at the tumor budding site. Immunohistochemical staining for vimentin was also done. **Results :** Tumor budding was seen in 52 tumors (90%). There were significant associations between NTB and cliniopathologic parameters such as tumor depth, nodal metastasis and clinical stage. Expression of cytoplasmic and nuclear β-catenin were significantly higher at tumor budding sites than in the tumor center. In contrast, expression of membranous and cytoplasmic E-cadherin were significantly higher in the tumor center than at the tumor budding sites. Vimentin was expressed at tumor budding foci of only 2 cases (3%). **Conclusions :** This study suggests that EMT occurs at tumor budding, and that NTB may be a good marker for predicting a poor prognosis in CRC.

Key Words : Colorectal carcinoma; Tumor budding; E-cadherin; Beta-catenin; EMT

Tumor budding has been defined as the presence of isolated single cells or small cell clusters scattered in the stroma at the invasive margin.[1] This histologic feature is related to aggressiveness in colorectal carcinomas (CRC).[1-5] At the invasive front of dimethylhydrazine-induced murine colonic carcinomas, a dissociation of the organized tumor cell complexes into isolated tumor cells was found together with loss of the cytological features of differentiation.[6] This corresponds to tumor budding in CRC. The disintegration of cell adhesion molecules is closely related to this process. Down-regulation of E-cadherin is accompanied by a loss of epithelial characteristics and the acquisition of mesenchymal properties, a process often referred to as the epithelial-to-mesenchymal transition (EMT).[7] During an EMT, carcinoma cells become more motile and invasive by acquiring characteristics similar to embryonic mesenchymal cells, thereby allowing penetration of the stroma surrounding the initial neoplastic focus.[8] Beta-catenin is bound to membrane-associated E-cadherin and is essential for its correct position and function.[9] Inactivation of the adenomatous polyposis coli (*APC*) gene is the earliest frequent event in CRC and leads to a cytoplasmic and subsequent nuclear accumulation of β-catenin.[10] Nuclear β-catenin activates genes necessary for cell proliferation, differentiation and invasive growth.[11-15] Thus, the intracellular distribution of β-catenin has a strong impact on the phenotype and behavior of tumor cells.[16] Several studies have shown that nuclear β-catenin occurs at tumor buds at the invasive margin of CRC.[16-18] Overall, it is plausible that modulation of E-cadherin-mediated adhesion is involved in tumor budding. The aim of this study was to evaluate the role of tumor budding in the progression of CRC and to compare the expression of E-cadherin and β-catenin between tumor budding sites and the tumor center whether EMT occurs at tumor budding foci.

MATERIALS AND METHODS

Patients

The present study included 58 patients who were among those who underwent curative surgery for colorectal adenocarcinoma

at Dongguk University Kyongju Hospital from January 2000 to December 2007. We selected patients whose paraffin embedded tissues were relatively well preserved and whose medical records were complete. We excluded patients who underwent preoperative chemotherapy and emergency surgery, and the patients who were diagnosed with mucinous adenocarcinoma. Patient ages ranged from 31 to 92 years. The male to female ratio was 13:16.

Microscopic examination and immunohistochemistry

Differentiation and depth of tumor, and status of lymph node metastasis were assessed after reviewing each tumor slide. The stage was defined according to the TNM staging system of the American Joint Committee on Cancer.[19] The presence of budding was determined according to the criteria proposed by Ueno *et al.*[1] The authors defined an isolated single cancer cell and a cluster composed of fewer than five cancer cells as tumor budding. The number of tumor budding (NTB) foci was counted in a field in which budding intensity was considered maximal at high power magnification.

For immunohistochemical staining, formalin fixed paraffin embedded tissue sections of 4 μm thickness were made and spread on poly-L-lysine coated slides. The sections were deparaffinized and hydrated in a graded series of alcohol solutions. Antigen retrieval was routinely performed by immersing the sections in 0.01 M citrate buffer (pH 6.0) in a pressure cooker and autoclaving for 15 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 min and slides were then incubated with primary antibody for 2 h at room temperature. The primary antibodies used were: anti-E-cadherin (1:1,000, Transduction Laboratories, Lexington, KY, USA), anti-β-catenin (1:500, Transduction Laboratories) and anti-vimentin (DAKO, Santa Barbara, CA, USA). Staining was done with an EnVision kit labeled with peroxidase (DAKO) and developed with 3,3′-diaminobenzidine tetrahydrochloride (Zymed Laboratories, South San Franciso, CA, USA) as the chromogen. Sections were counterstained for 3 min with Mayer's hematoxylin and then mounted. As a negative control, rabbit and mouse IgG isotypes were used instead of the primary antibody.

Immunoreactivity of β-catenin and E-cadherin were separately analysed for the tumor center and the tumor budding sites. Staining intensity of membranous, cytoplasmic and nuclear β-catenin was graded as absent (0), mild (1), moderate (2), or severe (3). Intensity of membranous and cytoplasmic E-cadherin was also graded into four categories.

Statistical analysis

Pearson correlation, one-way ANOVA and t-test were used. Statistical significance was assumed if a p-value was less than 0.05. Data are expressed as mean ± standard error.

RESULTS

Tumor budding was seen in 52 tumors (90%) and NTB sites was 14.62 ± 1.47. The relationship between NTB and clinicopathologic parameters is shown in Table 1. NTB was progressively increased as the tumor deepened (p=0.000). NTB was significantly higher in tumors infiltrating the subserosa and through the serosa (T3 and T4) than in tumors of Tis and T1 (p=0.001). NTB was also significantly higher in tumors with nodal metastasis than in tumors without nodal metastasis (p=0.013). NTB in tumors with stage II-IV was significantly higher compared with those of stage 0 and I (p<0.009). In addition, NTB in stage IV was significantly higher than for stage II and III (p<0.003). There were no statistically significant associations between NTB and other parameters such as gender, age and tumor differentiation.

Immunoreactivity of β-catenin and E-cadherin are compared between tumor center and tumor budding sites in Fig. 1. Immunoreactivity of nuclear and cytoplasmic β-catenin was higher at tumor budding sites than in the tumor center. In contrast, immunoreactivity of membranous and cytoplasmic E-cadherin was lower than at tumor budding sites than in the tumor center. As

Table 1. Relationship between the number of tumor budding and clinicopathologic parameters in colorectal adenocarcinomas

Parameter		Number	Number of tumor budding	p-value
Gender	Male	26	13.46 ± 2.16	0.481
	Female	32	15.56 ± 2.02	
Differentiation	Well	32	16.09 ± 2.13	0.261
	Moderate & poor	26	12.80 ± 1.96	
Depth	Tis	5	0.20 ± 0.20	0.000
	T1	4	0.75 ± 0.47	
	T2	5	10.40 ± 2.42	
	T3 & T4	44	18.00 ± 1.56	
Nodal metastasis	Absence	34	11.53 ± 1.73	0.013
	Presence	24	19.00 ± 2.37	
Stage	0	5	0.20 ± 0.20	0.000
	I	8	6.12 ± 2.42	
	II	21	16.23 ± 1.99	
	III	22	17.23 ± 2.16	
	IV	2	38.50 ± 3.50	

shown in Fig. 2, β-catenin and E-cadherin staining showed a statistically significant difference between the tumor center and tumor budding sites (p=0.000). Membranous β-catenin expression was significantly higher in the tumor center (2.02±0.13) than in tumor budding foci (0.53±0.10). In contrast, expression of cytoplasmic and nuclear β-catenin was significantly higher in tumor budding sites (cytoplasmic, 2.38±0.11; nuclear, 2.32±0.13) than in the tumor center (cytoplasmic, 1.86±0.08; nuclear, 1.09±0.11). Expression of membranous and cytoplasmic E-cadherin was significantly higher in the tumor center (mem-

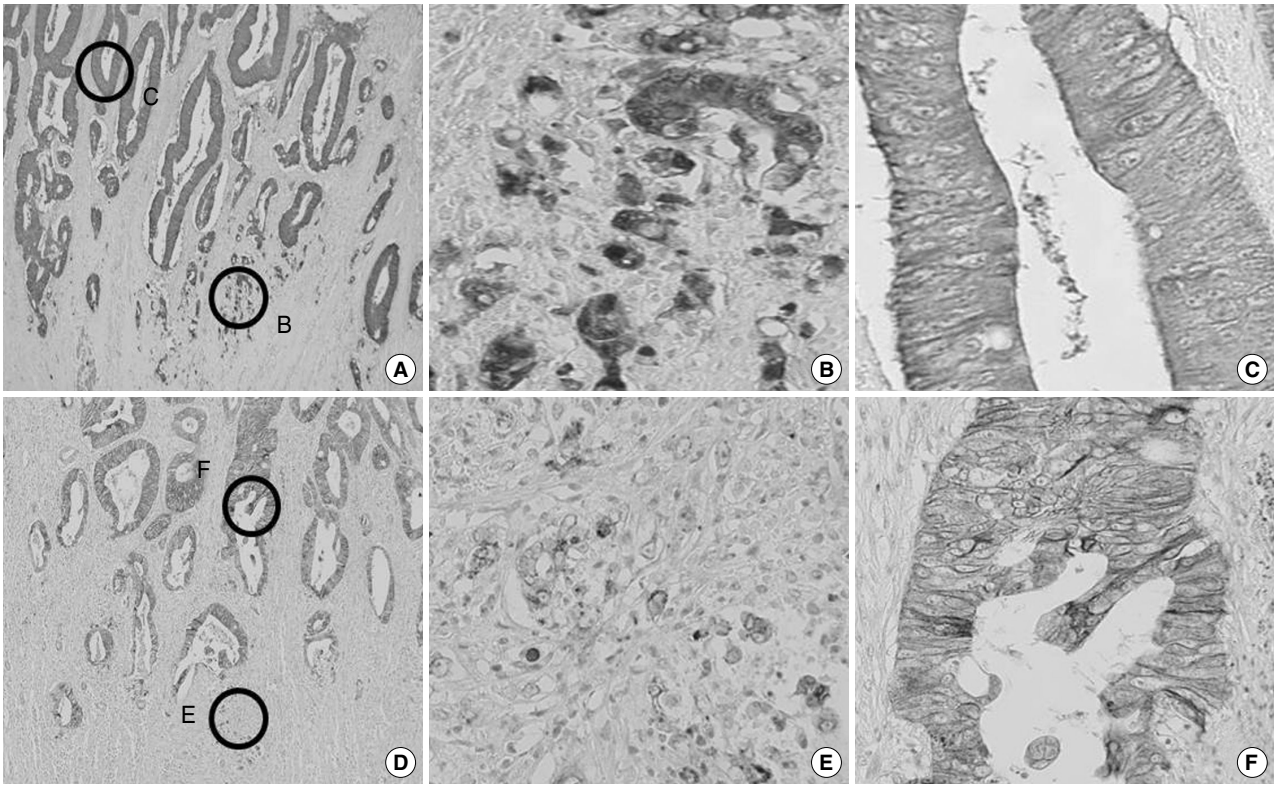

Fig. 1. Immunohistochemical staining of β-catenin (A-C) and E-cadherin (D-F) in colorectal adenocarcinomas. Immunoreactivities of nuclear and cytoplasmic β-catenin are higher in tumor budding (B) than in tumor center (C). Immunoreactivities of membranous and cytoplasmic E-cadherin are lower than in tumor budding (E) than in tumor center (F).

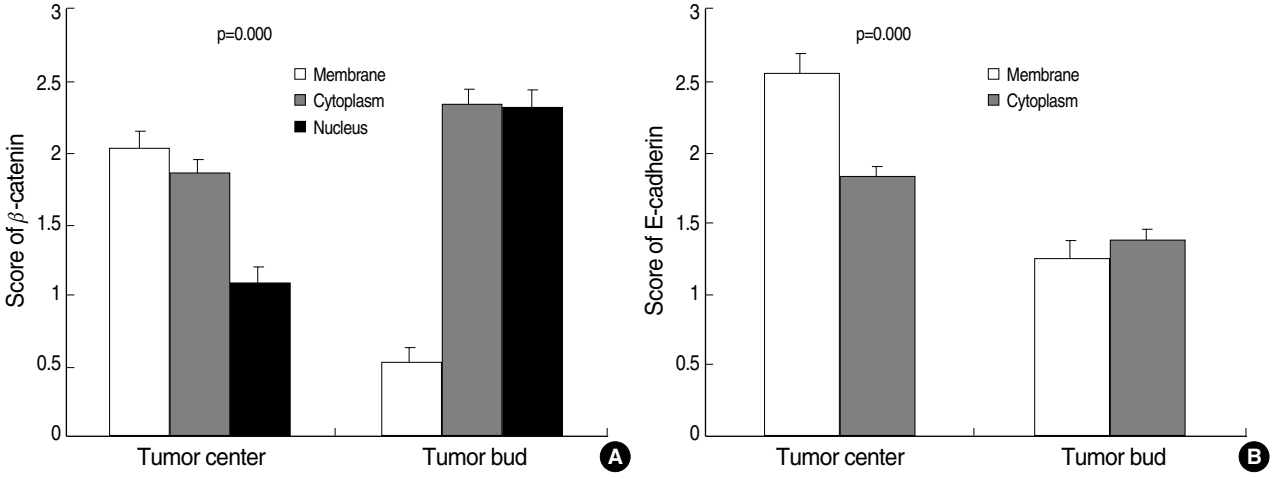

Fig. 2. Immunohistochemical scores of β-catenin (A) and E-cadherin (B) in colorectal adenocarcinomas. There is a significant difference in the scores of β-catenin (A) and E-cadherin (B) between tumor budding and tumor center (p=0.000).

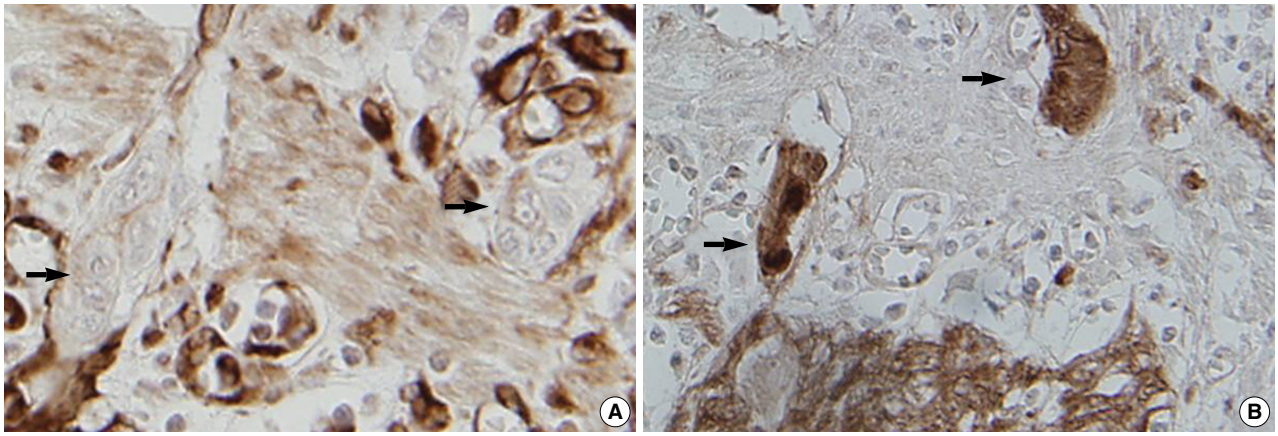

Fig. 3. Immunohistochemical staining of vimentin (A) and β-catenin (B) at the same area of colorectal adenocarcinoma. Tumor cells expressing nuclear and cytoplasmic β-catenin (B, arrows) at budding area show weak expression for vimentin (A, arrows).

branous, 2.56±0.11; cytoplasmic, 1.82±0.07) than in tumor budding sites (membranous, 1.24±0.13; cytoplasmic, 1.37±0.08).

Immunohistochemical staining for vimentin was done to examine its expression at tumor budding foci. As shown in Fig. 3, vimentin was expressed at tumor budding foci of only 2 cases (3%).

DISCUSSION

This study investigated the role of tumor budding in the progression of CRC, and compared expression of β-catenin and E-cadherin between tumor budding and tumor center sites to evaluate whether EMT occurs in tumor budding. Imai *et al.*[20] initially described tumor sprouting along the invasive margin and suggested that it was related to aggressiveness of the malignant tumor. The term 'tumor budding' was first described by Morodomi *et al.*,[2] who showed that the degree of budding was closely associated with lymph node metastasis in rectal cancers. Several recent studies have emphasized tumor budding as an index of tumor aggressiveness in CRC.[1-5] In our study, NTB was closely associated with tumor depth, nodal metastasis and clinical stage. Prerequisites for any parameter to be valid as a prognostic marker include simplicity, reproducibility and objectivity. Tumor budding can be determined simply by routine histopathology using hematoxylin-eosin stained sections. The reproducibility among histopathologists of budding measured either by intraobserver semiquantitative agreement or by interobserver agreement with regard to the presence or absence was high.[1,21]

Invasion and dissemination of well-differentiated carcinomas are often associated with loss of epithelial differentiation and gain of mesenchyme-like capabilities of the tumor cells at the invasive front.[16] EMT occurs via 2 distinct biological events: the breaking of cell-cell adhesions and a consequent increase in cell motility.[22] When β-catenin is bound to the cytoplasmic domain of E-cadherin, it enables E-cadherin to function as a cell-cell adhesion molecule and mediates the interplay of adherens junction molecules with the actin cytoskeleton.[9] Aberrations of E-cadherin expression can lead to abundant cytoplasmic β-catenin and its subsequent nuclear localization. Nuclear β-catenin activates genes necessary for invasive growth, like *matrilysin fibronectin, CD44,* and *uPAR*.[12-15] Brabletz *et al.*[16] have shown that disseminating tumor cells at the invasive front express nuclear β-catenin accompanied by either loss of membranous E-cadherin or cytoplasmic expression in CRC. In addition, they suggested that the tumor microenvironment regulates intracellular β-catenin distribution and the expression of E-cadherin.[16] In this study, nuclear β-catenin expression was increased to a greater extent in tumor budding than in tumor center sites. In contrast, expression of membranous and cytoplasmic E-cadherin was more decreased in tumor budding compared with the tumor center. However, a recent study suggested that nuclear β-catenin was often found at tumor budding sites in CRC, but was unlikely to be the sole cause of tumor budding.[5] Masaki *et al.*[3] did not find a correlation between nuclear β-catenin and changes in E-cadherin expression in the formation of tumor budding sites in patients with T1 CRC. In addition, the loss of membranous epithelial cell adhesion molecules (Ep-CAM) is associated with nuclear β-catenin localization and contributes to the formation of tumor budding.[23]

The present study shows that EMT might occur at tumor budding sites because aberrations of E-cadherin leading to nucle-

ar localization of β-catenin were observed at tumor budding sites. Immunohistochemical staining for vimentin was thus done to examine vimentin expression at tumor budding foci. However, vimentin was expressed at tumor budding in only 2 cases. Laminin-5 γ2 or β3 subunit-reactive budding colon carcinoma cells did not express vimentin.[24] Brabletz *et al.*[16] have shown weak expression of vimentin at tumor budding, but did not describe its frequency.

In summary, this study suggests that EMT occurs at tumor budding sites, and that NTB may be a good marker for predicting a poor prognosis in CRC.

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