A multicenter randomized study comparing 5-fluorouracil continuous infusion (ci) plus 1-hexylcarbamoyl-5-fluorouracil and 5-FU ci alone in colorectal cancer

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Abstract. To verify the effectiveness of oral 1-hexylcarbamoyl-5-fluorouracil (HCFU) in improving the surgical cure rate in advanced colorectal cancer, a multicenter randomized comparative study was conducted. A total of 429 patients who had had curative resection for stage II and III colorectal cancer were randomly assigned to a study group receiving a 14-day course of 5-FU continuous infusion (320 mg/m^2/day) followed by oral HCFU for a year (300 mg/day), or to the control group receiving a 14-day course of 5-FU continuous infusion alone. In terms of background factors, no significant differences were found between the 214 patients in the study group and the 215 in the control group. Adverse reactions during the treatment were more frequently seen in the study group. But with few exceptions, the toxicities were mild and the compliance was acceptable. The 5-year overall survival rate of the study group was similar to that of the control group. The 5-year disease-free survival rate of the study group was better than that of the control group in the patients with colon cancer (hazard ratio = 1.87; 95% confidence interval 1.03-3.38; p=0.037). However, this benefit was not seen in the patients with rectal cancer. A significant improvement in the disease-free survival rate was demonstrated through the addition of HCFU to 5-FU continuous infusion for the patients with colon cancer. The usefulness of oral fluoropyrimidine as an adjuvant for curative surgery for colon cancer was further warranted.

Introduction

Colorectal cancer was the fourth most common cancer and cause of cancer death in the world in the year of 2000 (1). At the present time, even if curative resection were carried out, cancer death would follow in about 40% of colorectal cancer patients (2). Although the surgical techniques have been advancing for decades, surgical treatment is inevitably limited in regard to the elimination of recurrence of the disease. Under such circumstances, randomized trials have shown that adjuvant therapy could improve the surgical cure rate of colorectal cancer (3-5). Currently, 5-FU with leucovorin (FU-LV) tends to be regarded as the standard adjuvant therapy for colorectal cancer (Haller DG, Proc ASCO 17: abs. 256, 1998; 6,7), and many studies are under way in search of a more active regimen. One direction of these studies might be the combined usage of the active agents FU-LV, oxaliplatin, and irinotecan. The efficacy of the combined regimens in an adjuvant setting and in advanced diseases has been investigated (8). Another alternative could be oral fluoropyrimidines such as capecitabine and UFT with or without oral leucovorine (9-11).

1-hexylcarbamoyl-5-fluorouracil (HCFU), a highly lipophilic derivative of 5-FU, was developed in Japan in 1981. When HCFU is given orally, the serum concentration of 5-FU is maintained approximately as high as that on 5-FU continuous infusion (12). Koyama *et al* reported a 43.3% response rate of HCFU for advanced colorectal cancer in a phase II study (13). The advantages of adjuvant chemotherapy using HCFU for patients with colorectal cancer in terms of overall or disease-free survival rate, or both, were reported in several randomized trials (14-17).

In order to verify the effectiveness of HCFU as adjuvant chemotherapy in patients who had histologically proven curative resection for colorectal cancer, we organized the East-Japan Colorectal Cancer Chemotherapy Study Group, and conducted a joint multicenter randomized comparative study.

Patients and methods

Eligibility. Patients with colorectal cancer conforming to the following criteria based on intraoperative findings were tentative candidates for registration: i) TNM stage II or III; ii) treated with curative resection; iii) an age of 75 years or

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younger; and iv) an ECOG performance status of 0-2. Patients who met the above criteria were given continuous infusion of 5-FU (320 mg/m²/day) for 14 postoperative days. Among the tentative candidates, cases histologically confirmed to be stage II or III, and treated with 5-FU continuous infusion for at least 10 days were finally registered as eligible. This study was approved by the participating institutions, and informed consent was obtained from each participant.

Randomization. Assignment to the groups was conducted by a center-based system using telephones (EPS Tokyo, Japan). After stratification into colon and rectal cancer, the patients were assigned to the groups by a minimization method in terms of histologically proven nodal status and depth of tumor invasion into the bowel wall.

Treatment and follow-up. In the study group, after 24-h continuous intravenous infusion of 5-FU (320 mg/m²/day) for 14 days, oral HCFU (300 mg/day) was given from the fourth post-operative week for one year. Patients who were assigned to the control group received continuous intravenous infusion of 5-FU for 14 days alone, and were not permitted further chemotherapy unless recurrence was seen. Follow-up examinations were made for at least 5 years after surgery. Compliance of HCFU was confirmed by interviews conducted by attending doctors or nurses in outpatient clinics. Clinical examinations, complete blood cell counts, serum biochemical examinations, serum CEA, chest X-ray, and liver ultrasound studies were performed periodically (clinical examination and blood tests: 1 and 2 weeks, and 1, 3, 6, 9, 12 and 15 months after surgery; serum CEA, chest X-ray and liver ultrasound study: every 3 months for two years, twice in the third year, and once a year thereafter).

Statistical analysis. A target size of 400 patients was calculated for this study, based on the assumption that the 5-year disease-free survival rates in the control group were 80% in colon cancer and 70% in rectal cancer. This sample size was needed in order to show a 8% difference for colon cancer and a 5% difference for rectal cancer with α=0.05 and ß=0.20. The endpoints of the study were overall survival rate, disease-free survival rate, adverse reactions and pattern of recurrence. Statistical analysis was performed using the Statistical Analysis System software package (SAS version 6.12, SAS Institute, Cary, NC). Intention to treat analysis was carried out. The time from the day of surgery to the event in each case was derived by the Kaplan-Meier method (18). Differences in distribution of patients' background factors were examined by the χ^2 test or the Mann-Whitney U test, and that of the survival rates was calculated by the log-rank test.

Results

Patient population. Between March 1995 and February 1998, 512 patients were temporarily enrolled, and 83 were ineligible. The remaining 429 patients were randomly allocated to two groups. A total of 214 were assigned to the study group and 215 to the control group. Two patients in each group (0.9%) were found to be ineligible after randomization. The violations of the protocol were a positive surgical margin in three, and disease of stage I in one. No statistically significant differences were found in the background factors of the two groups (Table I).

Table I. Patient characteristics.

	Study group (%)	Control group (%)
No. of cases	214	215
Median age in years (range)	60 (34-75)	61 (28-75)
Gender		
Male	107 (50.0)	122 (56.7)
Female	107 (50.0)	93 (44.3)
Stage (histologic)		
I	0	1 (0.4)
II	96 (44.9)	96 (44.7)
IIIa	86 (40.2)	94 (42.3)
IIIb	32 (14.9)	27 (12.6)
Primary tumor		
T2	12 (5.6)	13 (6.0)
T3	142 (66.4)	151 (70.2)
T4	60 (28.0)	51 (23.7)
Histologic grade		
Well differentiated	93 (43.4)	96 (44.7)
Moderately differentiated	110 (51.4)	108 (50.2)
Poorly differentiated	7 (1.9)	4 (1.9)
Mucinous	7 (3.3)	7 (3.2)
Lymph node metastasis		
Negative	102 (47.7)	102 (47.4)
Positive	112 (52.3)	113 (52.6)
Lymphatic invasion		
Negative	92 (43.0)	63 (29.3)
Positive	122 (57.0)	150 (69.8)
Unknown	0	2 (0.9)
Venous invasion		
Negative	92 (43.0)	95 (44.2)
Positive	122 (57.0)	118 (54.9)
Unknown	0	2 (0.9)
Curability		
Curative resection	212 (99.1)	214 (99.5)
Non-curative resection	2 (0.9)	1 (0.5)

Between 80% and 120% of the proposed dose of 5-FU was administered to 91% of the study group and to 88% of the control group. The mean total dosage of 5-FU was higher in the control group (6.62 g) than in the study group (6.57 g), but the difference was not statistically significant (p=0.679). The mean total dosage of HCFU in the study group was 99.1 g, and between 80% and 120% of the proposed dosage was administered in 58% of the patients.

Table II. Adverse reactions.

Adverse reactions	Study group (n=214) (%)	Control group (n=215) (%)	p-value
Hematologic			
RBC count	11 (5)	1 (0)	0.003
WBC count	10 (5)	4 (2)	0.112
Platelet count	9 (4)	1 (0)	0.010
Non-hematologic			
Total protein	17 (8)	4 (2)	0.003
BUN	8 (4)	1 (0)	0.020
Nausea, vomiting	17 (8)	8 (4)	0.067
Diarrhea	11 (5)	11 (5)	1.000
Anorexia	19 (9)	12 (6)	0.198
Hot feeling	10 (5)	4 (2)	0.112
Frequent desire to urinate	4 (2)	2 (1)	0.449
Overall	47 (22)	28 (13)	0.016

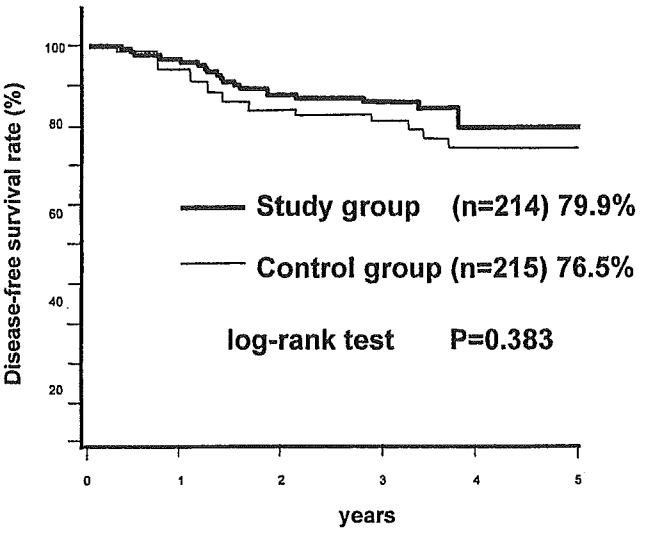

Figure 1. Disease-free survival rates.

Toxicity. The adverse reactions encountered in the two groups are presented in Table II. Adverse reactions were significantly higher in the study group (22%) than in the control group (13%) (p=0.016). Percentage increases in the levels of BUN and GTP, and percentage decreases in the levels of hemoglobin, hematocrit, platelets, erythrocytes and total protein were significantly larger in the study group. Grade 3/4 toxicity occurred in 6 patients of the study group and in 3 patients of the control group. No treatment-related death was encountered in the treatment period.

Efficacy. The median follow-up period of the entire cohort of patients was 54 months. Recurrences were recorded in 87 patients (40 in the study group; 47 in the control group). The recurrence rate of colon cancer in the study group was significantly lower than that in the control group (study group, 17; control group, 30; p=0.037). As shown in Table III, pulmonary recurrence in patients with colon cancer was significantly less frequent in the study group (p=0.031). Also, blood-borne recurrences (i.e., in the liver, the lung and the bone) were less frequent in the study group (p=0.036). Meanwhile, the recurrence rate of rectal cancer and its pattern did not differ between the two groups. Fig. 1 shows the disease-free curves for all patients. The 5-year disease-free survival rate did not differ significantly between the study group (79.9%) and the control group (76.5%) [hazard ratio (HR), 1.21; 95% confidence intervals (95%CI), 0.79-1.84; p=0.383]. However, in colon cancer, the 5-year disease-free survival rate of the study group (86.3%) was significantly higher than that of the control group (75.9%) (HR, 1.87; 95%CI, 1.03-3.38; p=0.037) (Fig. 2a). On the other hand, that of rectal cancer did not show a significant difference (HR, 0.71; 95%CI, 0.38-1.34; p=0.289) (Fig. 2b).

The 5-year overall survival rate of the study group was 83.5%, and that of the control group was 83.8% (HR, 0.96; 95%CI, 0.59-1.57; p=0.866) (Fig. 3). The 5-year overall survival rates of patients with both colon (HR, 1.40; 95%CI,

Table III. Pattern of recurrence.

	Blood-borne						
	Liver	Lung	Bone	All	Local	Others	Overall
Colon cancer							
Study group	11	2	0	13	1	3	17
Control group	14	9	2	25	1	4	30
p-value	0.529	0.031	0.156	0.036	1.000	0.702	0.037
Rectal cancer							
Study group	7	5	0	12	5	6	23
Control group	6	3	1	10	6	1	17
p-value	0.755	0.457	0.319	0.624	0.771	0.051	0.254

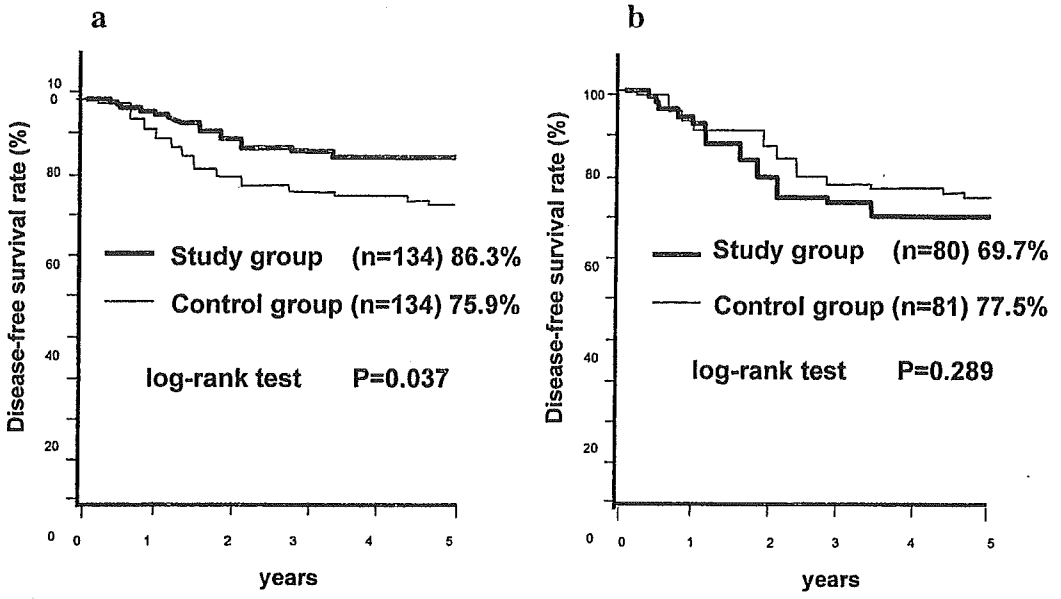

Figure 2. Disease-free survival rates by the tumor site, colon cancer (a) and rectal cancer (b).

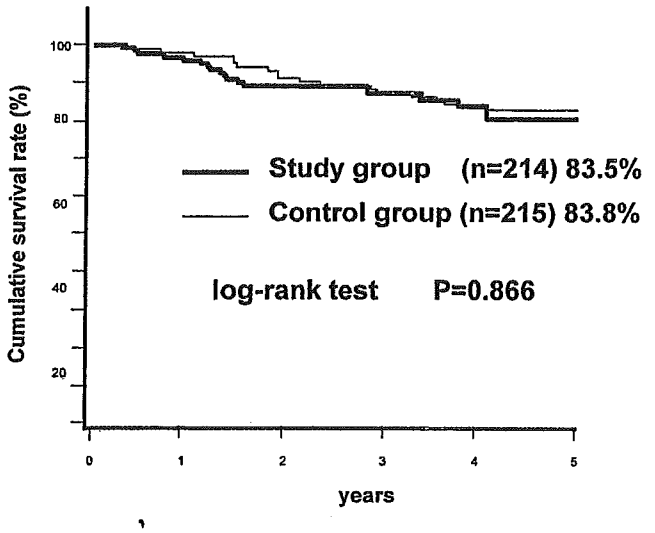

Figure 3. Overall survival rates.

0.72-2.72; p=0.317) and rectal cancer (HR, 0.58; 95%CI, 0.27-1.27; p=0.166) did not differ significantly between the two groups.

Discussion

HCFU, an oral derivative of 5-FU developed in Japan, has been considered to be effective against colorectal cancer with a high response rate of 43.3% (13). In many comparative clinical studies, HCFU has been shown to improve the overall or disease-free survival, or both, in colorectal cancer patients who underwent curative resection (14-17). Ito *et al* reported that the disease-free survival rate of patients with colon cancer who had HCFU-based chemotherapy was significantly better than that of patients who had surgery alone (16). However, the number of patients in the studies was too small to support a definite clinical consensus. In a comparative study for Dukes B and C colon cancer, Watanabe *et al* found that the disease-free survival rate of patients who had concurrent chemotherapy with HCFU, MMC, and 5-FU were significantly higher than those after surgery alone (Watanabe M, *et al*, Proc ASCO 19: abs. 1224, 2000). A significant decrease in liver recurrence among the patients who had HCFU-based chemotherapy was also reported (19,20). However, the study did not attempt to assess the efficacy of HCFU itself, but to make a comparison between adjuvant chemotherapy including HCFU and surgery alone.

In the present large-scale randomized comparative study, we assessed the usefulness of HCFU as an adjuvant to curative surgery for colorectal cancer. No differences were found in overall or disease-free survival rate when the patients with both colon and rectal cancer were analyzed altogether. However, when compared by the tumor site, namely colon and rectum, a statistically significant improvement in the disease-free survival rate was observed in patients with colon cancer.

The frequency of blood-borne recurrences, such as lung metastases, was significantly lower in the study group. This result coincided with reports by Ito *et al* (15,16) and the effectiveness of HCFU in reducing the recurrence rate in colon cancer was confirmed again in the present large-scale study.

There is no clear explanation, however, why HCFU is effective against colon cancer but not against rectal cancer. It seems that the efficacy of HCFU may be related to a certain inhibitory mechanism on blood-borne metastasis. Blood-borne recurrence is more common in colon cancer than in rectal cancer, in which local recurrence is frequent. Thus, the inhibitory effect of HCFU on blood-borne metastasis may not be as clear in rectal cancer as in colon cancer. The mechanism of the inhibition by HCFU of blood-borne metastasis is as yet unknown.

Although improvement of the disease-free survival rate was recognized in colon cancer, no increase in the overall survival rate accompanied it. We therefore examined in detail the treatments and outcomes of the patients with colon cancer

who developed recurrent diseases (study group, 17; control group, 30). In the study group, 2 patients survived after recurrence, whereas 10 survived in the control group. Recurrence patterns of the 12 survivors comprised 7 liver metastases, 3 lung metastases, one local failure, and 1 para-aortic nodal involvement. The treatments for recurrence were radical surgery in 9 (study group, local 1; control group, liver 6, lung 1; lymph node 1), hepatic artery infusion therapy in 1 (study group), systemic chemotherapy in 1 (control group, lung), and unknown in 1 (control group, lung). It was noteworthy that 6 of the 9 patients treated with liver resection survived through the end of study period. In recent years, aggressive resection has been carried out in patients with liver and lung recurrences, and there are many reports of favorable outcomes (21,22). In the present study, radical surgery was performed in patients with liver, lung, local and other recurrences, and we can observe a considerable number of long-term survivors. It is perhaps partly because most of these long-term survivors of recurrent disease were included in the control group, that there appears to be little difference between the overall survival rates of the two groups.

At the present time, numerous clinical trials are being conducted to establish a more effective adjuvant therapy against colorectal cancer. It is worthy of note that oxaliplatin and irinotecan together with FU-LV have recently produced some good results (8). The usefulness of oral anticancer derivatives of fluoropyrimidine such as capecitabine and UFT is also being studied. And UFT as adjuvant for rectal cancer is reported to offer a significantly higher survival rate, compared with surgery alone (Akasu T, *et al*, Proc ASCO 22: abs. 3524, 2004). Oral anticancer agents can be administered at home, and the adverse reactions are generally mild and well tolerated. If the efficacy of oral fluoropyrimidine is comparable with that of parenteral FU-LV, it could be considered an alternative. Trials to compare oral fluoropyrimidines with FU-LV are ongoing (11), and it may be necessary to evaluate adjuvant chemotherapy in terms of its cost-effectiveness and QOL, in comparison with those of already established regimens.

Postoperative one year administration of HCFU after 14-day 5-FU continuous intravenous infusion significantly improved the disease-free survival rate in patients with curatively resected colon cancer. HCFU, an oral fluoropyrimidine, is a safe, effective, and useful adjuvant therapy for colon cancer.

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Appendix

Patients in this study were registered from the following institutions: (Department of Surgery, unless otherwise specified): 1st Department of Surgery, Hokkaido University Graduate School of Medicine; Sapporo City General Hospital; Sapporo Kosei General Hospital; Sapporo Social Insurance General Hospital; Kushiro Rosai Hospital; Nikko Memorial Hospital; 2nd Department of Surgery, Hokkaido University Graduate School of Medicine; Tonan Hospital; Otaru Kyokai Hospital; Hakodate National Hospital; Shinnittetsu Muroran

General Hospital; Iwamizawa Rosai Hospital; Kusiro City General Hospital; Hakodate Medical Association Hospital; Hokkaido Gastroenterology Hospital; Hakodate Central General Hospital; Abashiri Kosei General Hospital; Shiogama City Hospital; Hachinohe City Hospital; Aomori Rosai Hospital; Nihonkai Hospital; Miyagi Cancer Center; Tohoku Employees' Pension Welfare Hospital; Sendai National Hospital; Tohoku Rosai Hospital; 1st Department of Surgery, Fukushima Medical University; 2nd Department of Surgery, Fukushima Medical University; Hoshi General Hospital; Social Insurance Nihonmatsu Hospital; Ohara General Hospital; Iwaki City Hospital of Joban; Iwaki Kyoritsu Hospital; Iwase General Hospital; Honjo Daiichi Hospital; International Medical Center of Japan; Japanese Red Cross Medical Center; Kanto Medical Center NTT EC; International Catholic Hospital; Showa General Hospital; Musashino Red Cross Hospital; Kanto Rosai Hospital; 2nd Department of Surgery, Teikyo University School of Medicine; School of Medicine, Keio University; National Tokyo Medical Center; Ida Municipal Hospital; Kawasaki Municipal Hospital; Kitasato Institute Hospital; 2nd Department of Surgery, Yokohama City University, School of Medicine; Yokohama Medical Center; Yokohama Municipal Citizen's Hospital; Yokosuka Kyosai Hospital; Fujisawa City Hospital; 1st Department of Surgery, Yokohama City University, School of Medicine; Kanagawa Cancer Center; 1st Department of Surgery, Teikyo University School of Medicine; Tokyo Medical and Dental University, School of Medicine; Tokyo Women's Medical University, School of Medicine; Nippon Medical School; Metropolitan Bokutoh Hospital; the Fraternity Memorial Hospital; Mitsui Memorial Hospital; The Jikei University School of Medicine; Yamanashi Prefectural Central Hospital; Misyuku Hospital; Tokyo Medical University Hachioji Medical Center; Aoto Hospital Jikei University School of Medicine; Yokohama City University Medical Center; Saiseikai Central Hospital; Hino Municipal Hospital; Saiseikai Kanagawaken Hospital; National Tokyo Hospital; Inagi Municipal Hospital; Tochigi Cancer Center; Jichi Medical School; Social Insurance Gunma Chuo General Hospital; Ibaraki Prefectural Central Hospital; Saiseikai Utunomiya Hospital; Utsunomiya Social Insurance Hospital; Gunma University, Graduate School of Medicine; Mito Medical Center; Nippon Medical School Chiba Hokusoh Hospital; Mito General Hospital; Nishigunma National Hospital; Tochigi National Hospital; Chiba Tokusyukai Hospital; Haga Red Cross Hospital; Ashikaga Red Cross Hospital; Niigata University School of Medicine; Niigata Cancer Center Hospital; Suibarago Hospital; Ojiya General Hospital; Koshigaya Hospital Dokkyo University School of Medicine; Saitama Social Insurance Hospital; Saitama Medical School; Saitama Red Cross Hospital; Aizawa Hospital; Komoro Kosei General Hospital; Nagano Red Cross Hospital.

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Prognostic implication of laminin-5 gamma 2 chain expression in the invasive front of colorectal cancers, disclosed by area-specific four-point tissue microarrays

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The laminin-5 gamma 2 chain (LN-5γ2) is known to be a marker of invasion in several cancer types. Our purpose was to examine the prognostic significance of LN-5γ2 expression in different areas of individual colorectal cancers (CRCs) by using tissue microarrays (TMAs), and to clarify the optimal areas for prognostic assessment. Using formalin-fixed paraffin-embedded tissue blocks of pT3 primary CRCs resected from 120 patients, we constructed TMA blocks of tissue core specimens taken from the submucosal invasive front, subserosal invasive front, central area, and rolled edge of each tumor. Using these four-point TMA sets, cytoplasmic LN-5γ2 expression was immunohistochemically surveyed, and the area-specific prognostic significance of LN-5γ2 expression was evaluated. The data revealed that 35, 30, 15 and 10% of the 120 CRCs showed high-grade LN-5γ2 expression in the submucosal invasive front, subserosal invasive front, central area and rolled edge, respectively. Disease-specific survival curves for the groups with high- and low-grade LN-5γ2 in the submucosal invasive front and subserosal invasive front were different significantly or of marginal difference (respective 5-year survival rates: 54 and 78% for submucosal invasive front ($P=0.030$) and 58 and 75% for subserosal invasive front ($P=0.055$)). Multivariate analysis revealed that the grades of LN-5γ2 expression in submucosal invasive front (hazard ratio = 2.0, $P=0.047$) and subserosal invasive front (hazard ratio = 2.9, $P=0.0033$) were independent prognostic factors. In contrast, the grades of LN-5γ2 expression in the central area and rolled edge did not have a significant impact on patient prognosis. Analysis using area-specific four-point TMAs clearly demonstrated that LN-5γ2 expression in the invasive front largely influences the degree of clinical aggressiveness of CRC and its tendency to metastasize.
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Keywords: area specific; colorectal cancer; four-point; immunohistochemistry; laminin-5γ2 chain; tissue microarray; tumor budding

Colorectal cancer (CRC) is a relatively homogeneous disease from a histological viewpoint, because most primary CRCs are well to moderately differentiated adenocarcinomas showing tubular patterns. However, the manner of spread and metastasis and patient prognosis vary among individual cases of CRC.

Invasion of cancer cells into their surrounding tissue is an essential step to metastasis. Recently, certain histological features of cancer-cell invasion into surrounding tissues have been shown to be important determinants of clinical behavior of CRC.[1,2] One such feature is tumor cell dedifferentiation in the invasive front, that is, where cancer cells invade into surrounding tissue at the periphery of a primary tumor. The grade of 'tumor budding', which is the relative number of clusters of 5 or fewer cancer

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cells in the invasive front, has been proposed as a scoring system for tumor cell dedifferentiation,[3] and correlates with regional and/or distant metastasis and poorer patient prognosis.[3–5] Furthermore, tumor cell dedifferentiation in primary CRC has been observed not only in the vertical invasive front but also in the lateral invasive front in the submucosal layer.[6]

Although the molecular mechanisms involved in the invasion of CRC are still not completely clear, invasion is known to be correlated with the overexpression of certain molecules. For example, the laminin-5 gamma 2 (LN-5γ2) chain was recently found to be expressed frequently in the cytoplasm of cancer cells that are invading into surrounding tissue as small clusters.[7–9] LN-5, which is composed of α3, β3 and γ2 chains, is a component of the basement membrane of normal intestinal mucosa.[10,11] The presence of the γ2 chain is a characteristic feature of LN-5, because no other laminins have this chain.[12–15] An increasing number of reports have demonstrated a correlation between LN-5γ2 overexpression and clinical behavior of cancers including CRC.[16–19] However, it is unknown whether the clinical significance of LN-5γ2 expression differs among specific areas within a tumor, for example, the central area, rolled edge, and invasive front.

Tissue microarray (TMA) is a recently developed technique for high-throughput evaluation of protein expression in a large number of archival tissue blocks used for routine histopathological diagnosis. A cohort of tissue core specimens obtained from tissue blocks are arranged into a single recipient paraffin block.[20] The utility of TMAs has been proved in a number of immunohistochemical studies of various cancer types.[21–24] However, from the viewpoint of tumor heterogeneity, it is still doubtful whether each tissue core specimen measuring 0.6–2.0 mm in diameter in a tumor represents the characteristics of the whole tumor.

In the present study, we investigated the prognostic significance of LN-5γ2 expression in four different specific areas of a CRC, and attempted to clarify the optimal area for prediction of patient prognosis. To complete this trial, we performed immunohistochemistry (IHC) on both a whole tissue section and TMA sections for 120 CRCs at an identical level of local extension (pT3). We examined the area-specific prognostic significance of LN-5γ2 expression, and found that the prognosis of patients with pT3 CRC was strongly associated with high-grade LN-5γ2 expression in the submucosal and subserosal invasive fronts, but not in the other two areas examined.

Materials and methods

Patient Characteristics

This study was carried out after approval by the internal review board. Among the 613 patients who underwent surgical therapy for primary CRCs at the National Defense Medical College Hospital between 1987 and 1993, we retrieved 120 patients with pT3 CRC, whose tumors had histologically invaded into the subserosal layer or nonperitonealized pericolic or perirectal tissues, according to the TNM classification, 5th edition.[25] These 120 patients were selected almost consecutively, but they did not include patients for whom there were insufficient data regarding outcome and histopathology, or an insufficient volume of archival paraffin-embedded tissue blocks for TMA construction. Patient characteristics and the clinicopathological features of the CRCs are presented in Table 1.

Features related to 'tumor budding' were divided into two grades, as described previously.[3] Briefly, we defined the focus of 'tumor budding' as an isolated single cancer cell or a cluster composed of fewer than five cancer cells, then classified cancers as low-grade if 0–9 foci were found in a ×200 microscopic field, or high grade if 10 or more foci were found.

Potentially curative surgical procedures, defined as the resection of all macroscopically identifiable tumors, were performed on 104 patients (86.7%), and palliative surgical procedures on 16 patients (13.3%).

All the patients were regularly followed up at our outpatient clinic and monitored for postoperative recurrence using chest X-ray films and measurements of serum carcinoembryonic antigen and carbonhydrate antigen 19-9 levels every 3 months, abdominal ultrasonography every 6 months, and colonoscopy every year. Contrast-enhanced computed tomography was performed when cancer recurrence was suspected. Whenever any findings suggestive of cancer relapse did not appear during 5 years, the follow-up procedure was changed to an annual physical check without any other detailed examinations. If patients did not visit our clinic, we confirmed their health condition by telephone once a year. At the last time of follow-up, 38 patients had died of cancer, with a median interval of 26.3 months (range 1.8–72.7 months) from surgery to death. Overall, 10 patients died of other diseases, with a median interval of 30.9 months (range 1.3–70.1 months) after surgical treatment. The median follow-up period of the 72 survivors was 79.0 months (range 40.0–135.9 months).

With regard to adjuvant therapies, systemic chemotherapy was performed on only three (3.0%) patients who had distant metastasis at the time of initial surgery. No patient free of distant metastasis received systemic chemotherapy in this period. None of the patients received chemotherapy or radiotherapy preoperatively.

Tissue-Microarray Construction

For each of the 120 cases of CRC, a representative hematoxylin and eosin (HE)-stained section was

Table 1 Clinicopathological features and their correlations with the grade of LN-5γ2 expression in whole tissue sections of 120 colorectal cancers

	Total	*No of cases (%)*		
		LN-5γ2 High grade (n=45)	*LN-5γ2 Low grade (n=75)*	P-*value*
Age, mean±s.d. (years)	60.1±11.5	62.3±10.9	58.7±11.8	0.29
Sex				
Male	75	29 (39)	46	0.88
Female	45	18 (40)	27	
Primary locus of tumor				
Cecum	9	4 (44)	5	0.87
Ascending colon	11	5 (45)	6	
Transverse colon	9	3 (33)	6	
Descending colon	5	3 (60)	2	
Sigmoid colon	43	15 (35)	28	
Rectum	43	15 (35)	28	
Distant metastasis				
Positive	25	12 (48)	13	0.22
Negative	95	33 (35)	62	
Nodal metastasis				
Positive	59	28 (47)	31	0.027
Negative	61	17 (28)	44	
Histological type[a]				
Poorly, mucinous	4	2 (50)	2	0.63[b]
Well, moderately	116	43 (37)	73	
Tumor budding				
High grade	51	31 (61)	20	<0.0001
Low grade	69	14 (20)	55	
Operation				
Potentially curative	104	35 (34)	69	0.027
Palliative	16	10 (63)	6	

[a]Poorly, poorly differentiated adenocarcinoma; mucinous, mucinous adenocarcinoma well; well-differentiated adenocarcinoma; moderately, moderately differentiated adenocarcinoma.
[b]Fisher's exact test.

selected by reviewing routine histopathological sections microscopically, and the corresponding tissue blocks stored in the hospital were used for this study. Before construction of the TMA blocks, whole sections were prepared from the 120 representative tissue blocks of CRC for HE staining and IHC. A whole tissue section included both tumor tissue and adjacent normal colorectal mucosal tissue.

In order to construct TMA blocks, a single tissue core was taken from each area of the submucosal invasive front, subserosal invasive front, central area, and rolled edge of a 'donor' block, and the core specimens from approximately 10 CRCs were transferred to a 'recipient' block using a Tissue Microarrayer (Beecher Instruments, Silver Spring, MD, USA) (Figure 1). By referring to a HE-stained whole section, we identified the submucosal invasive front, subserosal invasive front, central area, and rolled edge of the viable tumor tissue in each section, and core specimens of each area were taken from a point in the submucosal invasive front close to the mucosa, a point in the deepest subserosal invasive front, the mid point between the subserosal invasive front and the surface, and one of the highest points of the rolled edge, respectively. We used cores 2.0 mm in diameter and arranged them 0.7–0.8 mm apart in a recipient block. One TMA block contained a maximum of 50 tissue cores, and 15 TMA sets, comprising 480 core specimens, were prepared for the present study.

Immunohistochemistry

Sections, 4-μm-thick, were cut from both whole-tissue blocks and constructed TMA blocks and mounted on silane-coated glass slides. These sections were deparaffinized with xylene, rehydrated with ethanol and reacted with 5% hydrogen peroxidase in methanol for 10 min to quench endogenous peroxidase activity. The sections were then treated

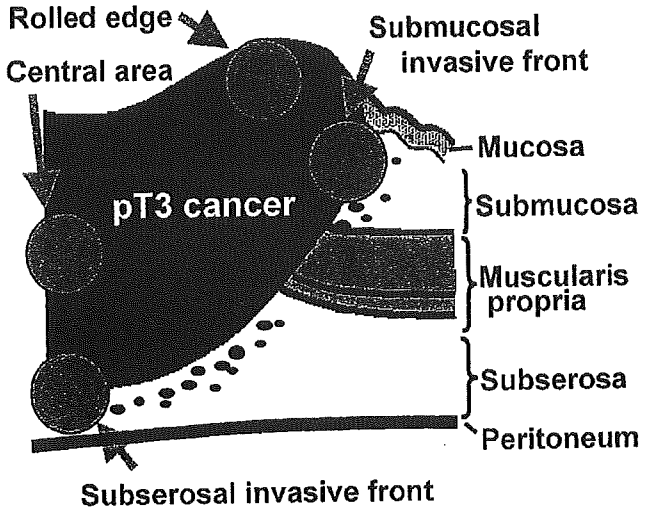

Figure 1 Illustration of four areas of a pT3 colorectal carcinoma and sampling sites used for TMA construction in the present study. The right half of the tumor cut surface is presented. The four areas comprise the submucosal invasive front, subserosal invasive front, central area and rolled edge. In the invasive front, 'tumor budding' can occur.

with 0.1% trypsin (DIFCO, Detroit, MI, USA) for 30 min, incubated in 2% normal swine serum (Dakocytomation, Grostrup, Denmark) for 10 min to block nonspecific binding of the antibody, and incubated with mouse monoclonal anti-LN-5γ2 IgG (clone D4B5; dilution 1:200; Chemicon, Temecula, CA, USA) at 4°C overnight. Subsequently, the sections were incubated with a secondary antibody covalently linked to dextran polymers containing multiple peroxidase molecules (EnVision System; Dako) for 2 h at room temperature. For visualization of the antigen, the sections were immersed in 0.05% diaminobenzidine tetrahydrochloride solution containing 0.01% hydrogen peroxidase for 8 min, and counterstained lightly with Mayer's hematoxylin.

LN-5γ2 staining in the cytoplasm of cancer cells was regarded as positive immunoreactivity and classified as high- or low-grade. With regard to whole-tissue sections, the grade of LN-5γ2 staining was evaluated in terms of the percentage of immunopositive cancer cells among the cancer cells at the invasive front. The grade was classified as low if the percentage was less than 20%, and as high if 20% or more. On the other hand, with regard to the tissue core sections of TMAs, the grade of LN-5γ2 staining was evaluated in terms of the percentage of immunopositive cancer cells among all the cancer cells included, and the grade was classified as low if the percentage was less than 20%, and as high if 20% or more.

Evaluation of Interobserver Agreement

Results of IHC staining were evaluated by two observers (ES and HT) independently, and cases with discrepant grades were re-evaluated with discussion. The degree of interobserver agreement for evaluating the LN-5γ2 immunoreaction of whole-tissue sections was measured using the generalized κ test for two or more observers.[26] In accordance with the criteria of Landis and Koch,[27] κ values were assigned to a scale of strength of agreement. When the κ value was <0.00, 0.00–0.20, 0.21–0.40, 0.41–0.60, 0.61–0.80 and 0.81–1.00, the strength of agreement was judged as poor, slight, fair, moderate, substantial, and almost perfect, respectively.

Statistical Analysis

Comparisons between groups were performed using the χ^2-test or Fisher's exact method. We used the unpaired *t*-test for comparison of groups with continuous variables following a normal distribution. Survival curves of patients were drawn according to the Kaplan–Meier method.[28] Differences between curves were calculated using the log-rank test.[29] The Cox proportional hazard model was used for multivariate analysis.[30] All statistical analyses were performed using Statview 5 software (SAS Institute, Cary, NC, USA) and differences at $P<0.05$ were considered significant.

Results

Expression Pattern of LN-5γ2 in Whole-Tissue Sections of CRC

Cytoplasmic LN-5γ2 staining was detected to a variable degree in dedifferentiated carcinoma cells at the invasive front (Figure 2). High-grade LN-5γ2 expression was detected in whole-tissue sections in 45 of 120 CRC cases (37.5%). The level of interobserver agreement in the evaluation of LN-5γ2 immunostaining was substantial: 87.5% ($\kappa=0.73$). In all discrepant cases, agreement was finally reached upon re-evaluation by the two observers using a discussion microscope.

The correlation of LN-5γ2 immunoreactivity with clinicopathological features is shown in Table 1. High-grade LN-5γ2 expression was detected more frequently in the node-metastasis-positive (28/59, 47.5%) and high-grade 'tumor budding' (31/51, 60.8%) groups than in the node-metastasis-negative (17/61, 27.9%) ($P=0.027$) and low-grade 'tumor budding' (14/69, 20.3%) ($P<0.0001$) groups (Figure 2). The curativity of surgical procedures for a primary CRC was also correlated with the grade of LN-5γ2 expression ($P=0.027$). Patient age and sex, histological type and synchronous distant metastasis were not correlated with LN-5γ2 immunoreactivity.

Disease-specific survival curves obviously differed between CRC with high-grade LN-5γ2 (5-year survival 46.0%) and CRC with low-grade LN-5γ2 (5-year survival 85.0%) ($P=0.0001$) (Figure 3).

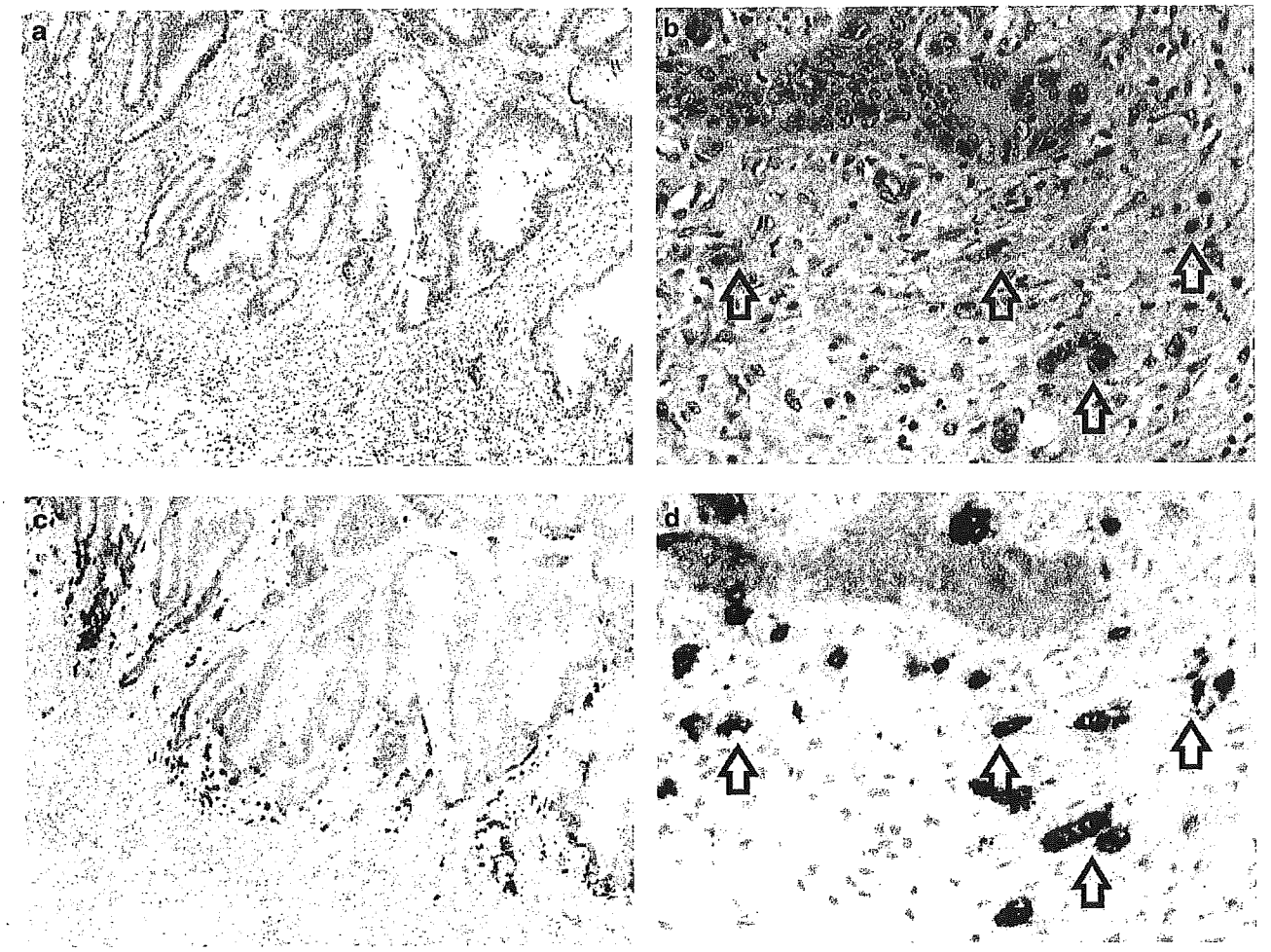

Figure 2 Characteristic microscopic appearance of 'tumor budding' and LN-5γ2 immunostaining in colorectal cancer. (**a**, **b**) Histological appearance (HE stain). (**a**) A low-power view. In the central area of the tumor (upper side), tubular structures are mostly preserved. On the other hand, the foci of 'budding' are located in subserosal invasive front (lower side). (**b**) A high-power view of the subserosal invasive front. Tumor cell dedifferentiation and a lot of foci of 'tumor budding', which are isolated single cancer cells or clusters of fewer than five cancer cells, are observed (arrows). (**c**, **d**) LN-5γ2 immunostaining (immunoperoxidase stain). (**c**) Same field as (**a**) LN-5γ2 is expressed zonally in cancer cells constituting the subserosal invasive front. In the central area (upper side), cancer cells are almost negative for the LN-5γ2. (**d**) Same field as **b**. LN-5γ2 is strongly positive in the entire cytoplasm of cancer cells in the foci of 'budding' (arrows). (magnifications **a**, **c**: ×40 and **b**, **d**: ×200).

Univariate analysis revealed that distant metastasis, nodal metastasis and the grade of 'tumor budding' were also significant prognostic factors ($P<0.0001$ each). Multivariate analysis using Cox's model, including these prognostic indicators as variables, revealed that LN-5γ2 expression was an independent prognostic factor (hazard ratio 3.5, 95% confidence interval (CI) 1.8–6.9, $P=0.0003$), in addition to distant metastasis (hazard ratio 10.9, 95% CI 5.3–22.2, $P<0.0001$) and nodal metastasis (hazard ratio 2.3, 95% CI 1.1–5.0, $P=0.037$) (Table 2). The grade of 'tumor budding' was not included in the multivariate analysis because the grade was strongly correlated with LN-5γ2 expression ($P<0.0001$).

Among 104 patients who underwent potentially curative surgery, 28 (26.9%) suffered postoperative recurrence. Disease-free survival curves after surgical therapy tended to differ between patients with CRC showing high-grade LN-5γ2 expression (3-year survival 66.7%) and those with CRC showing low-grade LN-5γ2 expression (3-year survival 81.0%), although the difference was not significant ($P=0.067$).

Of the 28 patients who suffered CRC recurrence, the postrecurrent disease-specific survival rate was significantly lower for the 13 patients whose primary cancers showed high-grade LN-5γ2 (5-year survival 0%) than for the 15 patients whose primary cancers showed low-grade LN-5γ2 (5-year survival 34.9%) ($P=0.0082$). In this set of patient groups, other clinicopathological parameters of the primary tumors, that is, the presence of distant metastasis or regional lymph node metastasis, did not have a significant influence on postrecurrence patient survival (data not shown). Cox's proportional hazard model including these parameters selected LN-5γ2

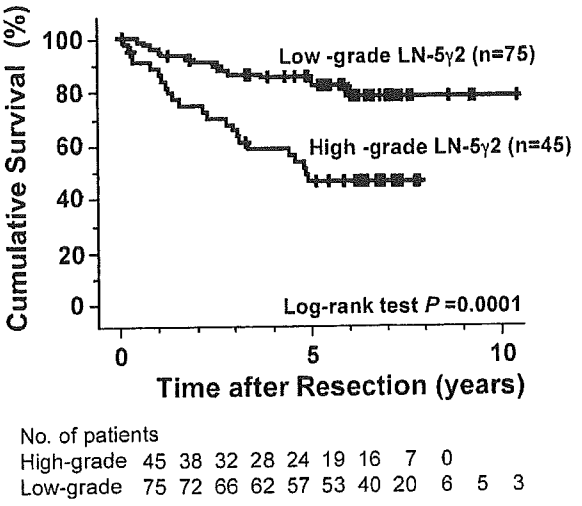

Figure 3 Cumulative disease-specific survival curves for 120 patients with primary colorectal cancers stratified by the grade of LN-5γ2 expression in the analysis of whole-tissue sections. The two curves for patients with high-grade LN-5γ2 expression and low-grade LN-5γ2 expression are significantly different ($P=0.0001$).

Table 2 Independent significance of clinicopathological parameters for disease-specific survival of 120 patients with colorectal cancer by the Cox's proportional hazard model

Parameter	*Hazard ratio*	*95% Confidence interval*	P-*value*
Distant metastasis			
Positive to negative	10.9	5.3–22.2	<0.0001[a]
Nodal metastasis			
Positive to negative	2.3	1.1–5.0	0.037[a]
LN-5γ2			
High- to low grade	3.5	1.8–6.9	0.0003[a]

[a]Statistically significant difference.

expression (hazard ratio 4.6, 95% CI 1.7–12.3, $P=0.0026$) as well as distant metastasis (hazard ratio 3.3, 95% CI 1.2–9.3, $P=0.023$) as independent postrecurrence prognostic indicators (Table 3).

Comparison of Incidence and Prognostic Implication of LN-5γ2 Status among Four Different Sites in Primary CRC Using TMA

Among tissue core sections from the submucosal invasive front and subserosal invasive front areas, high-grade LN-5γ2 expression was detected in 34.8% (40 of 115) and 30.3% (36 of 119), respectively. On the other hand, among tissue core specimens from the central area and rolled edge areas, high-grade LN-5γ2 expression was detected in only 15.0% (18 of 120) and 10.1% (12 of 119), respectively. The rates of high-grade LN-5γ2 immunostaining differed markedly between the specimens from

Table 3 Independent significance of clinicopathological parameters of primary tumors for postrecurrent disease-specific survival of 28 patients with recurrent colorectal cancer by the Cox's proportional hazard model

Parameter	*Hazard ratio*	*95% confidence interval*	P-*value*
Distant metastasis			
Positive to negative	3.3	1.2–9.3	0.023[a]
Nodal metastasis			
Positive to negative	1.3	0.48–3.3	0.64
LN-5γ2			
High- to low grade	4.6	1.7–12.3	0.0026[a]

[a]Statistically significant difference.

invasive fronts and the specimens from other areas ($P<0.0001$, submucosal invasive front and subserosal invasive front *vs* central area and rolled edge). Submucosal invasive front specimens from five CRC cases, a subserosal invasive front specimen from one case and a rolled edge specimen from one case were not informative because of tissue loss or sampling error. The difference in the percentages of high-grade LN-5γ2 among specimens from four different areas of a tumor resulted from the fact that the LN-5γ2 was characteristically expressed in the invasive front. Usually, rolled edge and central area specimens did not contain part of the invasive front. If the areas of the rolled edge and central area were relatively small, the tissue cores of central area and rolled edge specimens contained part of the invasive front where LN-5γ2 was usually positive, and such specimens tended to show high-grade LN-5γ2 expression. The grades of LN-5γ2 expression in tissue core specimens from the submucosal invasive front, subserosal invasive front, central area and rolled edge were concordant with the grade of LN-5γ2 expression in the corresponding whole-tissue sections in 70% (80 of 115), 67% (80 of 119), 66% (79 of 120) and 68% (81 of 119) of cases, respectively. The concordance rate of LN-5γ2 expression grades between submucosal invasive front and subserosal invasive front was 75% (86 of 114).

The prognostic significance of high-grade LN-5γ2 immunostaining differed markedly between specimens from the invasive front and specimens from other areas. With regard to specimens from the submucosal invasive front and subserosal invasive front, survival curves differed or marginally differed between the high- and low-grade LN-5γ2 groups ($P=0.030$ and 0.055, respectively), in a similar way to the prognostic results for whole-tissue sections. For specimens from the submucosal invasive front, 5-year survival rates after surgical therapy were 54 and 78% in the patients with CRC showing high- and low-grade LN-5γ2 expression, respectively. For specimens from the subserosal invasive front, the 5-year survival rates were 58 and 75% in these two patient groups, respectively (Figure 4a, b). Cox's

multivariate analysis revealed the independent significance of LN-5γ2 status in the submucosal invasive front and subserosal invasive front specimens as a prognostic indicator ($P=0.047$ and 0.0033, respectively) (Table 4).

Furthermore, the grade of LN-5γ2 expression in the submucosal invasive front specimens was a prognostic indicator in 27 patients with recurrent CRC. Postrecurrent disease-specific survival rate was significantly lower in the eight patients with primary cancers showing high-grade LN-5γ2 expression (5-year survival 0%) than in the 19 patients with primary cancers showing low-grade expression (5-year survival 27.2%) ($P=0.048$). Cox's proportional hazard model selected LN-5γ2 expression ($P=0.027$) as an independent postrecurrent prognostic indicator (Table 5). However, the grade of LN-5γ2 expression in the subserosal invasive front specimens was not associated with the outcome of patients with recurrent CRC.

In contrast, with regard to specimens from the central area and rolled edge, survival curves did not differ between CRC showing high- and low-grade LN-5γ2 expression ($P=0.21$ and 0.60, respectively). For specimens from the central area and rolled edge, 5-year survival rates after surgical therapy were 57 and 64% in the patients with high-grade LN-5γ2 expression, compared with 72 and 72% in the patients with low-grade LN-5γ2 expression, respectively. Multivariate analysis revealed no independent significance of LN-5γ2 status in the central area and rolled edge specimens as a prognostic indicator ($P=0.25$ and 0.70, respectively).

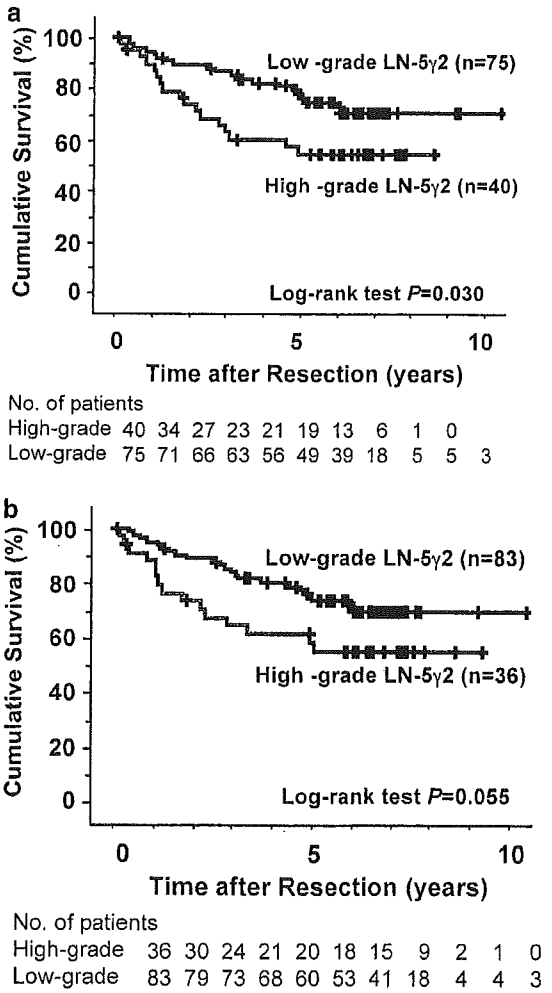

Figure 4 (**a**) Cumulative disease-specific survival curves for 115 patients with primary colorectal cancer stratified by the grade of LN-5γ2 expression in the analysis of core specimens from submucosal invasive front in TMA. The two curves for patients with high-grade LN-5γ2 expression and low-grade LN-5γ2 expression are significantly different ($P=0.030$). (**b**) Cumulative disease-specific survival curves for 119 patients with primary colorectal cancer stratified by the grade of LN-5γ2 expression in the analysis of core specimens from subserosal invasive front in TMA. The two curves for patients with high-grade LN5-γ2 expression and low-grade LN-5γ2 expression tend to be different ($P=0.055$).

Table 4 Independent significance of the grade of LN-5γ2 expression in submucosal invasive front and subserosal invasive front areas for disease-specific survival of 120 patients with colorectal cancer by the Cox's proportional hazard model

Parameter	*TMA taken from submucosal invasive front*		*TMA taken from subserosal invasive front*	
	Hazard ratio	P-*value*	*Hazard ratio*	P-*value*
Distant metastasis				
Positive to negative	10.9	<0.0001[a]	10.6	<0.0001[a]
Nodal metastasis				
Positive to negative	2.8	0.011[a]	2.6	0.021[a]
LN-5γ2				
High- to low grade	2.0	0.047[a]	2.9	0.0033[a]

[a]Statistically significant difference.

Table 5 Independent significance of the grade of LN-5γ2 expression in submucosal invasive front and subserosal invasive front areas for post-recurrent disease-specific survival of 28 patients with recurrent colorectal cancer by the Cox's proportional hazard model

Parameter	*TMA taken from submucosal invasive front*		*TMA taken from subserosal invasive front*	
	Hazard ratio	P-*value*	*Hazard ratio*	P-*value*
Distant metastasis				
Positive to negative	2.4	0.085	2.2	0.11
Nodal metastasis				
Positive to negative	1.2	0.77	0.99	0.98
LN-5γ2				
High- to low grade	2.9	0.027[a]	1.6	0.34

[a]Statistically significant difference.

Discussion

In the present study, we immunohistochemically examined LN-5γ2 expression in surgically resected CRCs showing the same extent of local spread, that is, pT3. Using whole-tissue sections, Lenander *et al*[18] and Aoki *et al*[19] showed that LN-5γ2 expression was correlated with an unfavorable outcome in patients with CRC. Using both univariate and multivariate analyses, we also demonstrated that the grade of LN-5γ2 expression had a great effect on patient outcome. Furthermore, we found that high-grade LN-5γ2 expression in primary CRCs was an independent postrecurrence prognostic indicator. These novel findings may be clinically applicable for patient follow-up after resection of primary tumors and for choosing adjuvant therapies or postrecurrent therapies.

In addition, by means of TMA devised to reveal the significance of LN-5γ2 expression in different areas of a tumor, we clearly demonstrated intratumoral heterogeneity of LN-5γ2 expression with regard to both positivity rate and prognostic implication. High-grade LN-5γ2 expression was detected more frequently in specimens taken from the submucosal invasive front and subserosal invasive front than in those taken from the rolled edge and central area. The grades of LN-5γ2 expression in the submucosal invasive front and subserosal invasive front were significantly correlated with patient outcome, whereas those in the rolled edge and central area were not.

In the present study, by employing TMA, we actually verified that dedifferentiated cancer cells in tissue cores from the invasive front, both the vertical invasive front in the subserosal layer and the lateral invasive front in the subserosal layer, showed overexpression of LN-5γ2, and confirmed that the grade of LN-5γ2 expression in the invasive front was of prognostic significance in patients with primary CRCs. Additionally, such overexpression in submucosal invasive front specimens had a prognostic influence in patients with recurrent CRC. In contrast, high-grade LN-5γ2 expression in tissue core specimens from the central area and rolled edge was less frequent and had no prognostic significance. Consequently, we clearly demonstrated that high-grade LN-5γ2 expression in dedifferentiated cells at the invasive front largely influenced the clinical aggressiveness of CRC and its potential to metastasize.

It is well recognized that TMA is efficient for screening of molecular alterations in a large number of tumor cases. In contrast, major drawback proper to TMA analysis consists in that the characteristics of sampled tissue do not always represent those of whole tumor. However, in this study, we showed that LN-5γ2 expression in invasive fronts, but not in central area or rolled edge, correlated with clinical aggressiveness. Therefore, area-specific TMA was concluded to be effective not only for high-throughput screening of molecular alterations but also for the identification of prognostic factors in CRC, at an approximately similar level with whole-tissue section analysis.

Abnormally strong expression of the LN-5γ2 has been characteristically detected by IHC in dedifferentiated cells dissociated from the neoplastic tubules of CRCs in whole-tissue sections,[7,8] and a tight correlation between the grade of LN-5γ2 expression and tumor dedifferentiation has been proved.[18,31] These pathological findings were supported by recent molecular studies which revealed that the cleavage of LN-5 chains by membrane-type 1 matrix metalloproteinase induced epithelial migration,[32] and that both LN-5γ2-coding gene and membrane-type matrix metalloproteinase gene were targets of the Wnt-β-catenin-T-cell factor signaling.[33–35] In Hlubek's studies, nuclear β-catenin accumulation, which was detected at the invasive front of CRC, enhanced transactivation of these two genes.[34,35] It is conceivable that the Wnt-β-catenin-T-cell factor signaling pathway triggers the enhancement of cancer cell motility and invasion through the interaction with these two molecules.

In the present study, we confirmed that the grade of LN-5γ2 was highly correlated with the grade of 'tumor budding', which is a morphological index of tumor dedifferentiation at the invasive front of primary CRCs, and with patient prognosis in primary CRC and recurrent CRC. The grade of 'tumor budding' has also been shown to be a powerful prognostic factor for patients with postoperative liver recurrence as well as patients with primary CRC.[36] Because of our favorable interobserver agreement for the judgment of LN-5γ2 expression, its evaluation was indicated to be useful as an objective marker of tumor cell dedifferentiation at the invasive front of CRC in a clinicopathological setting.

TMA may be useful for investigating the incidence and clinical significance of the expression of multiple different molecules in a large number of tumor cases, and the validity of TMA analysis has been reported to be acceptable.[21,23] However, previous studies did not emphasize the importance of taking tissue core samples from specific areas in the tumor. In CRC, Chung *et al*[37] and Hoos *et al*[38] employed TMA for the detection of β-catenin, phospho-β-catenin, p53, mdm-2, p21, Bcl-2, p27, cyclin D1 and Ki-67, but in those studies, the sites from which the tumor tissue core specimens were sampled were not rigorously specified. They showed that only the levels of phospho-β-catenin and p27 were associated with survival rate, although the alterations of these eight molecules had already been shown to be prognostic factors by other investigators.

Other than LN-5γ2, a number of proteins have been reported to show a difference in expression pattern between the invasive front and other areas in individual primary CRCs. These proteins comprise proliferating cell nuclear antigen,[39,40] CEA,[41] NF-

kappa B,[42] matrilysin[43] and trypsin.[44] The expression level of these proteins in the invasive front was shown to predict the malignant potential of CRC. Therefore, the area-specific multiple sampling method employed for the construction of TMAs in the present study seemed particularly applicable for analyzing the clinical and histopathological significance of molecules associated with cancer invasion and metastasis. Furthermore, we suggested that sampling of tissues from parts of the invasive front would be essential for TMA analysis of CRCs, irrespective of the volume of the invasive front.

A majority of TMA examinations have adopted a 0.6-mm-diameter punch in order to achieve high-throughput potential.[20–24] However, we preferred a 2.0-mm-diameter punch in order to obtain tissue core samples that would be guaranteed to contain the areas of the invasive front. In fact, the line of the invasive margin often showed a shift of approximately 0.5 mm from the core center, because of the accumulation of delicate shifts resulting from the manual punch and/or an unexpected tumor-margin shift due to tissue loss during section preparation. From such experience, we considered that a 0.6-mm-diameter punch would often fail to sample the areas of the invasive front that we intended to obtain. In 115 of 120 specimens from the submucosal invasive front and in 119 of 120 specimens from the subserosal invasive front in the present cohort, 2-mm-diameter tissue cores contained discrete samples of the invasive front with surrounding normal connective tissue. Therefore, we believe that a 2.0-mm-diameter punch is a more appropriate procedure for TMA in studies of the CRC invasive front.

In conclusion, we have found that the grade of LN-5γ2 expression in whole-tissue sections is strongly correlated with the prognosis of patients with primary CRC and that of patients with recurrent CRC. Moreover, the present TMA analysis has revealed that LN-5γ2 expression in samples taken from the invasive front in both the submucosal and subserosal layers is an independent prognostic factor of CRC. This is the first report to emphasize the importance of constructing area-specific multipoint TMAs for the study of CRC.

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A novel classification of tumour budding in colorectal cancer based on the presence of cytoplasmic pseudo-fragments around budding foci

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A novel classification of tumour budding in colorectal cancer based on the presence of cytoplasmic pseudo-fragments around budding foci

Aims: Tumour budding is an adverse prognostic factor in colorectal cancer (CRC). We have investigated the significance of cytoplasmic fragments occurring in the immediate vicinity of tumour budding foci.

Methods and results: Seventy-three CRCs with high-grade budding (> 10 budding foci in a ×20 objective field) were classified according to extent of budding (10–19 versus 20+ foci) and by the presence or absence of cytoplasmic fragments identified by immunostaining for cytokeratin. In serial sections, cytoplasmic fragments were shown to be dendritic cell processes in continuity with budding tumour cells and were renamed pseudo-fragments. Cytoplasmic pseudo-fragments, but not extent of budding, were associated with aberrant expression of β-catenin ($P = 0.045$) and laminin-5 γ2 ($P < 0.0001$), and with absent peritumoral lymphocytic infiltration ($P = 0.0077$). Cytoplasmic pseudo-fragments had a stronger association with infiltrating growth pattern ($P = 0.0014$) than extent of tumour budding ($P = 0.014$). There was no association between extent of budding and cytoplasmic pseudo-fragments ($P = 0.12$).

Conclusions: Cytoplasmic pseudo-fragments may be a marker for an activated budding phenotype that is associated with cell motility and increased invasiveness in CRC and is independent of the extent of budding.

Keywords: colorectal cancer, cytoplasmic pseudo-fragments, invasive marker, tumour budding

Abbreviations: CRC, colorectal cancer; MSI, microsatellite instability

Introduction

In colorectal cancer (CRC), tumour budding has been defined as the presence of isolated single cells or small cell clusters (up to four) scattered in the stroma at the invasive tumour margin.[1,2] Tumour budding has been established as a practical prognostic indicator, that can be assessed in slides stained by haematoxylin and eosin (H&E). Despite the modern emphasis on molecular prognostic markers, there are several recent reports highlighting the clinical value of tumour budding.[1–4]

Dysregulation of the Wnt signalling pathway plays a pivotal role in the development and progression of CRC. A key trigger is the translocation of β-catenin from the cell membrane to the nucleus where it binds with T-cell factor and promotes the transcription of cyclin D1, matrix metalloproteinase 7, CD44 and laminin-5 γ2 chain (LN-5γ2).[5,6] By means of immunohistochemistry, it has been shown that accumulation of nuclear β-catenin and overexpression of the preceding downstream molecules occur in dedifferentiated cancer cells (tumour buds) at the invasive margin of CRC.[6–9] It has therefore been proposed that activation of the Wnt signalling pathway plays a critical role in

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dedifferentiation of CRC and the development of budding foci.[10] Nevertheless, immunohistochemical observations are static and can provide only limited insight into the various dynamic cellular events that underlie tumour invasiveness. Therefore, an understanding of the cellular and molecular mechanisms explaining tumour budding lags behind the demonstration of potentially important clinical or prognostic implications.

Tumour budding foci are thought to arise through the processes of cellular dissociation or discohesion and/or active invasion into the surrounding stroma. When dissociation is the principal factor, tumour budding foci may comprise relatively inert cell clusters. By contrast, when there is active amoeboid movement, cytoplasmic processes and pseudopodia may extend from the cells for the purposes of attachment to surrounding structures and active movement. Such passive and active processes cannot be distinguished in H&E sections, in which the cytoplasmic features are unclear. Therefore, attempts at grading the aggressiveness of tumour budding have been limited to counting the number of budding clusters within a high-power field.

In order to visualize cytoplasmic features of tumour budding in greater detail, we performed immunohistochemistry for cytokeratin in a pilot study. This technique yielded a single novel observation associated with tumour budding, namely the presence of showers of cytoplasmic fragments at the invasive tumour margin. We hypothesized that this feature could serve as a marker for a more actively infiltrative and aggressive form of tumour budding. To test this hypothesis, we identified a series of CRCs with high-grade budding. CRCs were then stratified on the basis of cytoplasmic fragments to establish if this feature segregated with known adverse prognostic factors and would therefore identity a more aggressive form of budding. Additionally, we looked for correlation between cytoplasmic fragments and two markers of the Wnt signalling pathway. These comprised β-catenin nuclear expression which triggers this pathway[5] and the downstream protein LN-5γ2, which is expressed by dedifferentiated cancer cells at the invasive front and has been linked to tumour aggressiveness.[11–13]

Materials and methods

PATIENTS

Fifty-five CRCs were used in a preliminary study to correlate tumour budding and cytoplasmic fragments. This yielded 31 CRCs with high-grade budding. An additional 42 CRCs with high-grade tumour budding were identified, giving a total of 73 CRCs with high-grade budding. All CRCs had been surgically resected in the years 1983–1994 at McGill University related hospitals. The study was approved by the Institutional Review Board of the Faculty of Medicine of McGill University.

Tumour budding was assessed as described previously.[1] Briefly, we defined a focus of tumour budding as a single isolated cancer cell or a cluster composed of up to four cancer cells. Cancers were then divided into two groups according to the number of tumour budding foci in the densest field using a ×20 objective lens. Counts of 0–9 were termed low-grade, while counts of 10 + were termed high-grade budding. High-grade budding was further divided into counts of 10–19 (moderate) and 20 + (severe). Information about patient age, sex, tumour site, size, and stage was obtained from the pathological and clinical records. There were 29 male and 68 female subjects with an average age of 69.4 years (range 34–96 years). Cancers were located in the proximal colon ($n = 41$), distal colon ($n = 31$), and rectum ($n = 20$). There was some deliberate oversampling of proximal cancers in elderly females to ensure adequate representation of microsatellite instability-high (MSI-H) cancers in a subsequent study on the same material. All of the cancers showed at least submucosal invasion and ranged in size from 9 to 100 mm (mean size 40 mm). Tumours were staged according to the tumour node metastasis (TNM) staging system.[14] Tumour type was determined by the criteria of the World Health Organization.[15] Growth pattern and lymphocytic infiltration at the advancing tumour margin were evaluated according to the criteria of Jass *et al*.[16] Information on cancer location in six cases and tumour size for 11 cases was not available because of incomplete information.

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Ninety-seven cases were immunostained for β-catenin (clone β-Catenin-1, dilution 1 : 200; DakoCytomation, Grostrup, Denmark), LN-5γ2 (clone 4G1, dilution 1 : 50; DakoCytomation) and broad spectrum cytokeratin (clone MNF116, dilution 1 : 50; DakoCytomation). Four-micrometre-thick sections were cut from representative blocks and mounted on silane-coated glass slides. After dewaxing and rehydration to dH_2O, sections for immunostaining were subjected to heat antigen retrieval in an microwave oven (1200 W, 15 min) in 0.01 mol/l citrate buffer pH 7.0 for β-catenin or in purchased target retrieval solution pH 9.0 (S2367; DakoCytomation) for cytokeratin and

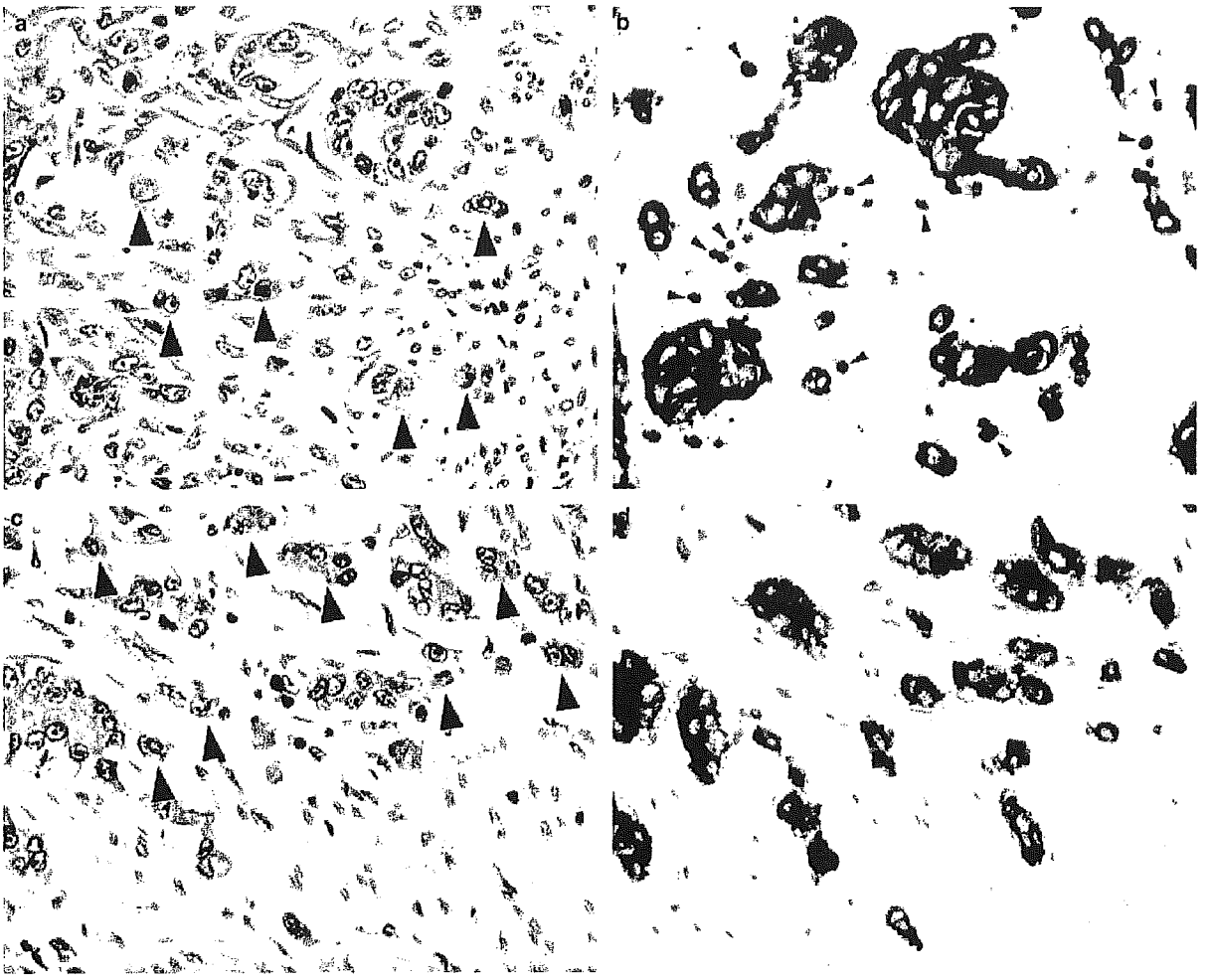

Figure 1. Characteristic high-power microscopic appearance of cytoplasmic fragments in colorectal cancer. **a,b**, High-grade cytoplasmic fragments with high-grade tumour budding. **c,d**, Low-grade cytoplasmic fragments with high-grade tumour budding. In (**a**) and (**c**), numerous foci of tumour budding (arrowheads) (an isolated single cell or a cluster of up to four cancer cells) occur at the invasive front (H&E staining). In (**b**), multiple cytoplasmic fragments (small arrowheads), which are round cytokeratin-immunostained spots without a nucleus at the invasive front, are demonstrated around tumour budding foci (cytokeratin immunostaining). In (**d**), in spite of abundant tumour budding foci, there are almost no cytoplasmic fragments (cytokeratin immunostaining).

LN-5γ2. After cooling, non-specific antibody binding was inhibited by incubating the sections in 4% skimmed milk. Endogenous peroxidase activity was blocked using 0.5% H_2O_2. After transfer to a humidified chamber, the sections were incubated with 10% normal goat serum swine serum (DakoCytomation) for 20 min and incubated with primary antibody at room temperature for 1 h. Subsequently, the sections were incubated with peroxidase-labelled polymer (K4005, EnVision+ System-HRP(AEC); DakoCytomation) for 30 min at room temperature. For visualization of the antigen, the sections were immersed in 3-amino-9-ethylcarbazole + substrate-chromogen (K4005, EnVision+ System-HRP(AEC); DakoCytomation) for 30 min, and counterstained lightly with Gill's haematoxylin.

Nuclear staining for β-catenin and cytoplasmic staining for LN-5γ2 in cancer cells were regarded as positive immunoreactivity, the grades of which were evaluated from the percentages of immunopositive cancer cells among the cancer cells at the invasive front. Immunoreactivity was evaluated semiquantitively and divided into immunopositive or immunonegative. The cut-off values used in this study were defined as follows: immunopositive for β-catenin if > 10% tumour cells demonstrated positive nuclear staining, and for LN-5γ2 if > 20% cells had cytoplasmic staining.

DEFINITION OF CYTOPLASMIC FRAGMENTS

Using cytokeratin-immunostained sections, small non-nucleated cytoplasmic fragments were detected around tumour budding foci at the invasive tumour margin. To be counted, fragments had to be: at least 2 μm in diameter, non-nucleated, lacking in evidence of nuclear fragmentation, uniformly positive for cytokeratin, smoothly contoured and free of surrounding inflammatory cells. On the basis of these features and the large numbers of fragments seen in some cases, it was inferred that neither degenerating nor apoptotic cells were being counted. Scores for each case were the highest number of fragments in a ×20 objective lens field (3.2 mm^2). Low-grade cancers had 0–9 fragments, and high-grade cancers had 10 + fragments (Figure 1).

To clarify the morphological features of cytoplasmic fragments, 3 μm-thick serial sections were cut from blocks of cancers with high-grade cytoplasmic fragments. Cytokeratin immunostaining was performed as described above and we investigated the continuity between cytoplasmic fragments and budding foci in consecutive sections.

STATISTICAL ANALYSIS

Comparisons between groups were performed using the χ^2 test or Fisher's exact method.

Results

The serial section study disclosed that some cytoplasmic fragments were clearly connected with budding foci (Figure 2). This provided evidence that cytoplasmic fragments represented dendritic cell processes rather than isolated cell fragments. They were therefore renamed cytoplasmic pseudo-fragments. There was a very strong relationship between cytoplasmic pseudo-fragments and tumour budding. Cytoplasmic pseudo-fragments were observed in only 8% (2/24) of low-grade tumour budding cancers (0–9 budding foci) but in 55% (17/31) of high-grade tumour budding cancers (10 + budding foci) ($P = 0.0004$) (Table 1). The remaining findings relate only to 73 CRCs with high-grade tumour budding stratified as 10–19 (moderate) and 20 + (severe) budding foci. Severe budding was associated with two adverse pathological prognostic factors, vascular invasion ($P = 0.0009$) and infiltrative growth pattern ($P = 0.014$). There was no association with aberrant expression of either β-catenin or LN-5γ2 (Table 2). When the same 73 CRCs were stratified on the basis of the presence of cytoplasmic pseudo-fragments, this feature was shown to segregate differently with pathological prognostic factors. There was a significant though weaker association with vascular invasion ($P = 0.021$). On the other hand, cytoplasmic pseudo-fragments were more strongly associated with an infiltrative growth margin ($P = 0.0014$) and were also associated with absent peri-

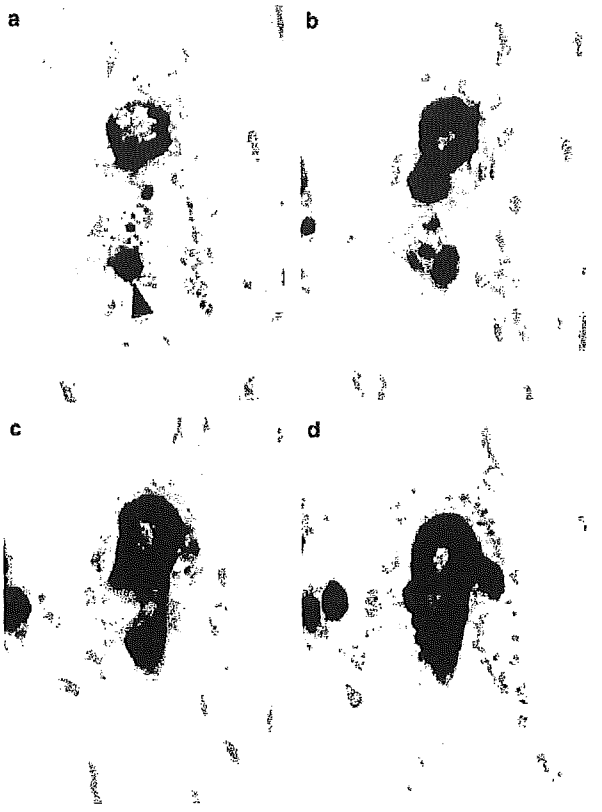

Figure 2. Serial section analysis for morphological features of cytoplasmic fragments (cytokeratin immunostaining). In (a), a cytoplasmic fragment (arrowhead) is identified near budding foci. In (b–d), the cytoplasmic fragment is demonstrated to be connected with one budding focus. Based on this finding and similar observations in serial sections, cytoplasmic fragments were interpreted as dendritic cell processes and renamed pseudo-fragments.

Table 1. Relationship between tumour budding and cytoplasmic pseudo-fragments

		Low-grade budding ($n = 24$)	High-grade budding ($n = 31$)	P-value
Cytoplasmic pseudo-fragments	Low-grade	22	14	0.0004
	High-grade	2	17	