Diseases of the Colon & Rectum

Differential Prognostic Significance of Morphologic Invasive Markers in Colorectal Cancer: Tumor Budding and Cytoplasmic Podia

Eiji Shinto, M.D.,[1,3] Jeremy R. Jass, M.D.,[3] Hitoshi Tsuda, M.D.,[1] Taichi Sato, M.D.,[2] Hideki Ueno, M.D.,[2] Kazuo Hase, M.D.,[4] Hidetaka Mochizuki, M.D.,[2] Osamu Matsubara, M.D.[1]

[1] *Department of Pathology II, National Defense Medical College, Tokorozawa, Japan*
[2] *Department of Surgery I, National Defense Medical College, Tokorozawa, Japan*
[3] *Department of Pathology, McGill University, Montreal, Canada*
[4] *Department of Surgery, Self Defense Forces Central Hospital, Tokyo, Japan*

PURPOSE: In colorectal cancer, the presence of cytoplasmic podia around tumor budding foci may be a morphologic marker for an activated budding phenotype that is associated with cell motility. In this study, we have investigated the prognostic significance of cytoplasmic podia. METHODS: A total of 136 pT3 colorectal cancers were classified according to extent of budding and cytoplasmic podia as identified by immunostaining for cytokeratin. The prognostic significance of budding and cytoplasmic podia was then assessed. RESULTS: The overall survival curves between the groups with high-grade and low-grade cytoplasmic podia were different (5-year survival rates were 60.5 and 83.8 percent respectively, $P = 0.0003$). Similar results were shown for tumor budding (59.8 and 87.7 percent, $P < 0.0001$). Multivariate analysis showed that the grades of cytoplasmic podia (hazards ratio, 2.4; $P = 0.012$) and budding (hazards ratio, 2.3; $P = 0.024$) were independent prognostic factors. Additionally, among colorectal cancers with high-grade budding, the grade of cytoplasmic podia was selected as an independent prognostic factor (hazards ratio, 2.4; $P = 0.042$). CONCLUSIONS: Cytoplasmic podia and budding are related but independent pathologic predictive markers in patients with resected pT3 colorectal cancer. [Key words: Colorectal cancer; Tumor budding; Cytoplasmic podia; Cytoplasmic pseudofragments; Prognosis]

Although colorectal cancer has traditionally been regarded as showing a relatively uniform morphology, a subset shows a distinctive structural change at the invasive margin that may be described as dedifferentiation. This morphologic change is characterized not only by loss of glandular differentiation but also by loss of intercellular cohesion. The term "tumor budding" has been applied to this feature when there are single cells or small clusters of up to four cells within the stromal tissue at the invasive margin.[1,2] Importantly, tumor budding has been shown to be an independent prognostic marker.[1–5] Furthermore, the assessment may be achieved in an inexpensive and reproducible manner simply by counting tumor buds in histologic sections stained by hematoxylin and eosin (H&E).

Dedifferentiated epithelial cells at the invasive margin of colorectal cancer may sometimes have a mesenchymal appearance that precludes their identification. We immunostained representative sections of colorectal cancer for epithelial cytokeratin to facilitate the detection of dedifferentiated cells.[6] In

Correspondence to: Eiji Shinto, M.D., Department of Pathology II, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-8513, Japan, e-mail: eiji-c@zf6.so-net.ne.jp

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some cases, cellular dedifferentiation was noted to be accompanied by the presence of scattered "cytoplasmic fragments." By reconstructing serial sections, the cytoplasmic fragments were shown to be in continuity with budding tumor cells and were termed "cytoplasmic pseudofragments."[6] However, these structures are likely to represent cytoplasmic projections or podia at the leading edge of migrating cells. We showed that the presence of the cytoplasmic fragments was closely associated with Wnt pathway activation as evidenced by nuclear translocation of beta-catenin and cytoplasmic expression of lamin-5 gamma2.[6] Importantly, *in vivo* experiments designed to explain the mechanisms underlying cancer cell motility have shown that Wnt signaling pathway activation is associated with the extension of cytoplasmic podia for the processes of cell attachment and locomotion.[7,8] For the purposes of this study, we will refer to the presence of cytoplasmic pseudofragments as cytoplasmic podia.

Although the development of cytoplasmic podia is a property of cells that have become dedifferentiated and is therefore strongly correlated with tumor budding, we were unable to demonstrate an association between the extent of tumor budding (stratified as moderate *vs.* severe) and the extent of cytoplasmic podia formation. We therefore suggested that the processes may arise through partially independent mechanisms and could have different biologic and clinical effects. Dedifferentiation and loss of cell cohesion are the global hallmarks of tumor budding, which was shown to be closely related to vascular invasion.[6] By contrast, cytoplasmic podia characterize a subset of colorectal cancers with tumor budding, and this feature was more strongly associated with a diffusely infiltrative tumor margin. This finding could be explained by the property of enhanced cell motility.[6] Although the adverse prognostic significance of tumor budding has been studied in detail,[1–5] there are no reports about the prognostic significance of cytoplasmic podia.

In this study, we have hypothesized that tumor budding and cytoplasmic podia may provide different and complementary prognostic information. As well as examining their prognostic significance in terms of survival, we assessed their relationship with the pattern of tumor recurrence because this impacts both clinical course and choice of adjuvant therapy.[9–11] Given that the detection of cytoplasmic podia depends on the use of immunohistochemistry (IHC), we also investigated the possibility that these structures could add further prognostic information in the previously highly malignant subset of colorectal cancer with high-grade tumor budding.

PATIENTS AND METHODS

This study was performed after approval by the Internal Review Board. Among 538 patients who underwent potentially curative surgical therapies for primary colorectal carcinomas (CRCs) at the National Defense Medical College Hospital between 1989 and 1993, we retrieved 136 patients with pT3 CRC, in which the tumors histologically invaded into the subserosal layer or into nonperitonealized pericolic or perirectal tissues, according to TNM classification.[12] Potentially curative surgical procedures implied resection of all macroscopically identifiable tumor. These 136 patients did not include patients who died from postoperative complications or patients in whom prognosis, histopathologic data, or sufficient volume of archival paraffin-embedded tissue blocks for IHC study were not available. Patient characteristics and clinicopathologic features of CRCs in the patient cohort are presented in Table 1.

All patients were regularly followed up at our outpatient clinic and were monitored for postoperative recurrence by chest x-ray, measurements of serum carcinoembryonic antigen and CA19-9 levels every three months, abdominal ultrasonography every six months, and colonoscopy every year. Contrast-enhanced computed tomography was performed when recurrence of cancer was suspected. When no findings suspicious of cancer relapse appeared by five years, the follow-up procedure was changed to an annual physical check-up without any other examinations. If patients did not visit our clinic, we confirmed their health status by telephone once per year. At the last time of follow-up, 33 patients had died of cancer, with the median interval of 36.8 (range, 5–113.6) months from the date of operation to death. Twelve patients died from other diseases or unknown causes with the median interval of 55.5 (range, 4.1–116.8) months after the surgical treatment. The median follow-up period of the 91 survivors was 115.2 (range, 46.9–142.5) months. In this study, we used overall survival as the measure of survival.

With regard to adjuvant therapies, systemic chemotherapy was offered to only four patients (3.1 percent) who had distant metastasis at the primary surgery. No patient free of distant metastasis received

Table 1.
Clinicopathologic Features and Correlations With Grades of Tumor Budding and Cytoplasmic Podia

		Tumor Budding			Cytoplasmic Podia		
	Total	High-Grade (n = 55)	Low-Grade (n = 81)	*P* Value	High-Grade (n = 43)	Low-Grade (n = 93)	*P* Value
Age (yr)	60 ± 11.7	60.9 ± 11.2	59.4 ± 12.1	0.49	60 ± 11.1	60 ± 12.1	0.97
Gender							
Male	79	33 (42)	46 (58)	0.71	26 (33)	53 (67)	0.7
Female	57	22 (39)	35 (61)		17 (30)	40 (70)	
Tumor location							
Right-sided	34	17 (50)	17 (50)	0.19	7 (21)	27 (79)	0.11
Left-sided	102	38 (37)	64 (63)		36 (35)	66 (65)	
Distant metastasis							
Positive	13	6 (46)	7 (54)	0.66	5 (38)	8 (62)	0.58[b]
Negative	123	49 (40)	74 (60)		38 (31)	85 (69)	
Nodal metastasis							
Positive	66	39 (59)	27 (41)	<0.0001	28 (42)	38 (58)	0.0085
Negative	70	16 (23)	54 (77)		15 (21)	55 (79)	
Venous invasion[a]							
≥3	38	22 (58)	16 (42)	0.0098	16 (42)	22 (58)	0.1
0–2	98	33 (34)	65 (66)		27 (28)	71 (72)	
Lymphatic invasion[a]							
≥1	45	20 (44)	25 (56)	0.50	15 (33)	30 (67)	0.76
0	91	35 (38)	56 (62)		28 (31)	63 (69)	
Tumor budding							
High-grade	55				31 (56)	24 (44)	<0.0001
Low-grade	81				24 (15)	69 (85)	
Cytoplasmic podia							
High-grade	43	31 (72)	12 (28)	<0.0001			
Low-grade	93	24 (26)	69 (74)				

Data are means ± standard deviations or numbers with percentages in parentheses unless otherwise indicated.
[a]Cutoff values of venous invasion and lymphatic invasion were provided to lead to the biggest difference in survival curves between each two categories.
[b]Fisher exact test.

a systemic chemotherapy during this period. None of the patients received chemotherapy or irradiation therapy preoperatively.

Immunohistochemistry

For each of the 136 cases, an H&E-stained section that included the most deeply invading component of the tumor was selected after microscopic review of the routine histopathologic sections. The corresponding tissue block was then used for the IHC study. All cancers were immunostained for broad-spectrum cytokeratin (monoclonal mouse anti-human cytokeratin; clone MNF116; dilution 1:50; DakoCytomation, Grostrup, Denmark). Four-micrometer-thick sections were cut from representative blocks including the invasive front and mounted on silane-coated glass slides. After dewaxing and rehydration to dH_2O, sections for immunostaining were subject to heat antigen retrieval in an autoclave (120°C, 10 minutes) in purchased target retrieval solution, pH 9.0 (S2367, DakoCytomation). After cooling, nonspecific antibody binding was inhibited by incubating the sections in 4 percent skim milk. Endogenous peroxidase activity was blocked by using 0.5 percent H_2O_2. After transfer to a humidified chamber, the sections were incubated with 10-percent normal goat serum (X0501, DakoCytomation) for 20 minutes, and incubated with primary antibody at room temperature for 1 hour. Subsequently, the sections were incubated with peroxidase labeled polymer (K4001, EnVision™ + System-HRP; DakoCytomation) for 30 minutes at room temperature. For visualization of the antigen, the sections were immersed in 0.05 percent diaminobenzidine tetrahydrochloride solution containing 0.01-percent hydrogen peroxidase for eight minutes, and counterstained lightly with Mayer's hematoxylin.

Staining for Assessment of Vessel Involvement

Sections were cut from the same blocks to perform double staining combining CD34 immunostaining

and elastica staining. After IHC staining for CD34 (monoclonal mouse anti-human CD34 class II; clone QBEnd10; dilution 1:50; DakoCytomation) following the same procedure as above, elastic fibers were stained in Resorsin-Fuchsin solution (Muto pure chemicals, Tokyo, Japan) for 20 minutes. Sections were then immersed in 95 percent alcohol for one minute, and counterstained lightly with Mayer's hematoxylin. By this combined staining method, it was possible to identify lymph vessels with immunostained endothelium and venous vessels with round elastic fibers on the same slide. The grades of lymph vessel invasion and venous vessel invasion were derived on the basis of the number of vessels infiltrated by carcinoma on one slide. Cutoff values for venous invasion (0–2, and ≥3) and lymphatic invasion (0, and ≥1) were those that lead to the largest difference in survival for the two categories.

Tumor Budding and Cytoplasmic Podia

We undertook microscopic review of all routine H&E-stained sections to evaluate the grade of tumor budding at the invasive front. The number of sections ranged from two to six per tumor, depending on tumor size. Tumor budding was assessed as described previously.[1] Briefly, a focus of tumor budding was defined as a single isolated cancer cell or a cluster composed of up to four cancer cells (Fig. 1). Cancers were then divided into two groups according to the number of tumor budding foci in the densest field using a ×20 objective lens. Counts of 0 to 9 were termed low-grade, and counts of 10+ were termed high-grade budding. High-grade budding was further divided into counts of 10 to 19 (moderate) and 20+ (severe).

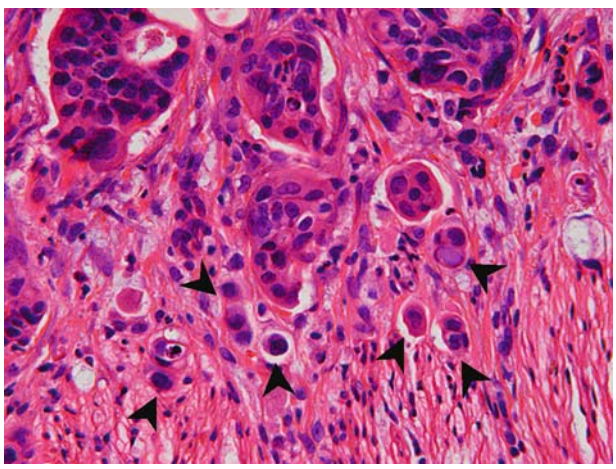


Figure 1. Characteristic high-power microscopic appearance of tumor budding (arrow heads; an isolated single cell or a cluster of up to four cancer cells) at the invasive front (hematoxylin and eosin staining).

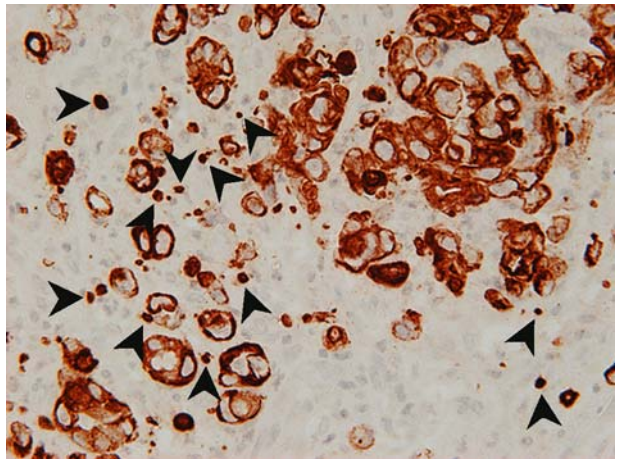


Figure 2. Multiple cytoplasmic podia (arrow heads; round cytokeratin-immunostained spots without nucleus) are demonstrated around tumor budding foci (cytokeratin immunostaining).

Cytoplasmic podia were evaluated as described previously.[6] Using cytokeratin-immunostained sections, small nonnucleated cytoplasmic fragments were detected around tumor budding foci at the invasive tumor margin (Fig. 2). To be counted, fragments had to be at least 2 μm in diameter, nonnucleated, lacking in evidence of nuclear fragmentation, uniformly positive for cytokeratin, smoothly contoured, and free of surrounding inflammatory cells. Scores for each case were the highest number of fragments in a ×20 objective lens field. Low-grade cancers had 0 to 9 fragments, and high-grade cancers had 10+ fragments.

Statistical Analysis

Comparisons between groups were performed by using the chi-squared test or Fisher's exact method. We used the unpaired *t*-test for the comparison of groups with continuous variables following a normal distribution. Survival curves of patients were obtained through the Kaplan-Meier method.[13] Differences between curves were calculated by using the log-rank test.[14] Cox proportional hazards regression analysis was used for multivariate analyses.[15] All statistical analyses were performed using StatView® 5 software (SAS® Institute, Cary, NC), and $P < 0.05$ was considered significant.

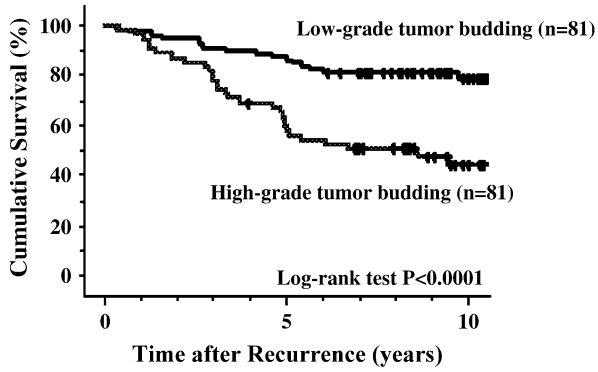
RESULTS

Relationship to Clinicopathologic Findings

Table 1 shows the distribution of budding and cytoplasmic podia according to clinicopathologic features. There were no significant differences in age, gender, tumor location, distant metastasis, or lymphatic invasion. However, the incidence of lymph node metastasis was higher in the high-grade budding group and the high-grade cytoplasmic podia group than in the low-grade groups ($P < 0.0001$ and $P = 0.0085$, respectively). The grade of tumor budding was positively associated with the grade of venous invasion ($P = 0.0098$). Tumor budding and cytoplasmic podia were strongly associated with each other ($P < 0.0001$).

Relationship to Survival

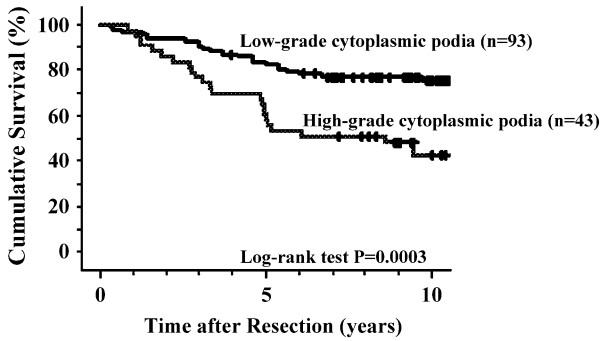
Figures 3 and 4 show the Kaplan-Meier overall survival curves according to the grade of tumor budding and the grade of cytoplasmic podia, respectively. The high-grade tumor budding and the high-grade cytoplasmic podia groups (5-year survival of 59.8 and 60.5 percent) had significantly worse survival ($P < 0.0001$, $P = 0.0003$) than the low-grade groups (5-year survival of 87.7 and 83.8 percent), respectively. The prognostic significance also was assessed by multivariate analysis (Table 2), using all parameters in Table 1 as variables. Cutoff values of age (75 or older, 74 or younger), venous invasion (0–2, ≥3) and lymphatic invasion (0, ≥1) were used since they showed the largest survival difference between each of the two categories. Tumor budding (hazard ratio, 2.3; $P = 0.024$) and cytoplasmic podia (hazard ratio, 2.4; $P = 0.012$) were independent prognostic factors, in addition to distant metastasis (hazard ratio, 4.3; $P = 0.0001$) and age (hazard ratio, 4; $P < 0.0001$).



No. of patients

High-grade	55	53	48	43	37	32	29	25	23	17	10
Low-grade	81	79	77	74	73	71	67	62	55	45	28

Figure 3. Cumulative overall survival curves for 136 patients with pT3 colorectal cancers stratified by the grade of tumor budding. Two curves for patients with high-grade tumor budding and low-grade tumor budding are significantly different ($P < 0.0001$).



No. of patients

High-grade	43	42	37	33	30	26	23	22	20	14	6
Low-grade	93	90	88	84	80	77	73	65	58	48	32

Figure 4. Cumulative overall survival curves for 136 patients with pT3 colorectal cancers stratified by the grade of cytoplasmic podia. Two curves for patients with high-grade cytoplasmic podia and low-grade cytoplasmic podia are significantly different ($P = 0.0003$).

Relationship to Postoperative Recurrence

Forty-four patients (32.4 percent) had postoperative recurrence (Table 3). The overall recurrence rates of the high-grade budding and high-grade cytoplasmic podia groups were higher than those of low-grade groups ($P = 0.0022$, $P = 0.0052$). With respect to primary recurrence sites, recurrence linked to hematogenous spread was more frequent in the high-grade budding group than in the low-grade group ($P = 0.040$). In contrast, peritoneal and/or local recurrence was more frequent in the high-grade cytoplasmic podia group than in the low-grade group ($P = 0.025$). With respect to pattern of organ recurrence, overall recurrence (excluding patients with recurrence limited to liver) occurred more frequently in the high-grade budding and high-grade cytoplasmic podia groups than in the low-grade groups ($P = 0.0004$, $P = 0.0008$ respectively). Neither budding nor cytoplasmic podia were associated with recurrence limited to liver (Table 3).

In the 44 patients with recurrent cancer, survival for the high-grade *vs.* low-grade budding groups differed significantly (5-year survival of 23.1 and 66.7 percent, $P = 0.0032$). Survival difference for high-

Table 2.
Significance of Clinicopathologic Parameters for Overall Survival

Parameter	Comparison of Survival		Multivariate Analysis by Cox Proportional Hazard Model		
	Five-Year Survival Rate (Kaplan-Meier Method)	*P* Value (Log-rank Test)	Hazard Ratio	95% Confidence Interval	*P* Value
Age (yr)					
75 or older *vs.* 74 or younger	50% *vs.* 81%	0.0001[a]	4	2–7.9	<0.0001[a]
Gender					
Female *vs.* male	80.6% *vs.* 73.4%	0.29		Not selected	
Tumor location					
Right-side *vs.* left-side	76.5% *vs.* 76.4%	0.8		Not selected	
Distant metastasis					
Positive *vs.* negative	46.2% *vs.* 79.6%	0.0003[a]	4.3	2–9	0.0001[a]
Nodal metastasis					
Positive *vs.* negative	68.1% *vs.* 84.3%	0.0056[a]		Not selected	
Venous invasion					
≥3 *vs.* 0–2	68.4% *vs.* 79.5%	0.011[a]		Not selected	
Lymphatic invasion					
≥1 *vs.* 0	68.9% *vs.* 80.2%	0.26		Not selected	
Tumor budding					
High-grade *vs.* low-grade	59.8% *vs.* 87.7%	<0.0001[a]	2.3	1.1–4.5	0.024[a]
Cytoplasmic podia					
High-grade *vs.* low-grade	60.5% *vs.* 83.8%	0.0003[a]	2.4	1.2–4.9	0.012[a]

[a]Statistically significant.

Table 3.
Postoperative Recurrence

		Tumor Budding			Cytoplasmic Podia		
	Total	High-Grade (n = 55)	Low-Grade (n = 81)	*P* Value	High-Grade (n = 43)	Low-Grade (n = 93)	*P* Value
Overall	44	26 (47)	18 (22)	0.0022	21 (49)	23 (25)	0.0052
Primary recurrence site[a]							
Hematogenous							
Liver	21	9 (16)	12 (15)		9 (21)	12 (13)	
Lung	8	7 (13)	1 (1)		5 (12)	3 (3)	
Other	3	2 (4)	1 (1)		0 (0)	3 (3)	
Total	30	17 (31)	13 (16)	0.04	12 (28)	18 (19)	0.26
Lymphatic							
Lymph node	3	3 (5)	0 (0)	0.064[b]	3 (7)	0 (0)	0.03[b]
Nonhematogenous nonlymphatic							
Peritoneum	4	2 (4)	2 (3)		3 (7)	1 (1)	
Local	9	6 (11)	3 (4)		5 (12)	4 (4)	
Total	13	8 (15)	5 (6)	0.14[b]	8 (19)	5 (5)	0.025[b]
Pattern of organ recurrence							
Liver only	8	2 (4)	6 (7)	0.47[b]	1 (2)	7 (8)	0.44[b]
All sites[c]	35	23 (42)	12 (15)	0.0004	19 (44)	16 (17)	0.0008

Data are numbers with percentages in parentheses unless otherwise indicated.

[a]Primary recurrence was not limited to a single organ. Primary recurrence was found in liver and lung for two patients, in liver and lymph nodes for one patient, in liver and local area for one patient, and in peritoneum and local area for one patient. The information of recurrence sites was not available for one patient.

[b]Fisher's exact test.

[c]Excluding patients with involvement of liver in isolation.

Table 4.
Significance of Clinicopathologic Parameters for Overall Survival in Patients With Postoperative Recurrence

	Comparison of Survival		Multivariate Analysis by Cox Proportional Hazard Model		
Parameter[a]	Five-Year Survival Rate (Kaplan-Meier Method)	*P* Value (Log-rank Test)	Hazard Ratio	95% Confidence Interval	*P* Value
Age (yr)					
75 or older *vs.* 74 or younger	16.7% *vs.* 50%	0.0044[b]	2.2	1.1–4.6	0.035[b]
Distant metastasis					
Positive *vs.* negative	36.4% *vs.* 42.4%	0.55		Not selected	
Nodal metastasis					
Positive *vs.* negative	38.7% *vs.* 46.2%	0.33		Not selected	
Venous invasion					
≥3 *vs.* 0–2	36.8% *vs.* 44%	0.081		Not selected	
Tumor budding					
High-grade *vs.* low-grade	23.1% *vs.* 66.7%	0.0032[b]	2.6	1.2–5.5	0.017[b]
Cytoplasmic podia					
High-grade *vs.* low-grade	28.6% *vs.* 52.2%	0.089		Not selected	

[a]Tumor location, gender, and lymphatic invasion were not included. Prognostic significance of these parameters could not be found.
[b]Statistically significant.

grade *vs.* low-grade cytoplasmic podia groups was just short of significance (5-year survival of 28.6 and 52.2 percent, $P = 0.089$; Table 4). In this affected patient group, no other clinicopathologic features in the primary tumors affected survival except for age (5-year survival of 16.7 and 50 percent, $P = 0.0044$). Using the Cox proportional hazard model, tumor budding (hazard ratio, 2.6; $P = 0.017$) was selected as an independent prognostic indicator, as well as age (hazard ratio, 2.2; $P = 0.035$; Table 4).

Significance of Cytoplasmic Podia in CRCs with High-Grade Budding

In the 55 CRCs with high-grade budding, the distribution of variables listed in Table 1 was compared across severe and moderate budding groups (n = 11, n = 44; respectively) and across high-grade and low-grade cytoplasmic podia groups (n = 31, n = 24). There were no significant differences in age, gender, tumor location, distant metas-

Table 5.
Significance of Clinicopathologic Parameters for Overall Survival in Patients With High-Grade Budding

	Comparison of Survival		Multivariate Analysis by Cox Proportional Hazard Model		
Parameter[a]	Five-Year Survival Rate (Kaplan-Meier Method)	*P* Value (Log-rank Test)	Hazard Ratio	95% Confidence Interval	*P* Value
Age (yr)					
75 or older *vs.* 74 or younger	33.3% *vs.* 65%	0.013[b]	3.3	1.4–7.9	0.0081[b]
Distant metastasis					
Positive *vs.* negative	16.7% *vs.* 65%	<0.0001[b]	4.7	1.8–11.8	0.0012[b]
Nodal metastasis					
Positive *vs.* negative	56.2% *vs.* 68.8%	0.28		Not selected	
Venous invasion					
≥3 *vs.* 0–2	45.5% *vs.* 69.2%	0.038[b]		Not selected	
Tumor budding					
Severe *vs.* moderate	53.0% *vs.* 61.4%	0.77		Not selected	
Cytoplasmic podia					
High-grade *vs.* low-grade	51.6% *vs.* 70.6%	0.087	2.4	1–5.5	0.042[b]

[a]Tumor location, gender, and lymphatic invasion were not included. Prognostic significance of these parameters could not be found.
[b]Statistically significant.

tasis, venous invasion, or lymphatic invasion. Tumor budding was positively associated with nodal metastasis ($P = 0.023$). The correlation between severity of tumor budding and grade of cytoplasmic podia was of marginal significance only ($P = 0.089$).

A survival analysis of the subgroup of 55 patients with high-grade tumor budding is shown in Table 5. Old age, distant metastasis, and high-grade venous invasion were adverse prognostic factors in univariate analysis ($P = 0.013$, $P < 0.0001$, $P = 0.038$). High-grade cytoplasmic podia showed a trend toward reduced survival ($P = 0.087$). In multivariate analysis, cytoplasmic podia (hazard ratio, 2.4; $P = 0.042$) was an independent prognostic factor, as well as age (hazard ratio, 3.3; $P = 0.0081$) and distant metastasis (hazard ratio, 4.7; $P = 0.0012$).

With respect to primary recurrence sites, peritoneal and/or local recurrence occurred in seven cases (22.6 percent) with high-grade cytoplasmic podia but only in one (4.2 percent) low-grade case, the difference falling short of significance ($P = 0.12$). With respect to global organ recurrence, the high-grade cytoplasmic podia group showed trend toward more frequent extrahepatic recurrence than the low-grade group (51.6 and 29.2 percent, $P = 0.094$).

DISCUSSION

In this study, we examined the clinicopathologic significance of two markers of dedifferentiation at the invasive front in CRC: tumor budding and cytoplasmic podia. These markers cosegregated and both were associated with lymph node metastasis, although only budding was associated with venous invasion. Their prognostic significance was shown by both univariate and multivariate analysis (Tables 1, 2, and 4). Importantly, the presence of cytoplasmic podia was shown to be an independent prognostic parameter even among CRC cases with high-grade budding (Table 5).

We used a double-staining technique, combining CD34 immunostaining and elastica staining to examine vessel invasion. The grade of tumor budding correlated with the extent of venous invasion, and this correlation was consistent with the postoperative recurrence pattern (Table 3). Specifically, the extent of tumor budding was positively associated with a recurrence pattern consistent with hematogenous spread. By contrast, the grade of cytoplasmic podia was positively associated with peritoneal or local recurrence. Whereas tumor budding is a marker of venous invasion, cytoplasmic podia may indicate the development of an additional property, tumor cell motility, which would explain direct invasion into local tissues. Multivariate analysis showed that tumor budding and cytoplasmic podia serve as independent prognostic indicators. The data support our underlying hypothesis that tumor budding and cytoplasmic podia represent differential features of tumor aggressiveness, each providing complementary insight into malignant potential.

Neither tumor budding nor cytoplasmic podia were correlated with lymph vessel invasion, yet both were positively correlated with lymph node metastasis. These data indicate that the extent of lymph vessel invasion does not provide a direct measure of the potential to metastasize to lymph nodes. Tumor budding and cytoplasmic podia instead may explain the potential of malignant cells to implant and grow within lymph nodes.

The liver is the primary target organ for hematogenous metastasis in CRC. Hepatectomy and hepatic arterial infusion chemotherapy have been shown to be effective therapies for liver metastasis from CRC, providing the metastasis is confined in the liver.[9–11] However, the presence of extrahepatic spread obviates the use of aggressive curative therapy for liver metastasis, and systemic chemotherapy becomes the treatment of choice. In this study, we have shown that CRCs with high-grade tumor budding or high-grade cytoplasmic podia have a strong predisposition to spread extrahepatic sites. It is possible, therefore, that the finding of the phenotypes of high-grade tumor budding and/or cytoplasmic podia could condition the decision to treat hepatic metastases by aggressive approaches with curative intent.

With respect to survival after postoperative recurrence, tumor budding and possibly cytoplasmic podia were shown to be prognostic factors by univariate analysis. On the other hand, neither synchronous (with initial diagnosis) distant metastasis nor lymph node metastasis predicted survival (Table 4). These results indicate that the findings at the invasive front in primary tumor may provide a remarkably early warning of the subsequent post-recurrent behavior of the tumor. In other words, the biologic properties of the primary tumor may be more directly linked to future aggressive behavior than the stage at the time of diagnosis. Additionally, tumor budding was selected as an independent prognostic marker in patients who developed post-

operative recurrence by multivariate analysis. Presumably the link between high-grade venous invasion and high-grade budding may explain multiorgan recurrence and, therefore, a rapidly fatal course.

The analyses showed that although cytoplasmic podia occur in the context of budding cells, the presence of these structures provides additional clinically important information. Importantly, in CRCs with high-grade budding, the finding of cytoplasmic podia was an independent prognostic factor. On the other hand, we did not demonstrate an independent prognostic effect by subclassifying high-grade budding into moderate *vs.* severe budding. In this regard, our results differ from the larger study by Ueno *et al.*,[1] which was based only on rectal cancers. Our findings suggest that the assessment of dedifferentiation at the invasive margin of CRC should heed both budding (or cellular discohesion) and the development of cytoplasmic podia (a marker of cell motility).

CONCLUSIONS

This study has demonstrated the differential and independent prognostic significance of tumor budding and cytoplasmic podia in CRC. Cytoplasmic podia were shown to be an independent prognostic marker in patients with high-grade budding. We propose that the combination of budding and cytoplasmic podia provides a high index of aggressiveness. Apart from the prognostic information that may be gained from the assessment of these features, it is important to understand the mechanisms underlying a phenomenon that is likely to be the principal morphologic alteration that precedes invasion and metastasis in CRC.

REFERENCES

1. Ueno H, Murphy J, Jass JR, Mochizuki H, Talbot IC. Tumour 'budding' as an index to estimate the potential of aggressiveness in rectal cancer. Histopathology 2002;40:127–32.
2. Hase K, Shatney C, Johnson D, Trollope M, Vierra M. Prognostic value of tumor "budding" in patients with colorectal cancer. Dis Colon Rectum 1993;36:627–35.
3. Tanaka M, Hashiguchi Y, Ueno H, Hase K, Mochizuki H. Tumor budding at the invasive margin can predict patients at high risk of recurrence after curative surgery for stage II, T3 colon cancer. Dis Colon Rectum 2003; 46:1054–9.
4. Jass JR, Barker M, Fraser L, *et al.* APC mutation and tumour budding in colorectal cancer. J Clin Pathol 2003;56:69–73.
5. Okuyama T, Oya M, Ishikawa H. Budding as a useful prognostic marker in pT3 well- or moderately-differentiated rectal adenocarcinoma. J Surg Oncol 2003; 83:42–7.
6. Shinto E, Mochizuki H, Ueno H, Matsubara O, Jass JR. A novel classification of tumour budding in colorectal cancer based on the presence of cytoplasmic fragments around budding foci. Histopathology 2005;47:25–31.
7. Muller T, Bain G, Wang X, Papkoff J. Regulation of epithelial cell migration and tumor formation by beta-catenin signaling. Exp Cell Res 2002;280:119–33.
8. Conacci-Sorrell ME, Ben-Yedidia T, Shtutman M, Feinstein E, Einat P, Ben-Ze'ev A. Nr-CAM is a target gene of the beta-catenin/LEF-1 pathway in melanoma. Genes Dev 2002;16:2058–72.
9. Tsalis K, Vasiliadis K, Christoforidis E, *et al.* Current treatment of colorectal liver metastases. Tech Coloproctol 2004;8:174–6.
10. Berber E, Pelley R, Siperstein AE. Predictors of survival after radiofrequency thermal ablation of colorectal cancer metastases to the liver: a prospective study. J Clin Oncol 2005;23:1358–64.
11. Allen-Mersh TG, Earlam S, Fordy C, Abrams K, Houghton J. Quality of life and survival with continuous hepatic-artery floxuridine infusion for colorectal liver metastases. Lancet 1994;344:1255–60.
12. Sobin LH, Wittekind CH, eds. UICC TNM classification of malignant tumours. 5th ed. New York: Wiley-Liss, 1997.
13. Kaplan E, Meier P. Nonparametric estimation from incomplete observations. J Am Stat Assoc 1958;53:457–81.
14. Mantel N. Evaluation of surgical data and two new rank order statistics arising in its consideration. Cancer 1966; 50:163–70.
15. Cox DR. Regression models and life-tables. J R Stat Soc Ser B 1972;34:187–220.