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Original contribution

Tumor budding at the invasive front of colorectal cancer may not be associated with the epithelial-mesenchymal transition ☆

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Summary Tumor budding is thought to reflect the epithelial-mesenchymal transition (EMT). However, the molecular mechanism linking tumor buds and the EMT remains unclear. Here, we examined the induction of tumor budding and EMT and their association with EMT-related proteins (ZEB1, TWIST, SNAIL, and SLUG) in colorectal cancer (CRC). Immunohistochemical expression of pan-cytokeratin was examined for identification of tumor budding in 101 CRCs. Grading of tumor budding was classified into low- and high-grade groups. Tissue microarray was conducted to identify tumor budding sites. The expression of E-cadherin, ZEB1, TWIST, SNAIL, and SLUG was examined in areas of tumor budding and the surrounding tumor stroma using a double-immunostaining method. Specifically, pan-cytokeratin and EMT-related proteins were assessed by double immunostaining. Low or no expression of E-cadherin was found in areas of tumor budding. Moreover, ZEB1, TWIST, SNAIL, and SLUG were not expressed in regions of tumor budding. However, the expression level of ZEB1 in the stromal cells surrounding tumor budding was significantly more frequent than that of TWIST, SNAI, and SLUG. In addition, the expression of EMT-related proteins in surrounding stromal cells was significantly greater in areas of high-grade tumor budding than in low-grade areas. Our present results suggest that EMT-related proteins play a minor role in forming tumor buds. In addition, our findings suggest the existence of subtypes of stromal cells in CRC with phenotypical and functional heterogeneity.

1. Introduction

Worldwide, colorectal carcinoma (CRC) is the third most common cancer in men and the second most common cancer in women [1]. Invasion and metastatic dissemination of tumor

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cells are key factors in patient prognosis. Although understanding of the molecular mechanism underlying invasion and metastasis provides new insight for prediction of tumor prognosis, progress has been inadequate [2]. This limitation has motivated researchers to identify additional risk factors and biologic subgroups that might help to improve patient stratification and the resulting therapeutic decisions [2,3].

The epithelial-mesenchymal transition (EMT) is known to be a critical mechanism for the acquisition of a malignant phenotype by epithelial cells [2]. In CRC, tumor budding is defined as single cells or small clusters of de-differentiated tumor cells that are frequently observed at the cancer invasive front [2,4-6]. Tumor budding is reported to be a novel marker that predicts lymph node metastasis, distant metastasis, and patient survival [2,4-6]. Therefore, it has been adopted as a new prognostic factor by the International Union against Cancer [2,4,5].

The process of EMT includes the down-regulation of E-cadherin and the subsequent weakening of adherens junctions [7-9]. The changes in gene expression that inhibit E-cadherin and promote the mesenchymal transition are caused by master regulators, including ZEB1, TWIST, SNAIL, and SLUG [2,7-9]. ZEB1 is a transcriptional factor that induces the EMT and has been shown to down-regulate E-cadherin in epithelial cells. ZEB1 is induced by transforming growth factor β1 and nuclear factor $\kappa\beta$ [2,10-13]. TWIST is a helix-loop-helix transcriptional factor that promotes the EMT and down-regulates E-cadherin [14,15]. Recent studies have shown that although TWIST induces cell motility and abrogates cellular adhesion and metastasis, down-regulation of TWIST decreases metastatic potential and invasion of the tumor cells [7,13]. SNAIL and SLUG are also transcriptional factors that down-regulate the expression of E-cadherin and can contribute to the EMT [2,7,9,16]. Therefore, SNAIL and SLUG are known to play a role in tumor invasion [8,10]. Their expression is activated early in the EMT, and they thus have central roles in the progression of cancer cells [2,7-9,17,18]. In CRCs, the reduction of E-cadherin produces the histologic feature that is seen as tumor budding at the cancer invasive front [2]. Although the mechanism(s) underlying the EMT in cancer development has been identified, the mechanism(s) underlying tumor budding remains unclear.

Despite such close association of the EMT with tumor budding, little is known about the events promoting a tumor budding phenotype. The aim of this study is to identify the molecular mechanism underlying EMT that is associated with tumor budding at the invasive front in CRC.

2. Materials and methods

2.1. Patients

The study included 101 patients diagnosed as having CRC at Iwate Medical University Hospital during the period of 2005-2010. Information about patient age, sex, site, macroscopic type, histologic type, tumor grade, and stage was obtained from the hospital medical records. Only patients with surgical resections were included. All of the patients were interviewed to exclude a positive family history of CRC. Histologic classification of the tumors was performed according to the typing scheme of the Japanese Classification of Colorectal Cancer [19]. Dukes stage was used to determine tumor stage [19]. Clinicopathological findings are listed in Table 1.

Resected specimens were fixed in 20% buffered formalin and cut into 3-mm slices parallel to the major axis of the tumor. The representative sliced tissue specimens were embedded in paraffin and cut into 3-μm-thick sections.

The study was approved by the Ethical Committee of Iwate Medical University (No. H27-166).

2.2. Immunohistochemistry

Tumor budding was defined as an isolated cancer cell or a small cluster of tumor cells (<5) at the invasive margins [2,4]. The number of buds was counted along the entire invasive front using immunostaining of pan-cytokeratin (CK, AE1/AE3; Dako, Glostrup, Denmark). The method is described below. Areas with the greatest amount of budding were used for tissue microarray (TMA). After selecting an area in which budding was most intensive, the buds were counted in a field measuring 0.950 mm^2 through an objective lens (WHK 22× ocular lens; Olympus, Tokyo, Japan). For each sample, all neoplastic cells of the tumor bud were counted.

Immunohistochemical evaluation was performed using a TMA. Formalin-fixed, paraffin-embedded tumor blocks were

Table 1 Demographic and pathological characteristics of the patients with CRC

Variables	Frequency (%)
Total	101
Man/woman	62 / 39
Age (y; range)	66 (38-94)
Size (mm; range)	57 (15-120)
Locus	
Cecum	5 (5.0)
Ascending	9 (8.9)
Transverse	3 (3.0)
Descending	6 (5.9)
Sigmoid	18 (17.8)
Rectum	60 (59.4)
Differentiation	
Well	22 (21.8)
Moderate	71 (70.3)
Poor	6 (5.9)
Mucinous	2 (2.0)
Dukes classification	
A	2 (2.0)
B	44 (43.6)
C	55 (54.4)
D	0