of Wnt pathway dysregulation on the basis of our findings.

Promoter methylation has been linked with the epigenetic inactivation of several genes, including *LN-5γ2*.[22–25] However, there are no reports on the status of *LN-5γ2* promoter methylation in CRC. We found that *LN-5γ2* promoter methylation occurred only in 5 of 38 cases (13 percent) in which it was tested. Furthermore, *LN-5γ2* was not correlated with CIMP insofar as there was no association with MLH1-loss CRCs. Therefore, inactivation of LN-5γ2 by methylation of its promoter region is unlikely to explain the less aggressive behavior of sporadic MLH1-loss CRC. Although HNPCC CRCs do not show CIMP, some genes, including *HPP1* and *CDKN2A* (p16), may be methylated in HNPCC as epigenetic changes outside CIMP.[42] *LN-5γ2* promoter methylation may be categorized in a similar way. With respect to the possible biologic effects of *LN-5γ2* methylation, four of five cases with this change showed negative *LN-5γ2* immunopositivity and in two of these this was despite a positive finding for nuclear β-catenin. Therefore, whereas *LN-5γ2* promoter methylation is uncommon, it may explain some instances of gene silencing and nonexpression of LN-5γ2 by immunohistochemistry.

Our results are consistent with the concept that high-grade budding in MLH1-expressing cancers is a marker for invasiveness. On the other hand, tumor bud formation in MLH1-loss cancers may be a marker of cellular discohesion in isolation. Despite the differing features of tumor budding according to the status of DNA mismatch repair protein expression, lymph node metastasis and vascular invasion were not significantly different between the two groups of CRC. Therefore, although MLH1-loss cancers with high-grade budding may show less evidence of local invasiveness, the potential for vessel involvement and nodal metastasis by MLH1-loss cancer may be increased merely by the increased tendency for dedifferentiation through loss of cell cohesion.

CONCLUSIONS

We have shown that tumor budding in MLH1-expressing CRC shows enhanced invasiveness as evidenced by expression of LN-5γ2 and development of cytoplasmic podia (cytoplasmic pseudofragments), which is correlated to Wnt signaling pathway activation as indicated by nuclear translocation of β-catenin. By contrast, sporadic MLH1-loss CRC and probably CRCs in HNPCC undergo budding by mechanisms that are independent of Wnt pathway dysregulation. Accordingly, genetic changes related to DNA mismatch repair status are likely to be of importance in determining the biologic properties of tumor buds in CRC. *LN-5γ2* promoter methylation is uncommon and is not associated with particular CRC subtypes but may explain some instances of non-expression of *LN-5γ2* in CRCs with high-grade tumor budding.

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Diseases of the Colon & Rectum

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

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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

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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).

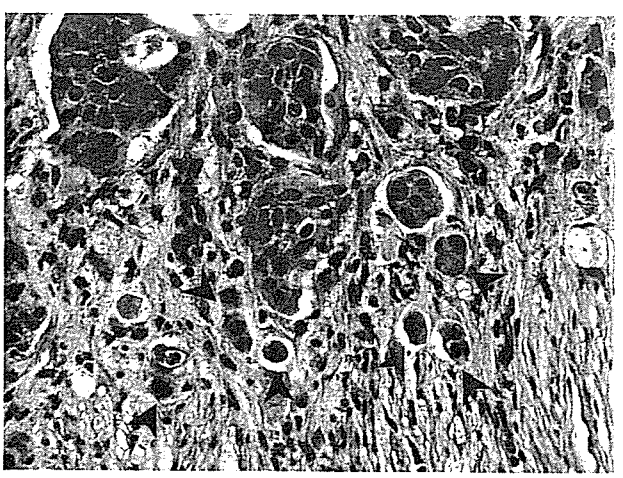


Figure 1. Characteristic high-power microscopic appearance of tumor budding (arrow heads; an isolated single cell or a cluster of up to four cancer cells) at the invasive front (hematoxylin and eosin staining).

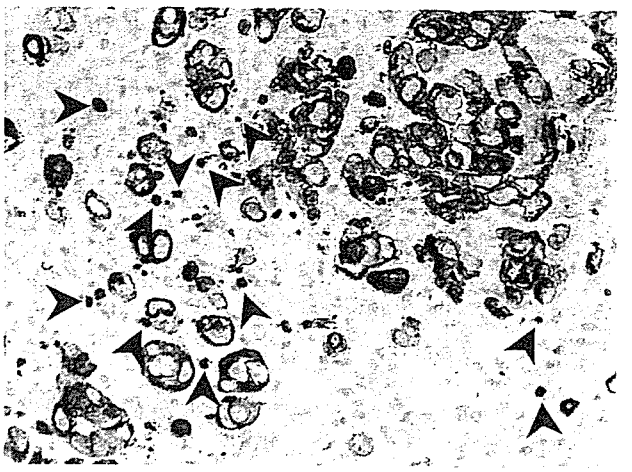


Figure 2. Multiple cytoplasmic podia (arrow heads; round cytokeratin-immunostained spots without nucleus) are demonstrated around tumor budding foci (cytokeratin immunostaining).

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.

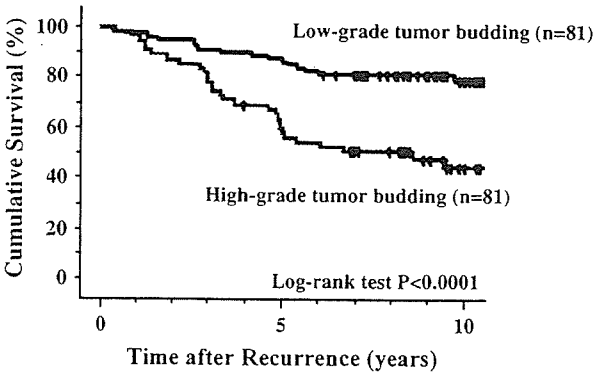
RESULTS

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

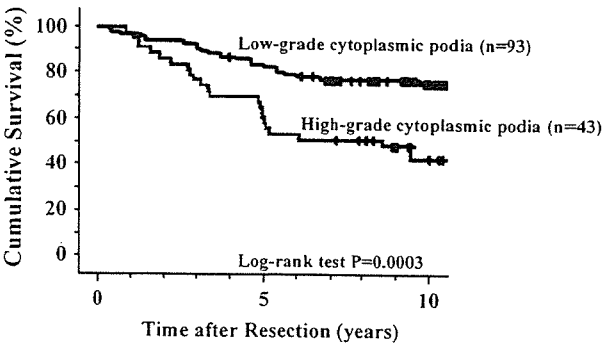
Figures 3 and 4 show the Kaplan-Meier overall survival curves according to the grade of tumor budding and the grade of cytoplasmic podia, respectively. The high-grade tumor budding and the high-grade cytoplasmic podia groups (5-year survival of 59.8 and 60.5 percent) had significantly worse survival ($P < 0.0001$, $P = 0.0003$) 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

Parameter[a]	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	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

Parameter[a]	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	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 postrecurrent 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.

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特集 主題II：大腸癌術後のフォローアップ

I. 大腸癌術後再発に関するフォローアップ —大腸癌研究会プロジェクト研究「大腸癌術後のフォローアップに関する研究」の検討結果より—

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大腸癌研究会「大腸癌術後のフォローアップに関する研究プロジェクト」における検討結果について概説する．対象は上記プロジェクト研究参加14施設にて1991～96年に治癒切除を行った大腸癌5,317例である．再発時期，発見動機，治療成績につき検討した．stage Iでは，比較的一定の割合で5年間再発する一方，他のstageでは術後3年は高い頻度で再発を来し，その後の再発増加率は緩徐であった．術後5年以降の再発は全体の1%未満と稀であった．再発臓器の頻度では肝臓が最も多く，肺，局所の順であった．発見動機では，問診，身体検査，腫瘍マーカー検査の組み合わせが約半数を占めていた．再発巣に対して治癒切除が施行できた症例の予後はできなかったものより有意に良かったが，再発巣切除後の予後には再発時期による差を認めなかった．今後，術後フォローアップにおける検査方法や間隔などにつき，更なる検討が必要と考えられる．

索引用語：大腸癌，術後フォローアップ，再発

はじめに

厚生労働省の統計によれば，現在日本における死亡原因の第一位は悪性新生物であり，心疾患，脳血管疾患がこれに続く．アメリカ合衆国では大腸癌は癌死亡原因の第二位，本邦では第三位であり，生活様式の欧米化にともない本邦においては近年最も増加傾向を示す疾患の一つである[1-3]．大腸癌に対する最も確実な治療方法は，根治的に外科的切除を行うことであるが，そのような治癒切除を行った症例においてもある一定の割合で再発が生じる．このような再発症例に対する治療成績を向上させることが，大腸癌全体の治療成績向上につながると考えられる．

大腸癌の術後フォローアップの第一の目的は，このような再発症例を治療可能な状態で発見し，治癒に結びつけることである．現在のところ，再発大腸癌に対し最も治癒が期待できる治療方法は外科的切除であるが，近年では様々な薬剤の開発とともに化学療法も目覚しく発達しており，従来の化学療法に比し良好な成績が報告されている[4,5]．よって予後改善の観点からも，これまで以上に術後フォローアップの果たす役割が重要になってくる可能性がある．

また術後フォローアップの他の役割として，大腸癌術後における機能障害に対する対応や精神的ケアとしての側面も挙げられるであろう．事実，患者の大部分はintensiveなフォローアップを求めるとの報告もある[6]．

本邦においては従来より欧米諸国と比し，よりintensiveなフォローアップがなされてきたが，その検査内容や間隔については施設によって異なっており，統一されたフォローアップ方法は存在していないのが実情であった．そこで大腸癌研究会（会長：武藤徹一郎）において14施設（表1）からなる，「大腸癌術後のフォローアップに関する研究プロジェクト」（委員長：望月英隆）が組織され，大腸癌術後の至適フォローアップ方法について検討を重ねてきた．そこで討議され得られた結果の一部が，昨年発刊された大腸癌治療ガイドラインの術後サーベイランスの項に掲載された[7]．

本稿では，上記プロジェクト研究にて得られた結果を中心に述べることとする．

I　参加施設における大腸癌治癒切除後フォローアップの現況

参加14施設中，各施設における大腸癌術後フォローアップ方法に関するアンケートに回答のあった13施設での現況を表2に提示する．表中の数字は，各時点で検査を実施している施設数が示されている．およそ3年まではどの施設もある程度intensiveにフォローアップしていたが，その後に関しては施設によって相違が認められた．術後フォローアップの期間としては，術後5年までとした施設が7施設，10年とした施設が4施設，可能な限り永続的とした施設も2施設あった．また，stageによって術後フォローアップ方法を変えているのは6施設，変えていないのが7施設であった．

表1　術後フォローアップに関する研究プロジェクト参加14施設一覧

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敬称略

II　大腸癌治癒切除後の再発

上記プロジェクト研究参加14施設にて1991～96年に治癒切除を施行した大腸癌5,317例を対象として，stage別・再発臓器別に再発の特徴につき検討した．観察期間の中央値は，再発症例で術後3.5±2.9年，非再発症例において7.1±3.1年であった．再発906例中，死亡原因不明症例および生存症例で追跡期間5年未満であった症例を合わせた割合は2.9%であり，各施設にて十分なフォローアップが行われていたと考えられる．ただし，本稿におけるstageは前規約である大腸癌取扱い規約第6版におけるものであることをあらかじめお断りしておく．

上記5,317例における各stageの内訳はstage I：1,368例，stage II：1,835例，stage IIIa：1,421例，stage IIIb：693例である．そのうち再発を来したのは全体で906例（17.0%）であった．各stageにおける再発率はstage I：3.7%，II：12.5%，IIIa：24.1%，IIIb：40.8%とstageが高くなるにつれて再発率も高くなっており，大腸癌取扱い規約におけるstage分類の有用性が証明される形となった．また，再発臓器の頻度としては，肝臓，肺，局所の順であった．術後経過年数と再発の状況について図1，2にstage別，再発臓器別にまとめた．術後比較的早い段階では，stageが高いほど，早期より再発が生じていることがわかる．stage Iでは術後3年での累積再発出現率は68.6%であるのに対して，それ以外のstageでは再発のおよそ80%以上が3年で出現していた．ただし，術後5年目における累積再発出現率はいずれのstageにおいてもおよそ95%程度であった（図1）．以上のことより，stage Iにおいては比較的一定の頻度で再発例が生じるのに対し，それ

表2　参加施設における大腸癌術後フォローアップの現況

経過年数	1年目				2年目				3年目				4年目				5年目
経過月数	3-4	6	8-9		3-4	6	8-9		3-4	6	8-9		3-4	6	8-9		3-4	6	8-9	
外来受診 腫瘍マーカー	13	12	13	13	13	11	13	13	10	10	10	13	5	12	5	13	5	10	5	13
胸部レントゲン検査	3	9	1	10	2	9	2	10	2	7	2	9	1	5	1	9	1	5	1	9
胸部CT	1	3	1	6	1	2	1	6		3		7		2		7		3		7
腹部超音波検査	4	7	4	8	4	7	4	8	3	6	3	8		8		8		7		8
腹部CT	2	5	2	9	1	5	2	9		4		10		3		10		3		10
骨盤CT（直腸癌）	2	8	2	12	2	7	2	12	1	5	1	12		4		12		4		9
大腸内視鏡検査				10				7				9				7				10

CT，コンピューター断層撮影

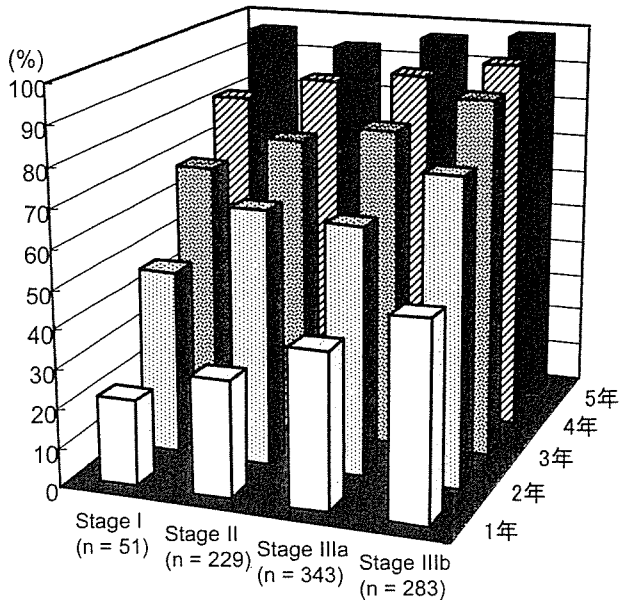

図1　stage別再発累積出現率の推移

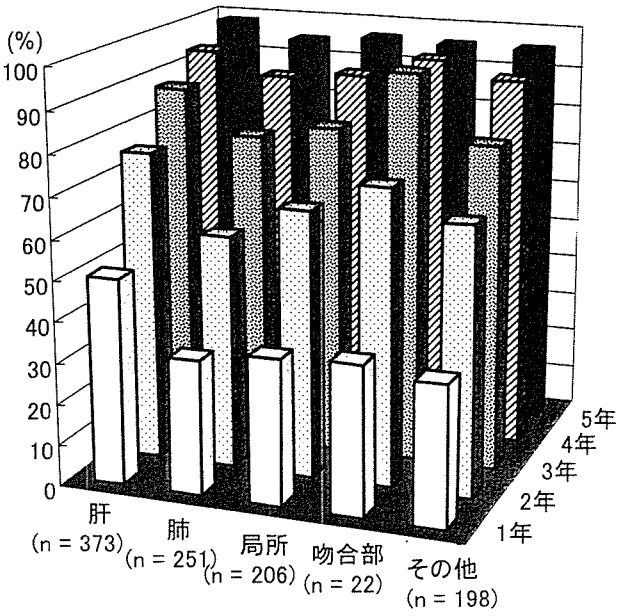

図2　臓器別再発累積出現率の推移

以外のstageにおいては術後3年までは急速に再発が生じ，その後再発は緩やかに増加する傾向にあることがわかった．これを臓器別に検討すると，いずれの臓器においても術後3年で再発の80%内外が，5年で再発の約95%が生じていた．ただし，吻合部再発については術後3年の時点で95.5%が生じていた．

それでは，大腸癌治癒切除後いったい何年まで再発例は生じうるのであろうか．今回の検討においては，stage Iにおいて術後10年2カ月で脳転移が生じた症例が存在し，これが術後再発までの最長期間であった．それ以外のstageについてはstage IIで8年，IIIa：9年，IIIb：8年となっていた．ただし，術後5年以降生じた再発症例数が全体に占める割合は各stage・臓器において1%未満となっており，頻度自体は非常に低いものであった．すなわち，術後5年以降も患者のフォローアップを行っても，それは患者100人中少なくとも99人においては，再発を発見するという点において益するところがない，ということになる．よって，大腸癌術後フォローアップの期間としては5年までが妥当と考えられる．ただし，5年でフォローアップを打ち切るにあたっては，非常に低い確率ではあるが，今後も再発の可能性があることを患者に対しインフォームドコンセントし，医師もまたそれを自覚する必要があるであろう．また，最近の報告によれば，直腸癌において放射線化学療法を施行した患者は，通常に比べより晩期の再発が多かった，との報告もある[8]．今後，術後補助化学療法や直腸癌に対する放射線化学療法の発達とともに，そのような症例に対する検討も必要となるかもしれない．

III　再発発見動機

それでは，これらの再発はどのような検査によって発見されたのであろうか．再発発見動機となった検査法と，その検査で発見された再発がどの程度治癒切除に結びついたのかを表3に示す．術後フォローアップに際し，外来にて行う問診や身体検査，および腫瘍マーカー検査の組み合わせだけでも，肺を除くほとんどの臓器において半数以上の再発を発見できていた．各臓器についてみると，肝再発においては腹部超音波検査やコンピューター断層撮影（CT）といった画像検査で再発を発見された症例が43%であった．また，発見後の治療法については，腫瘍マーカー検査もしくは画像検査にて発見された症例の約半数が治癒切除に結びついており，非常に有効な手段であることが示唆された．肺については胸部レントゲン検査で約半数の再発例が発見されるとともに，その半数は治癒切除に結びついており，肺再発に対しては胸部レントゲン検査が重要であると考えられた．一方局所再発や吻合部再発においては問診や身体検査が約半数の患者の再発発見動機と

表3　再発発見動機と再発に対する治癒切除率

	肝臓	肺	局所	吻合部	その他
自覚症状					
身体所見	5%	8%	48%	50%	28%
	(22%)	(14%)	(40%)	(82%)	(27%)
腫瘍マーカー検査	47%	26%	23%	18%	42%
	(52%)	(27%)	(29%)	(75%)	(18%)
肝画像検査	43%				
	(45%)				
胸部レントゲン検査		48%			
		(50%)			
CT		10%	18%	18%	19%
		(42%)	(29%)	(25%)	(14%)
大腸内視鏡検査			7%	9%	
			(71%)	(100%)	

( )内は治癒切除率
肝画像検査：腹部超音波検査もしくはCT

表4　再発臓器別再発巣に対する治癒切除率

再発臓器	再発巣治癒切除率	再発巣治癒切除後5年生存率
肝臓	46% (172/373)	45%
肺	38% (95/251)	55%
局所	38% (78/206)	27%
吻合部	68% (15/22)	33%

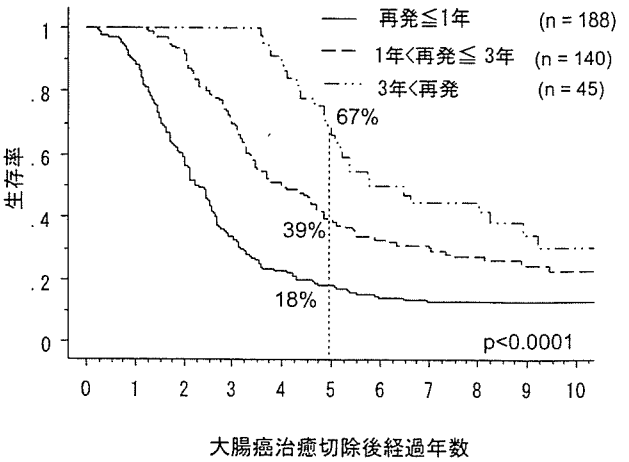

図3　肝再発時期別生存曲線（n = 373）

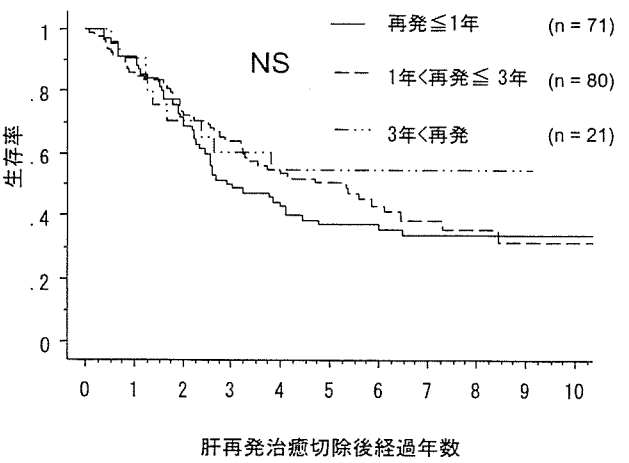

図4　肝再発時期別肝切除後生存曲線（n = 373）

なっていた．よって外来における基本的診療の重要性が再認識される形となった．

IV　再発に対する治療成績

大腸癌治癒切除後に再発を来した場合，それを治癒に結びつける最も確実な手段は，現在のところ外科的切除であると考えられる．事実，今回の検討では再発巣に対して治癒切除できた症例の予後は，できないものに比べていずれの臓器においても有意に予後が良好であった．表4に再発臓器別に再発巣に対する治癒切除率を示す．今回の検討では概ね40～50%の切除率となっていた．ただし，吻合部再発については他の再発に比べ68%と高い切除率であった．よって，吻合部再発は他の局所再発とは別に取り扱う方が妥当と考えられた．

再発巣に対する治癒切除後の治療成績であるが，肝転移や肺転移といった遠隔転移再発巣切除後の5年生存率は50%内外と良好な成績であった．一方，局所再発や吻合部再発についてはいずれも30%内外となっており，前述の遠隔転移に比べやや劣った結果となった．よってこれらの治療成績を改善することが今後の課題であると考えられる．

次に，再発までの期間と予後についての検討を行った．初回手術から再発までの期間を1年以内，1年以降3年以内，3年以降に分け，各時期に再発した症例の予後をみた．一例として肝再発における予後の比較を図3，4に示す．初回手術から再発までの期間が長いほど，全体としての予後は良くなっていた（図3）．しかしながら，再発巣に対して外科的に治癒切除できた症例における再発巣切除術後の成績としては，3つの再発までの期間において有意差を認めなかった（図4）．すなわち，いずれの時期に再発したとしても，それが治癒切除に結びつけば，成績としては変わらないということになる．よって術後フォローアップによってこのような治癒切除可能な再発を見つけることは，再発時期にかかわらず重

表5　再発906症例の予後

死亡	734例	原癌死 695例
		他病死 30例
		不明死 9例
生存	172例	追跡期間5年未満 17例
		追跡期間5年以上 155例

要であると考えられる．図3，4に肝再発に関する成績を示したが，これは他の再発臓器に関しても同様の傾向であった．

再発症例における最終的な予後を表5に示す．906例中172例（19.0%）が今回の研究プロジェクトにおけるフォローアップ終了時点で生存しており，これらの症例は術後フォローアップによる再発巣発見が根治的治療に結びついた集団である．今後このような症例を増やしていくことが，治療成績の向上につながると考えられる．

一方で，大腸癌術後再発が外科的手術の対象とならない場合，化学療法や放射線治療がその適応となる．特に近年の化学療法の進歩は目覚ましく，以前に比べて良好な成績が報告されている[4,5,9]．昨年春には本邦においても5-FU，ロイコボリン，オキサリプラチンを組み合わせたFOLFOXが認可されたことは記憶に新しい．今後，手術の対象とならないような再発を早期に発見することも予後延長に寄与する可能性があり，予後改善の観点より，術後フォローアップの重要性が益々大きくなる可能性がある．

おわりに

本邦において比較的intensiveに行われてきた大腸癌に対する術後フォローアップは，再発巣に対する高い治癒切除率に結びついており，有用である可能性が示唆された．ただし，その検査間隔や検査方法などについては今後更なる検討が必要であると考えられる．また，intensiveな術後フォローアップが実際どれほどの予後改善効果があるかについては，randomized controlled trialによる結果を待たねばならない．

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Follow-up after Curative Resection for Colorectal Cancer : A Study from the Japanese Society for Cancer of the Colon and Rectum

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The aim of this study was to clarify the characteristics of relapse of patients followed up intensively after curative resection for colorectal cancer. We enrolled 5,317 patients who underwent curative resection at 14 hospitals from 1991 to 1996. The relapse rates of stage I, II, IIIa and IIIb were 3.7%, 12.5%, 24.1% and 40.8%, respectively. The relapse rate in patients with stage I colorectal cancer was constant during the 5-year follow-up. On the other hand, those in other stages increased rapidly during the first 3 years and gradually for the next 2 years. The relapse rate 5 years or later was less than 1%. As for surveillance tools, the combination of clinical visits, physical examination and tumor marker measurement detected approximately half of the cases of relapse. The earlier the relapse occurred, the worse the prognosis after curative resection for colorectal cancer. The prognoses of the patients with curative resection for relapse were better than those without resection. However, the time to relapse made no difference in prognosis after curative resection for relapse. Further studies will be necessary to validate the efficacy of these intensive follow-up and surveillance tools.

（依頼原稿）

Overexpression in Colorectal Carcinoma of Two Lysosomal Enzymes, CLN2 and CLN1, Involved in Neuronal Ceroid Lipofuscinosis

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BACKGROUND. Lysosomal proteases are implicated in cancer progression and metastasis. In the current study, using subtraction cloning for genes that are differentially expressed in metastasis, the authors isolated a clone encoding ceroid lipofuscinosis, neuronal 2 (CLN2), which is a lysosomal serine protease defective in neuronal ceroid lipofuscinosis (NCL). Increased CLN2 activity has been reported in breast carcinoma and the antiapoptotic effect of another causative gene of NCL, ceroid lipofuscinosis, neuronal 1 (*CLN1*), is known.

METHODS. The mRNA levels of *CLN2, CLN1*, and cathepsins B, D, H, and L were investigated in colorectal carcinoma patients with different clinical stages using real-time quantitative reverse transcriptase polymerase chain reaction. A polyclonal antibody was raised against a recombinant CLN2 protein for immunoblotting and immunohistochemistry.

RESULTS. The mRNA levels of *CLN1* and cathepsins B, D, and L were significantly higher in metastatic lesions than in primary tumors. In the primary tumors, mRNA expressions of *CLN2* and cathepsin D were associated with advanced clinical stages ($P < .015$ and $P < .031$, respectively). Among the lysosomal enzymes examined, only the mRNA expression of *CLN2* in both the primary tumors of all patients and the pT3 tumors was correlated with the presence of liver metastases ($P < .0049$ and $P < .029$, respectively). The polyclonal antibody prepared in the current study demonstrated CLN2 overexpression by immunoblotting and immunohistochemistry.

CONCLUSIONS. The results indicate that there is a close correlation between *CLN2* and CLN1 expression and colorectal carcinoma progression and metastasis and suggest that they may be potential molecular targets. ***Cancer* 2006;106:1489–97.**

KEYWORDS: colorectal carcinoma; metastasis; ceroid lipofuscinosis, neuronal 2 (CLN2); ceroid lipofuscinosis, neuronal 1 (CLN1).

Proteases, including serine (plasminogen activators), cysteine (cathepsins B, L, and H), and aspartic (cathepsin D) proteases, and matrix metalloproteases, have been shown to play a major role in cancer progression and metastasis through their ability to degrade the basement membrane and extracellular matrix and their proteolytic activation on other proteases.[1–4] In colorectal carcinoma, many studies have shown that lysosomal proteases (cathepsins) are implicated in disease progression, metastasis, and the prognosis of patients.[5–18]

In the present study, which was concerned with the identification of the genes implicated in colorectal carcinoma metastasis, we performed subtraction cloning and isolated a clone corresponding to *CLN2* (ceroid lipofuscinosis, neuronal 2). *CLN2* encodes a lysosomal

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protease with tripeptidyl peptidase activity and a defect in this activity is associated with the late infantile neuronal ceroid lipofuscinosis (LINCL).[19] Neuronal ceroid lipofuscinoses (NCLs) are a group of inherited neurodegenerative disorders, characterized by an accumulation of autofluorescent material in the lysosomes of neurons and other cell types.[20] To our knowledge, to date, 6 causative genes of NCL (*CLN1, CLN2, CLN3, CLN5, CLN6,* and *CLN8*) have been cloned and characterized.[21] Among them, *CLN1* encodes lysosomal palmitoyl-protein thioesterase (PPT), which removes fatty acids from fatty-acylated cysteine residues in proteins,[22] and *CLN3* encodes a membrane-bound protein of lysosomes with an unknown function.[23] In addition, cathepsin D is responsible for another type of NCL in sheep.[24]

To our knowledge to date, several studies have revealed a relation between proteins involved in NCL and tumor growth. For example, it has been shown that the enzymatic activity of CLN2 is increased in breast carcinoma tissue, and that this increased activity correlates with other breast carcinoma biomarkers including cathepsin D and estrogen and progesterone receptors.[25] Furthermore, it has been reported that *CLN3* is overexpressed in a variety of cancer cell lines and colorectal carcinoma tissues.[26] In addition, it has been reported that the overexpression of *CLN1* in neuroblastoma cells protects against apoptosis[27] and that an inhibitor of CLN1 kills cultured tumor cells per se and also enhances their killing by chemotherapeutic drugs.[28]

In the current study, to evaluate the association of *CLN2* with colorectal carcinoma progression and metastasis, we examined the mRNA expression of *CLN2* in 124 samples of colorectal carcinoma at various clinical stages. We also studied the expression of *CLN1* and cathepsins B, D, H, and L in the same samples. In addition, a polyclonal antibody was raised against a recombinant CLN2 protein and was used for immunoblotting and immunohistochemistry.

MATERIALS AND METHODS

Samples

Tissue specimens were obtained from 124 patients who underwent resection of colorectal carcinoma at the Jichi Medical School (Tochigi, Japan) (Table 1). Written informed consent was obtained form all patients and the study was approved by the ethics committee of the Jichi Medical School. Paired samples were collected from the tumor and adjacent normal tissue of each surgical specimen. Immediately after resection, tissues were homogenized in guanidine isothiocyanate solution (4 M guanidine isothiocyanate, 50 mM Tris-HCl [pH 7.5], 25 mM of ethylenediamine tetraacetic acid [EDTA]) and stored in aliquots at −80 °C until analysis.

TABLE 1
Patient Characteristics

	No. (%) of patients	
	Primary tumor (n = 124)	pT3 tumor (n = 88)
Age in yrs		
Mean	61.2	61.3
Range	19-85	19-84
Median value	63	63
Gender		
Male	63 (50.8)	47 (53.4)
Female	61 (49.2)	41 (46.6)
TNM stage		
I	30 (24.2)	
II	33 (26.6)	32 (36.4)
III	33 (26.6)	29 (33.0)
IV	28 (22.6)	27 (30.7)
Histology		
Well	85 (68.6)	58 (65.9)
Moderately	30 (24.2)	23 (26.1)
Poor	9 (7.3)	7 (8.0)
Tumor size in mm		
< 20	5 (4.0)	2 (2.3)
≥ 20 to < 50	68 (54.8)	41 (46.6)
≥ 50	51 (41.1)	45 (51.1)
Location		
Right	34 (27.4)	28 (31.8)
Left	90 (72.5)	60 (68.2)
Depth of tumor invasion		
pT1	4 (3.2)	
pT2	28 (22.6)	
pT3	88 (71.0)	
pT4	4 (3.2)	

Well: well-differentiated adenocarcinoma; Moderately: moderately differentiated adenocarcinoma; Poor: poorly differentiated adenocarcinoma.

RNA Preparation and cDNA Synthesis

Total cellular RNA was extracted using an RNeasy Mini Kit (Qiagen K. K., Tokyo, Japan) according to the manufacturer's protocol. DNase treatment was performed on columns during RNA purification using an RNase-Free DNase Set (Qiagen). First-strand cDNA was synthesized from total cellular RNA using an oligo(dT) primer as a template and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA).

Subtraction Cloning

Subtraction cloning was performed using samples of primary tumor and liver metastasis of the same patient who underwent synchronous resection of the primary sigmoid colon cancer and liver metastasis. To