Prognostic Value of Immunohistochemical Analysis of Tumor Budding in Colorectal Carcinoma

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Abstract. *Background: Tumor 'budding' of colorectal carcinoma along the invasive margin has been associated with increased malignant potential. This study investigated the possible prognostic significance of budding in invasive colorectal carcinoma. Patients and Methods: Specimens resected from 149 patients who underwent potentially curative surgery for invasive colorectal carcinoma were studied. Budding was defined according to Ueno's criteria; budding intensity was assessed by examination of hematoxylin-eosin (HE)-stained specimens and immunohistochemical (IHC)-stained specimens using anti-cytokeratin antibody and anti-lymphatic vessel antibody. Results: Immunohistochemical analysis identified many more budding foci that were not detectable by examination of HE-stained specimens. Multivariate analyses revealed that budding identified using immunohistochemical staining was a significant prognostic marker for disease-free survival and there was significant correlation between budding and microlymphatic vessel infiltration at the invasive tumor front. Conclusion: Budding, particularly as assessed with immunohistochemical staining, is a useful predictor of poor prognosis in patients with invasive colorectal carcinoma.*

Depth of tumor penetration and degree of lymph node metastasis according to Dukes' classification are the two most important established prognostic indicators for colorectal carcinoma. Histological findings, such as tumor differentiation and presence of venous and/or lymphatic invasion have also been associated with recurrence and metastasis. Tumor 'budding' along the invasive margin, which is thought to reflect malignant potential, has attracted attention as a risk factor for nodal involvement (1-4) and as a marker for prognosis (5-12).

In previous studies, some criteria were used to define tumor budding and budding intensity by examination of hematoxylin-eosin (HE)-stained specimens. Recently several detailed examinations by immunohistochemical (IHC) analysis have been reported (13-15), but there was no study that compared budding counts by examination of both two methods. The purpose of the present study was to compare the budding assessment in HE-stained specimens and IHC-stained specimens, and to clarify the prognostic impact of budding in invasive colorectal carcinoma and the usefulness of IHC analysis to assess budding.

Patients and Methods

Colorectal carcinomas with wall penetration through the muscularis propria (T2, T3, and T4) from 149 patients [91 men, 58 women; median age, 66 years (range, 20-86 years)] who underwent potentially curative resection without neoadjuvant chemotherapy or preoperative radiation therapy from 1997 to 2000 at Nara Medical University Hospital were studied. Median follow-up was 70 months (range, 10-84 months). Patients with familial adenomatous polyposis, hereditary non-polyposis colorectal cancer syndrome, or inflammatory bowel disease were excluded. Clinicopathological data were recorded based on the TNM classification (16).

Definition of tumor budding. According to Ueno's criteria, budding foci were defined as isolated single cancer cells derived from a cluster composed of fewer than five undifferentiated cancer cells. Such scatted foci can be observed in the stroma of actively invasive frontal regions. The budding number was determined *via* counts in one microscopic field ×200 in an area of maximal budding. The degree of budding was classified as negative or positive corresponding to 0-4 and ≥5 budding foci in one field, respectively, using HE-stained specimens (Figure 1a), while for IHC-stained specimens budding was classified as 'group A' or 'group B', which corresponded to 0-15 and ≥16 budding foci in one field, respectively.

Immunohistochemical staining. Immunohistochemical staining was performed on 5-μm sections of formalin-fixed, paraffin-

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Table I. *Clinicopathological findings and recurrence in relation to budding.*

	HE (n=149)			IHC (n=149)		
	Negative (n=125)	Positive (n=24)	*p*-Value	group A (n=130)	group B (n=19)	*p*-Value
Wall penetration						
T2/T3	102	12	**0.0008**	103	11	**0.0404**
T4	23	12		27	8	
Differentiation						
Well	54	7	0.4269	54	7	0.9009
Moderate	64	15		68	11	
Poor or mucinous	7	2		8	1	
Lymph node metastasis						
Positive	50	15	**0.0418**	54	11	0.1793
Negative	75	9		76	8	
Lymphatic invasion						
Positive	101	22	0.2522*	106	17	0.5293*
Negative	24	2		24	2	
Venous invasion						
Positive	53	13	0.2878	53	13	**0.0234**
Negative	72	11		77	6	
Liver metastasis						
Positive	9	4	0.2262*	11	2	0.6724*
Negative	116	20		119	17	
Recurrence						
Present	27	12	**0.0037**	27	12	**<0.0001**
Absent	98	12		103	7	

HE, hematoxylin and eosin staining; IHC, immunohistochemistry. *P*-value determined using the Chi-square test; *Fisher's exact probability test.

embedded tissues. After protein blocking with 10% normal horse serum, the monoclonal lymphatic endothelial marker D2-40 (Dako Cytomation Inc, USA; 1:50 dilution) was applied to tissues which were then incubated for 60 minutes at 37.0˚C. The polymer detection system, Alkaline Phosphatase Detection (Nichirei, Japan), with permanent blue chromogen [Nitro Blue Tetrazolium (NBT), SIGMA Chemical Co, USA and 5-bromo-4-chloro-3-indolyphosphate-5-toluidine salt (BCIT), SIGMA Chemical Co] was used to visualize the signal. Following peroxidase blocking with 3% hydrogen peroxide, anti-cytokeratin antibody AE1/AE3 (Dako Cytomation Inc, 1:50 dilution) was applied to the same slides, which were then incubated for 60 minutes at 37.0˚C. A polymer detection system was used with DAB chromogen (Dako Cytomation Inc) for visualization (Figure 1b). Cancer cells surrounded by endothelial cells that stained positively for D2-40 at the invasive front were defined as microlymphatic vessel infiltration.

Statistical analysis. The chi-square test or Fisher's exact probability test was used to assess associations between categorized variables. Simple regression was used to assess correlation between budding counts by both methods. The postoperative survival rate was calculated using the Kaplan-Meier method. Multivariate analysis was carried out by Cox's proportional hazards regression analysis to determine which factors had an independent effect on postoperative survival. $P<0.05$ was considered statistically significant.

Results

By examination of HE-stained specimens, budding counts ranged from 0 to 25 buds with 2.56±3.66 [mean±standard deviation (SD)]. The number of specimens with budding counts 0, 1-4, 5-9 and ≥10 were 55, 70, 18 and 6 respectively, while these negative or positive for budding were 125 and 24 respectively. On examination of IHC-stained specimens, budding counts ranged from 0 to 50 buds with 6.21±8.131 (mean±SD). The number of specimens with budding counts 0, 1-4, 5-9 and ≥10 were 39, 45, 31 and 34 respectively. The number of specimens in group A and group B were 130 and 19 respectively. There was significant positive correlation between budding counts by both methods (Figure 2).

Clinicopathological variables and cancer recurrence in relation to budding are summarized in Table I. As analyzed with HE staining, budding was significantly associated with wall penetration, incidence of lymph node metastasis and cancer recurrence, while as analyzed with IHC staining, budding was significantly associated with wall penetration, venous invasion and recurrence. Disease-free survival rate in patients with specimens negative or positive for budding were 75.1% and 40.9% , respectively

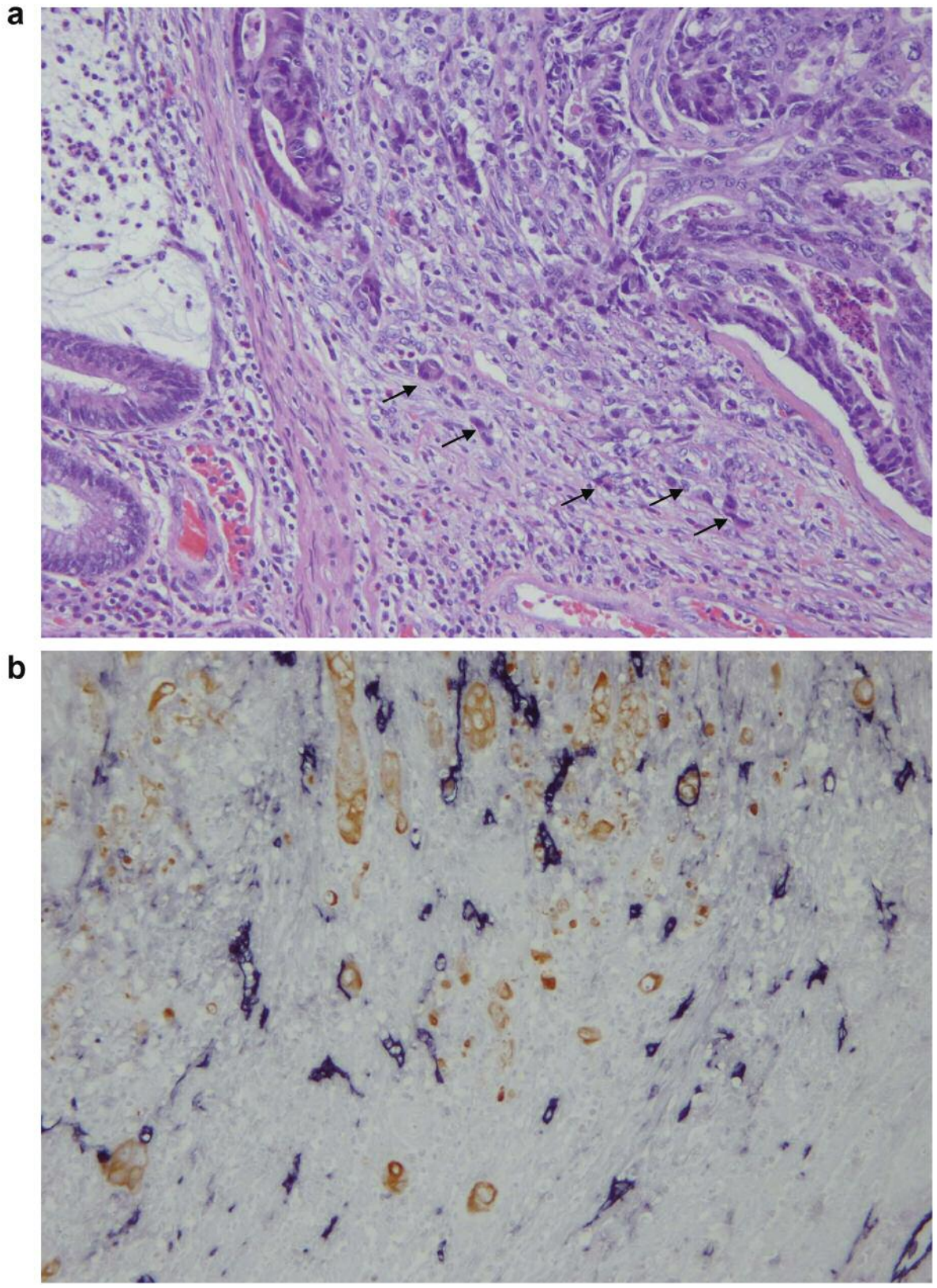

Figure 1. *Arrows indicate budding foci in HE staining for budding (a) (original magnification, ×200). Immunohistochemical staining for budding and microlymphatic vessel infiltration with AE1/AE3 and D2-40 double staining (b) (original magnification, ×200).*

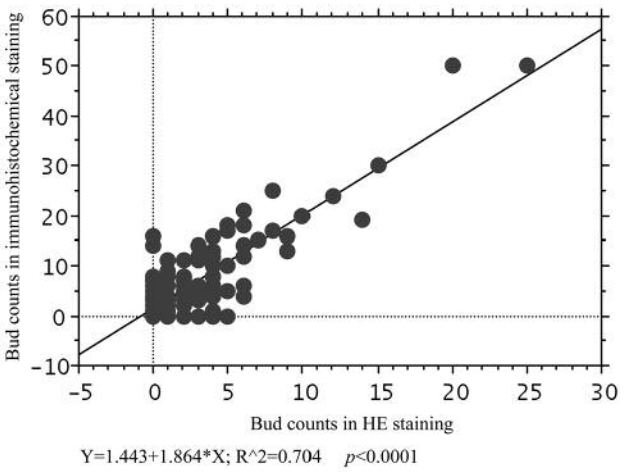

Figure 2. *Simple regression between bud counts in HE staining and immunohistochemical staining.*

and was significantly lower in patients with specimens positive for budding. By univariate analysis of HE staining assessment, wall penetration, lymph node metastasis, lymphatic invasion, venous invasion, liver metastasis and budding were selected as significant co-factors; however, by multivariate analysis, budding was not a significant independent co-factor (Table IIa).

By the examination of IHC-stained specimens, disease-free survival in group B was significantly shorter than in group A ($p<0.0001$). Lymph node metastasis and budding were independent prognostic factor for disease-free survival by multivariate analysis (Table IIb).

Of all 149 immunohistochemical-stained specimens, 39 cases (26.2%) had microlymphatic vessel infiltration. The number of microlymphatic-infiltrating cancer cells ranged from 0 to 6. The microlymphatic vessel infiltration rate in group B was significantly higher (57.9%) than in group A (21.5%). There was also a significant association between budding and microlymphatic vessel infiltration ($p=0.0008$) (Table III).

Table II. *Results of multivariate analysis on postoperative disease-free survival.*

a) HE staining (n=149)

Selected factor	Category	*P*-value*	Odds ratio
Wall penetration	T2, T3 *vs.* T4	**0.0047**	2.724
Lymph node metastasis	positive *vs.* negative	**<0.0001**	5.446
Lymphatic invasion	positive *vs.* negative	0.1091	5.287
Venous invasion	positive *vs.* negative	0.2580	1.494
Liver metastasis	present *vs.* absent	0.2660	1.617
Budding	negative *vs.* positive	0.1574	1.704

b) IHC staining (n=149)

Selected factor	Category	*P*-value*	Odds ratio
Wall penetration	T2, T3 *vs.* T4	0.0629	1.988
Lymph node metastasis	positive *vs.* negative	**<0.0001**	6.192
Lymphatic invasion	positive *vs.* negative	0.1145	5.129
Venous invasion	positive *vs.* negative	0.7106	1.143
Liver metastasis	present *vs.* absent	0.0820	2.104
Budding	group A *vs.* group B	**<0.0001**	5.469

*Cox proportional hazards regression model.

Table III. *Correlation with budding and microlymphatic vessel infiltration at the invasive front in IHC stained specimens.*

	Budding		
	Group A (n=130)	Group B (n=19)	
Microlymphatic vessel infiltration			
Positive	28	11	$p=0.0008$
Negative	102	8	

P-value determined using the Chi-square test.

Discussion

Budding, as initially proposed by Imai (17) in 1954, is a morphological phenomenon in colorectal carcinoma that occurs along the invasive margin and has a reported association with poor prognosis. Some investigators have questioned the usefulness of budding because of difficulties involved in its assessment, including the objectivity of quantitative analysis and complexities associated with detection. To address the former problem, Morodomi *et al.* and Ueno *et al.* provided an objective budding assessment index that is amenable to use as a routine index for histopathological diagnosis by examination of HE-stained specimens(2,9). To address the latter problem, some studies have reported examination by an immunohistochemical technique (13-15), but there was no study that compared budding counts by examination of both methods and, further, assessed budding and microlymphatic infiltration at the invasive tumor margin concurrently.

In the present study, we have demonstrated that anti-cytokeratin antibody (AE1/AE3) can be used to identify isolated cancer cells in intermediate tissue, and anti-lymphatic endothelial vessel antibody (D2-40) makes it possible to detect lymphatic endothelial vessels. As shown in Figure 1, the double-staining technique with DAB (AE1/AE3) and BCIP/NBT (D2-40) makes it possible to easily distinguish between a single cancer cell and lymphatic vessels in these tissues.

Using IHC, we were able to distinguish between microlymphatic infiltration and budding in 26.2% of cases, and detected many cases of occult budding that were not detected using HE staining. The difference in budding counts by the two methods might occur because of a lack of skill in the examination of HE-stained specimens. We suspected that isolated cancer cells assessed as 'budding foci' in HE-stained specimens included many microlymphatic vessel infiltrating cancer cells, but cancer cells which infiltrated microlymphatic vessels were not so many as to remarkably decrease budding counts. In any event, budding counts generally increased, consequently, indicating that previous classification systems might be revised.

Referring to previous reports (2, 9, 14), when we classified budding intensity into two groups with 0-4 buds and ≥5 buds, no significant difference was found in disease-free survival rate (70.8% and 65.3%, respectively). When classified into groups with 0-9 buds and ≥10 buds, there was a significant difference in disease-free survival rate (72.3% and 55.4% respectively, p=0.027). However, budding was not a significant independent co-factor by multivariate analysis. (p=0.3127, odds ratio 1.42) Therefore, we adopted a new classification system with a cut-off of 15 buds which revealed that tumor budding was a significant factor, as was nodal involvement, for disease-free survival.

Importantly, immunohistochemical analysis showed that budding was significantly associated with microlymphatic vessel infiltration at the invasive tumor margin, and the strict discrimination of budding foci and microlymphatic vessel infiltration was revealed in this study for the first time. We suggest that IHC analysis is equally important in the diagnosis detecting budding and microlymphatic vessel infiltration. Furthermore, as shown in Table I, budding was significantly associated with venous invasion by examination of IHC-stained specimens. These results suggest that budding may occur during the initial phase of microlymphatic vessel infiltration and venous invasion by cancer cells.

While some recent studies have reported the relationship between tumor budding and expression of matrix metalloprotease (MMP)-9, MMP-7, MMP-2 or cathepsin B(18-20), the mechanism of budding has not yet been fully elucidated. We suggest that this study provides some important clues for understanding the mechanism underlying budding.

In conclusion, tumor budding detected using HE staining remains a valuable prognostic predictor for patients with invasive colorectal carcinoma. However, IHC analysis using AE1/AE3 and D2-40 can readily detect single cancer cells and exclude lymphatic vessel infiltration, suggesting that this may be a more intensive method for predicting prognosis for these patients.

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