Table II. Relationship between the expression of claudin-1, -3, -4 or -7 genes and clinicopathological features.

Variables/categories	claudin-1 expression		P-value	claudin-3 expression		P-value	claudin-4 expression		P-value	claudin-7 expression		P-value
	low (n=102)	high (n=103)		low (n=102)	high (n=103)		low (n=102)	high (n=103)		low (n=102)	high (n=103)	
Age	65.6±11.3	66.0±10.3	0.775	65.6±11.1	66.0±10.5	0.805	65.7±11.2	65.8±10.4	0.917	65.1±11.0	66.5±10.6	0.344
Gender												
Male	58	54	0.524	51	61	0.160	50	62	0.108	50	62	0.108
Female	44	49		51	42		52	41		52	41	
Size												
≤5 cm	58	57	0.826	57	58	0.951	56	59	0.731	54	61	0.365
>5 cm	44	46		45	45		46	44		48	42	
Histological type												
Well differentiated	28	33	0.047	26	35	0.362	29	32	0.809	28	33	0.762
Moderately differentiated	54	62		60	56		60	56		60	56	
Poorly differentiated	20	8		16	12		13	15		14	14	
Depth of invasion												
T1	10	9	0.846	9	10	0.294	7	12	0.320	10	9	0.085
T2	44	50		41	53		52	42		38	56	
T3	41	39		44	36		36	44		46	34	
T4	7	5		8	4		7	5		8	4	
Lymph node metastasis												
Absent	50	45	0.930	46	49	0.722	51	44	0.296	42	53	0.140
Present	52	58		56	54		51	59		60	50	
Location												
Colon	61	51	0.139	62	50	0.784	66	46	0.039	56	56	0.940
Rectum	41	52		40	53		36	57		46	47	
Lymphatic invasion												
Absent	66	68	0.843	67	67	0.924	75	59	0.145	70	64	0.829
Present	36	35		35	36		27	44		32	39	
Venous invasion												
Absent	40	37	0.237	40	37	0.626	35	42	0.340	28	49	0.029
Present	62	66		62	66		67	61		74	54	
Liver metastasis												
Absent	70	69	0.802	69	70	0.802	72	67	0.396	59	80	0.022
Present	32	34		34	32		30	36		43	23	

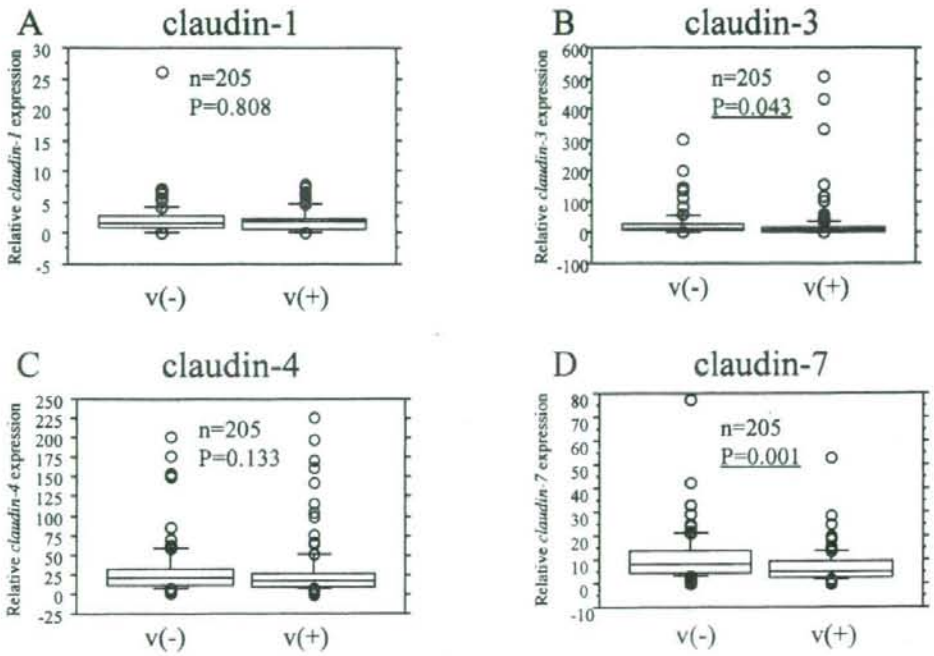

Figure 3. Associations of *claudin-1*, *-3*, *-4* and *-7* gene expression levels with venous invasion in 205 patients with colorectal cancer. Box boundaries, the 25th and 75th percentiles of the observed values; capped bars, the 10th and 90th percentiles; solid line, median. P-values were calculated by the Mann-Whitney U test. *Claudin-3* and *-7* gene expression levels were higher in the absence than in the presence of venous invasion (P=0.043, P=0.001).

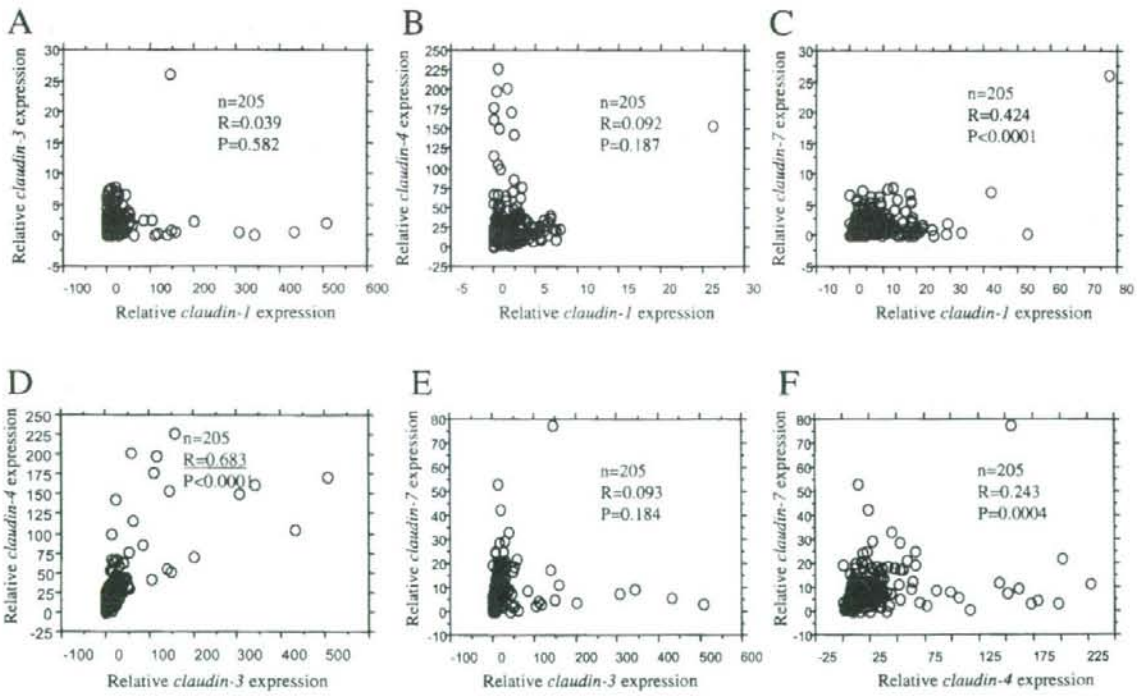

Figure 4. Correlation among *claudin-1*, *-3*, *-4* and *-7* gene expression levels in colorectal cancers. Each gene expression level is relative to that of the *β-actin* gene. The expression of the *claudin-3* gene correlated with that of the *claudin-4* gene (R=0.683).

Correlations among claudin-1, -3, -4 and -7 gene expression. Correlations between gene expression levels are shown in Fig. 4. The expression of the *claudin-3* gene correlated with that of the *claudin-4* gene (R=0.683).

Discussion

Cell-to-cell adhesiveness is generally reduced in various human cancers. The dissociation of cancer cells from primary cancer nests is a crucial step in metastasis. The suppression of cell-to-cell adhesiveness may trigger the release of cancer cells from primary cancer nests and increase tumor invasiveness (13). In this study, we examined the expression levels of the *claudin-1*, *-3*, *-4* and *-7* genes in colorectal cancer and the relationship of such levels to clinicopathological variables.

We compared the mRNA expression of each *claudin* gene between colorectal cancer tissue and adjacent normal mucosa. Dhawan *et al* (14) reported that the expression of claudin-1 is higher in human primary colon carcinoma and metastasis than in normal colorectal tissue. Pan *et al* (15) found that the expression of claudin-3 and -4 is significantly higher in human endometrial carcinoma than in normal endometrial tissue at the protein and mRNA levels. As for claudin-7, Kominsky *et al* (10) reported that this gene is down-regulated in breast cancers as compared with normal breast tissue. However, Sobel *et al* (16) found no significant difference in the expression of claudin-7 between human invasive cervical carcinoma and normal cervical tissue. In our study, expression levels of the *claudin-1*, *-3* and *-4* genes were higher in cancer than in normal adjacent mucosa, whereas the expression level of the *claudin-7* gene cancer did not differ significantly between cancer and normal adjacent mucosa.

We then examined the relationship between claudin gene expression levels and clinicopathological features. Sheehan *et al* (17) reported that a decreased expression of claudin-1 correlates with high tumor grade and biochemical disease recurrence in prostate carcinomas. Resnick *et al* (18) showed that a low expression level of claudin-1 is associated with a higher tumor grade and recurrence in patients with colorectal cancer. In our study, claudin-1 expression was associated with the histological type. As for claudin-3 and -4, Sheehan *et al* (17) reported that the expression of claudin-3 correlates with advanced-stage tumors and recurrence, whereas the expression of claudin-4 correlates with only advanced-stage tumors. Pan *et al* (15) found a slight though insignificant trend towards positive associations of claudin-3 and -4 levels with tumor grade and disease stage in patients with endometrial carcinoma. Our study found no significant relationship between the expression level of the *claudin-3* gene and any clinicopathological feature. The expression of the *claudin-4* gene correlated with only tumor location. As for claudin-7, Kominsky *et al* (10) reported that the loss of claudin-7 expression is associated with nodal metastasis in primary breast carcinomas. Sauer *et al* (19) found that a reduced expression of claudin-7 correlates with metastatic disease in breast carcinoma. Usami *et al* (12) demonstrated that a reduced expression of claudin-7 correlates with metastasis in squamous cell carcinoma of the esophagus. In our study, a reduced expression of the *claudin-7* gene correlated with venous invasion and liver metastasis in colorectal cancer.

When expression levels of the *claudin-1*, *-3*, *-4* and *-7* genes were contrasted with the presence or absence of lymph node metastasis, no correlation was noted for any gene. We also examined potential correlations of gene expression levels with the presence or absence of venous invasion. Sauer *et al* (19) reported that a reduced expression of claudin-7 correlates with metastatic disease. Usami *et al* (12) found that a reduced expression of claudin-7 correlates with tumor invasion in squamous cell carcinoma of the esophagus. In our study, *claudin-3* and *-7* gene expression levels were higher in the absence than in the presence of venous invasion. This finding suggested that reduced *claudin-3* or *-7* gene expression levels might contribute to venous invasion in colorectal cancer.

We then examined correlations among *claudin-1*, *-3*, *-4* and *-7* gene expression in colorectal cancers. Expression of the *claudin-3* gene was found to correlate with that of the *claudin-4* gene.

In conclusion, our results show that a reduced expression of the *claudin-7* gene correlates with venous invasion and liver metastasis in colorectal cancer. Reduced levels or the absence of claudin-7 expression may thus be a novel marker or predictor of metastasis.

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Preoperative Serum Carcinoembryonic Antigen Level as a Predictive Factor of Recurrence After Curative Resection of Colorectal Cancer

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Background: We evaluated the prognostic value of the preoperative serum carcinoembryonic antigen (CEA) level in patients with colorectal cancer (CRC).

Patients and Methods: The study group comprised 638 patients. The optimal cutoff value for the preoperative serum CEA level was determined. Predictive factors of recurrence were evaluated using multivariate analyses. The relapse-free time was investigated according to the CEA level.

Results: All patients underwent potentially curative resection for CRC without distant metastasis, classified as stage I, II, or III. The optimal cutoff value for preoperative serum CEA level was 10 ng/ml. Elevated preoperative serum CEA level was observed in 92 patients. Multivariate analysis identified tumor–node–metastasis (TNM) stage and preoperative serum CEA level as independent predictive factors of recurrence. The relapse-free survival between CEA levels >10 ng/ml and <10 ng/ml significantly differed in patients with stage II and III. However, there was no significant difference in relapse-free survival between CEA levels >10 ng/ml and <10 ng/ml in patients with stage I.

Conclusion: Preoperative serum CEA is a reliable predictive factor of recurrence after curative surgery in CRC patients and a useful indicator of the optimal treatment after resection, particularly for cases classified as stage II or stage III.

Colorectal cancer (CRC) is a common malignancy and the second commonest cause of cancer-related death in Japan; it was estimated that >92,000 new cases of CRC occurred in the year 2000 and that >40,000 people died of the disease in 2004.[1] The Japanese Society for Cancer of the Colon and Rectum reported 5-year survival rates, in 2004, of 94.3%, 90.6%, 81.2%, 71.4%, 56.0%, and 13.2%, respectively, for cases classified as stage 0, I, II, IIIa, IIIb, and IV based on the *Japanese General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum, and Anus*.[2] To further improve survival rates, it is important to identify predictive factors for relapse. Based on the predictive factors, it may be possible to improve survival by some treatments.

The International Union against Cancer (UICC) tumor–node–metastasis (TNM) classification is recognized as the best predictor of outcome, and precise

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staging is necessary to treat CRC. No residual tumor should be left if CRC is diagnosed as capable of potentially curative resection. Advances in chemotherapy for CRC have led to the development of several regimens to prevent relapse. Since the early 1990s, adjuvant chemotherapy with a 5-fluorouracil (5-FU) plus leucovorin (LV) regimen has been recognized as standard therapy for patients with UICC stage III colon cancer, and has resulted in a 30% decrease in the relapse rates compared with surgery alone.[3–6] Recently, the addition of oxaliplatin to the 5-FU/LV therapy regimen (the MOSAIC regimen) or the capecitabine regimen has further improved patient outcomes, and these approaches are being accepted as a new standard of care.[7,8] In cases of recurrence after colorectal surgery, several reports have indicated that a 5-FU-based regimen with the addition of oxaliplatin (FOLFOX) or irinotecan (FOLFIRI), or monoclonal antibodies such as cetuximab and bevacizumab, can improve patient outcome.[9–11] Thus, identification of predictive factors for recurrence and early detection of relapse are crucial to improve CRC treatment.

Several reports have suggested that the postoperative serum CEA level is a useful marker of recurrence after colorectal surgery.[12–15] Moreover, it can be measured cheaply and easily. Monitoring of the postoperative CEA level is thus commonly used in the follow-up of CRC patients.[15–19] However, there has been some controversy about the significance of the preoperative CEA level as a predictive factor of recurrence.[20–22] Furthermore, few previous reports have considered optimal cutoff values for CEA levels.[23,24]

The current study evaluated the optimal cutoff value for the CEA level in patients with CRC, and its efficacy as a predictive factor of recurrence.

PATIENTS AND METHODS

Between April 1992 and February 2003, 816 patients underwent colorectal surgery at the Gastroenterological Centre of Yokohama City University Medical Centre, Japan. Of these, a series of 638 patients with stage I, II, and III received potentially curative colorectal surgery. Curative resection was defined by the absence of any gross residual tumor from the surgical bed and a surgical resection margin that was pathologically negative for tumor invasion. One hundred seventy-eight patients were excluded from the present study because of stage IV (n = 150) and noncurative resection (n = 28). Data were retrieved from operative and pathological reports. Follow-up data were obtained from the outpatient clinical database.

The study group comprised 380 men and 258 women aged 26–92 years (median and quartiles 64 and 57–71 years, respectively). Patients with macroscopic peritoneal metastasis, positive peritoneal lavage cytology, unresectable bulky tumor, or distant metastasis were excluded from the study. Of these 638 patients, 169 were subsequently classified as stage I, 221 as stage II, and 248 as stage III. After 1997, 185 patients with good performance status who gave informed consent received adjuvant chemotherapies. Starting 12 weeks after curative surgery, pyrimidine-fluoride-based regimens were mainly used for 1–2 years in patients classified as stage III.

Preoperative imaging studies were routinely performed following a barium enema and colorectal fiber examination, using abdominal ultrasonography (US) and computed tomography (CT) to determine the location, macroscopic appearance, diameter, and depth of invasion of the tumor, as well as lymph node metastasis and distant metastasis.

Staging was principally based on the UICC/TNM classification of CRC. Experienced pathologists from our institution participated in this study and maintained the quality of the diagnosis. Of the 638 registered patients, 336 had tumors located in the colon and 302 had tumors located in the rectum. The pathologic tumor diameter indicated the maximum microscopic length of the tumor irrespective of the depth. Differentiated tumors were histologically observed in 578 patients, and undifferentiated tumors were seen in 60 patients. Lymphatic invasion was observed in 273 patients, and vascular invasion was observed in 362 patients.

All patients were followed up every 12–16 weeks for at least 5 years according to our standard protocol, which included tumor-marker studies, CT, colorectal fiber examinations, US, and chest radiography. Bone scans were performed when bone metastasis was indicated. The development of new or recurrent metastatic lesions following surgery was defined as a postoperative relapse. Median follow-up time was 78.9 ± 38.5 months for all registered patients. The study was retrospective and neither randomized nor controlled.

Detection of Serum CEA

Serum CEA was measured preoperatively by an Elecsys CEA electrochemiluminescence assay on a Modular Analytics E170 system (Roche Diagnostics

K.K, Tokyo, Japan). The normal range for serum CEA is 0–4.9 ng/ml at our institution.

Statistical Analysis

All data were analyzed using SPSS software version 10.0 for Windows (SPSS Inc., Chicago, IL). The clinical endpoint of this study was overall relapse-free survival. Relapse-free survival was calculated using the Kaplan–Meier estimation method and examined by the log-rank test. The chi-square test was used to evaluate the differences in proportions and the Student's *t*-test was used to evaluate the continuous variables. All data were expressed as the mean ± standard deviation SD. A multivariate analysis using a stepwise forward Cox proportional hazards regression procedure was performed for relapse-free survival. In this analysis, nine variables were employed as follows: age, sex, tumor location, tumor diameter, histological type, lymphatic invasion, hematological invasion, preoperative CEA level, and TNM stage. Probability (*P*) values were considered statistically significant at the 0.05 level. The clinicopathological terminology principally followed the UICC/TNM classification.

RESULTS

Stratification of Preoperative Serum CEA Level

To confirm the optimal classification of the serum CEA level, time to relapse was calculated at 5 ng/ml intervals. The relapse-free survival was compared between the groups with lower and higher CEA levels at each threshold. Multivariate Cox proportional hazards regression was used to compare the time to relapse between the two groups. The preoperative serum CEA level with the highest chi-square value was regarded as the optimal critical point of classification. The most significant difference in relapse-free survival was detected at a threshold value of 10 ng/ml [χ^2 = 35.310, hazard ratio (95% confidence interval) = 3.210 (2.185–4.715), $P < 0.0001$; Table 1]. The critical cutoff value of the CEA level was thus defined as 10 ng/ml.

Comparison of Clinicopathological Factors Between Patients with CEA Levels <10 ng/ml and >10 ng/ml

There were significant differences in tumor diameter, lymphatic invasion, and TNM stage between the two groups. The high CEA patients tended to have a larger tumor diameter, higher incidence of lymphatic invasion, and more advanced TNM stage (Table 2). There was no significant difference in the application of adjuvant chemotherapies between the two groups.

TABLE 1. *χ^2 values and hazard ratios according to serum CEA levels calculated by the Cox proportional regression hazard model*

Threshold (ng/ml)	χ^2	Hazard ratio (95% CI)	*P* value
<5, ≥5	27.505	2.631 (1.833–3.776)	<0.001
<10, ≥10	35.310	3.210 (2.185–4.715)	<0.001
<15, ≥15	30.941	3.201 (2.137–4.884)	<0.001
<20, ≥20	18.670	2.882 (1.783–4.657)	<0.001
<25, ≥25	18.379	3.073 (1.839–5.134)	<0.001
<30, ≥30	16.738	3.203 (1.834–5.594)	<0.001
<40, ≥40	4.314	2.138 (1.044–4.381)	0.038

CI, confidence interval.

TABLE 2. *Patient characteristics according to serum CEA levels*

Variables	CEA (<10 ng/ml) (*n* = 546)	CEA (≥10 ng/ml) (*n* = 92)	*P* value
Age (years)			
<75/≥75	473/73	79/13	0.843
Sex			
Male/female	321/225	59/33	0.334
Tumor diameter (cm)			
<5/≥5	344/202	32/60	<0.001
Location			
Colon/rectum	297/249	45/47	0.381
Histologic type			0.365
Differentiated	497	81	
Undifferentiated	49	11	
Lymphatic invasion			
Absence/presence	243/303	30/62	0.031
Vascular invasion			
Absence/presence	317/229	45/47	0.101
UICC/TNM staging			
I/II/III	163/185/198	6/36/50	<0.001
Curability			
R0/R1	533/13	87/5	0.102
Recurrence			
Absence/presence	462/84	54/38	<0.001

Comparison of Relapse-Free Survival According to Stage and Pattern of Recurrence Between Patients with CEA <10 ng/ml and >10 ng/ml

Overall, there were significant differences in relapse-free survival between the two groups (Fig. 1). There was no significant difference in relapse-free survival among patients classified as stage I (Fig. 2). However, there were significant differences among those classified as stage II and stage III ($P = 0.036$ and $P < 0.001$, respectively; Figs. 3 and 4). Recurrence was observed in 38 (41.3%) patients with CEA

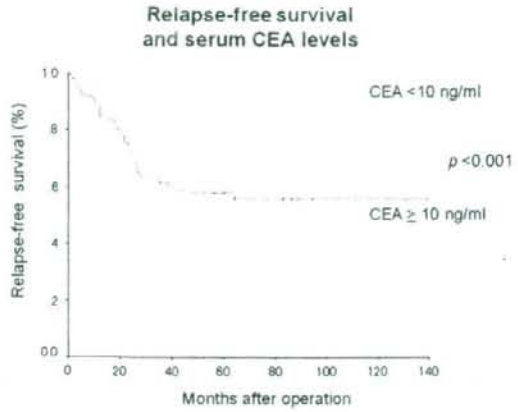

FIG. 1. Relapse-free survival and serum CEA levels. Comparison of relapse-free survival according to serum CEA level.

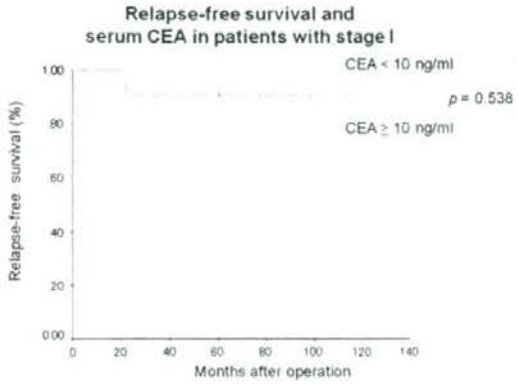

FIG. 2. Relapse-free survival and serum CEA in patients with stage I.

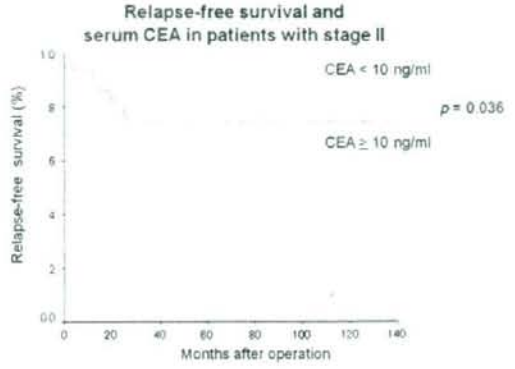

FIG. 3. Relapse-free survival and serum CEA in patients with stage II.

FIG. 4. Relapse-free survival and serum CEA in patients with stage III.

>10 ng/ml and in 84 (15.4%) patients with CEA <10 ng/ml ($P < 0.001$). However, there was no significant difference in the pattern of recurrence between the two groups. The local recurrence rates were 23.7% for CEA >10 ng/ml and 14.3% for CEA <10 ng/ml. The distant metastasis rates were 76.3% and 84.5%, respectively (Table 3).

Prognostic Factors for Relapse-Free Survival

The multivariate analysis showed that preoperative serum CEA level and TNM stage independently affected relapse-free survival. However, age, sex, tumor location, tumor diameter, lymphatic invasion, vascular invasion, and microscopic appearance were not independent prognostic factors on multivariate analysis (Table 4).

DISCUSSION

Serum CEA was originally reported in 1965 by Gold and Freedman.[22] This factor is cheap and easy to measure, and postoperative CEA is commonly assessed in the follow-up of CRC patients.[15,17-20] However, there has been controversy about the significance of the preoperative CEA level as a predictive factor of recurrence,[20-22] and only a few reports have evaluated optimal cutoff values.[23,24] Some previous reports have defined 5 ng/ml as the cutoff value for

TABLE 3. *Prognostic factors for relapse-free survival*

	Multivariate Cox regression result	
Variable	Hazard ratios (95% CI)	*P* value
Serum CEA level (ng/ml)		
<10/≥10	3.064 (1.839–5.105)	<0.001
UICC/TNM staging		
II/I	6.210 (1.281–7.924)	0.013
III/I	7.225 (3.792–21.584)	<0.001

CI, confidence interval.

TABLE 4. *Patterns of recurrence according to preoperative serum CEA levels*

			$n = 122$
	CEA (<10 ng/ml) ($n = 84$)	CEA (≥10 ng/ml) ($n = 38$)	*P* value
			0.214
Local recurrence	12 (14.3%)	9 (23.7%)	
Anastomotic	2	2	
Pelvic or tumor bed	6	4	
Nodal	4	3	
Distant metastasis	71 (84.5%)	29 (76.3%)	
Hepatic	41	11	
Pulmonary	35	17	
Peritoneal	8	2	
Osseous	7	2	
Brain	5	3	

One patient had an unknown recurrence pattern.

the CEA level.[16,25–27] However, applying this cutoff value selects too many patients (approximately 25%) as high risk (in the current report, 24.6% of patients had CEA >5 ng/ml). Moreover, in our study, the most significant difference in relapse-free survival was detected at a threshold value of 10 ng/ml. However, it is necessary to validation of the optimal threshold in an independent patient group. In the current study, 14.4% of patients had CEA level >10 ng/ml. Moreover, when we limited the analysis to patients with CEA >5 ng/ml, the optimal cutoff value as a predictive factor for relapse was also 10 ng/ml (data not shown). These results confirm that a cutoff value of 10 ng/ml is a more powerful prognostic factor for recurrence than the usual value of 5 ng/ml.

Even after potentially curative resection, patients with CEA > 10 ng/ml showed a high rate of recurrence (41.3%) compared with patients with CEA < 10 ng/ml (15.4%). However, there was no significant difference in relapse-free survival among patients classified as stage I. This might have resulted from the relatively small number of patients with high CEA levels among those classified as stage I ($n = 6$). However, this result suggested that patients classified as stage I could only be treated surgically.

Patients classified as having high and low preoperative CEA levels showed significant differences in relapse-free survival in stages II and III. These findings suggested that the patients with high CEA levels might have harbored undetectable distant metastatic disease around the time of the operation. Julia et al. reported that, of stage I or II patients, 32.8% tested positive for disseminated tumor cells after surgery, and patients who were marker-positive for disseminated cells in post-resection lavage samples showed significantly poorer prognosis.[28] These results suggested that, even though curative resection was performed, residual tumor cells were present. Furthermore, Sadahiro et al. reported that the presence of CEA messenger RNA-expressing cells in peripheral blood 7 days after curative surgery was a novel independent factor predicting recurrence in patients with CRC.[29] These reports suggested that conventional staging alone was not suitable for the postoperative treatment of such patients.

Although relapse-free survival was compared according to the preoperative serum level of CEA in this study, it is also important to compare disease-specific and overall survivals in these patients. Disease-specific survival and overall survival significantly differed between patients with CEA < 10 ng/ml and CEA > 10 ng/ml (5-year survival, 90.7% versus 77.2%, $P = 0.002$; 84.8% versus 72.2%, $P = 0.005$, respectively).

In the current study, 547 patients had CEA <10 ng/ml and 81 patients relapsed. We investigated the predictive factors of recurrence in these patients. Multivariate analysis showed that only TNM stage was a significant factor ($P < 0.001$), while lymphatic invasion tended to be a predictive factor for recurrence ($P = 0.061$ and data not shown). There was no difference in recurrence pattern between the two groups. Multivariate analysis identified the TNM stage and the preoperative CEA level as predictive factors for relapse. Previously, several reports suggested that preoperative CEA was a significant prognostic factor only in patients classified as stage II or stage III.[5,16,27] Park and Lee also mentioned the CEA level of 10 ng/ml as the cutoff value to predict recurrence in Dukes' C rectal cancer patients. They concluded that adjuvant therapy should be administered in patients with elevated preoperative serum CEA level.[30] Our results support their findings.

In 1993, the National Surgical Adjuvant Breast and Bowel Project (NSABP) reported the results of a surgical adjuvant clinical trail that indicated signifi-

cant prolongation of both disease-free survival and overall survival in stage II and III colon cancer patients who received FU plus LV compared with patients who received semustine, vincristine, and FU.[5] Following on from this work, there have been several studies comparing the efficacy of different regimens for adjuvant chemotherapy after curative resection of CRC. Recently, the European MOSAIC trial reported the efficacy of infused 5-FU, leucovorin, and oxaliplatin (FOLFOX4) compared with 5-FU/LV in an adjuvant setting in 2,246 patients with completely resected stage II and III colon cancer.[7] Based on the results, FOLFOX4 has been recommended as a treatment for early-stage colon cancer in the National Comprehensive Cancer Network (NCCN) guidelines.

However, even though these chemotherapies have been shown to be effective, they remain costly. There have been several reported economic analyses of adjuvant chemotherapy for CRC.[31–33] Adjuvant chemotherapy has been accepted as the standard treatment for stage III CRC. However, adjuvant chemotherapy for stage II CRC remains controversial and is not routinely recommended to all patients. According to the current American Society of Clinical Oncology guidelines, the criteria used to indicate adjuvant chemotherapy are poorly differentiated histology, T4 lesions, bowel perforation, and inadequate number of sampled lymph nodes (<13).

It is important to identify patients who are at high risk of relapse, especially in stage II. Our study suggests that a cutoff value for preoperative CEA of 10 ng/ml is a powerful marker of postoperative relapse. In patients with a high CEA level, adjuvant chemotherapy such as FOLFOX4 should also be recommended.

The introduction of chemotherapy for recurrence caused clinical bias when the outcome measure was overall survival time; we therefore used disease-free survival time as the outcome in the current study. After 1997, 185 patients receiving adjuvant chemotherapy were included in the study; however, we detected no significant difference due to the use of adjuvant chemotherapy based on the preoperative CEA levels. By restricting the outcome to disease-free survival time, this report provided additional evidence that the preoperative CEA level is a useful marker.

This study included relatively older patients for a long duration. Therefore, a further study of high-volume patients will be necessary to identify the optimal classification for preoperative CEA level in CRC.

In conclusion, the preoperative serum CEA level in patients with CRC, which can be measured easily prior to surgery, is a reliable predictive factor of recurrence. This measure might therefore be a candidate for use in the staging system, in addition to conventional factors such as lymph node metastasis or depth of invasion, and will be useful for treatment planning in patients undergoing curative resection of CRC, especially those classified as stage II or III.

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Clinicopathological significance of the gene expression of matrix metalloproteinases and reversion-inducing cysteine-rich protein with Kazal motifs in patients with colorectal cancer: *MMP-2* gene expression is a useful predictor of liver metastasis from colorectal cancer

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Abstract. Matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9) and membrane-type matrix metalloproteinase 1 (MT1-MMP) are involved in colorectal cancer invasion and metastasis. Reversion-inducing cysteine-rich protein with Kazal motifs (RECK) inhibits MMP-2, MMP-9 and MT1-MMP. We examined the clinicopathological significance of the relative expression of these genes in patients with colorectal cancer, especially with regard to metastasis. We studied surgical specimens of cancer tissue and adjacent normal mucosa obtained from 205 patients with untreated colorectal carcinoma. *MMP-2*, *MMP-9*, *MT1-MMP*, *RECK* and *β-actin* mRNA of cancer tissue and adjacent normal mucosa were measured by quantitative real-time reverse-transcriptase polymerase chain reaction. *MT1-MMP* gene expression was higher in cancer tissue than in adjacent normal mucosa. In contrast, *MMP-2*, *MMP-9* and *RECK* gene expression levels were lower in cancer tissue than in adjacent normal mucosa. As for the relationship between the gene expression and clinicopathological factors, *MMP-2* expression correlated with the depth of invasion, venous invasion and liver metastasis; *MMP-9* and *RECK* expression correlated with venous invasion. There were positive correlations among the gene expression levels of *MMP-2*, *MMP-9* and *RECK*. *MMP-2* gene expression was considered a useful predictor of liver metastasis from colorectal cancer.

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Key words: matrix metalloproteinase-2, matrix metalloproteinase-9, membrane-type matrix metalloproteinase 1, Kazal motifs, colorectal cancer

Introduction

Colorectal cancer, one of the most prevalent cancers worldwide (1), is the second leading cause of cancer-related mortality in developed countries (2). Tumor cell invasion and metastasis involve multiple steps, including proteolytic degradation of the basement membrane (BM) and extracellular matrix (ECM), altered cell adhesion and the physical movement of tumor cells. Among the many steps of tumor invasion and metastasis, excessive degradation of the matrix is one of the hallmarks of this process (3).

Matrix metalloproteinases (MMPs) are a key family of proteolytic enzymes involved in extracellular matrix degradation. In colorectal cancer, several MMPs have been found to be associated with tumor stage, prognosis, or both (4). Matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) have been implicated in the progression, invasion and metastasis of colorectal cancer in animal models and patients (5). MMP-2 and MMP-9 can degrade denatured collagen and type IV, V, VII, IX and X collagens. Type IV collagen is particularly abundant in basement membranes. These gelatinases are now also thought to be involved in cell differentiation, apoptosis, angiogenesis, immune response and cancer cell growth (6). The reversion-inducing cysteine-rich protein with Kazal motifs (RECK) gene was originally

Table I. PCR primers and conditions.

Gene	Primer	Temperature (C)	Product size (bp)
MMP-2	5'-CCCTCCCTTCAACCATTCCC-3' 5'-TTCCAGCAGACACCATCACC-3'	55.6	186
MMP-9	5'-TGGTCCTGGTGCTCCTGGTG-3' 5'-GCTGCCTGTCGGTGAGATTGG-3'	61.2	111
MT1-MMP	5'-AAGAGGAGAAGAGCAAACAG-3' 5'-CGGTAGGCACTGAACTTG-3'	55.1	91
RECK	5'-ACTGCCGAGAATACTGTCAAGCC-3' 5'-ACTATCCGTTGGGTTCCTCATTGG-3'	64.9	161
ß-actin	5'-AGTTGCGTTACACCCTTTCTTGAC-3' 5'-GCTCGCTCCAACCGACTGC-3'	60.0	171

discovered in an expression cloning screen designed to isolate the transformation of suppressor genes against activated ras oncogenes (5,7,8). The RECK gene encodes a membrane-anchored glycoprotein and is down-regulated during the malignant conversion of cells (9). Although RECK is widely expressed in normal tissues and non-neoplastic cell lines, its expression is strongly suppressed in oncogene-transformed fibroblasts and several tumor-derived cell lines (9,10). RECK inhibits MMP-2, MMP-9 and membrane-type matrix metalloproteinase 1 (MT1-MMP) secretion and activity, suggesting that it participates in the regulation of MMPs and tumor invasiveness (11). RECK is also vital to developmental vasculogenesis and its down-regulation has been implicated in tumor angiogenesis and progression (9,11,12).

In this study, we examined the clinicopathlogical significance of the relative expression of these genes in patients with colorectal cancer, especially with regard to metastasis.

Materials and methods

Patients and samples. We studied surgical specimens of cancer tissue and adjacent normal mucosa obtained from 205 patients with untreated colorectal carcinoma. The patients underwent surgery at the Yokohama City Medical Center, Gastroenterological Center and Kanagawa Cancer Center between 2002 and 2006. Informed consent was obtained from each patient and the Yokohama City Medical Center Committee and Kanagawa Cancer Center Committee approved the study. Each sample was embedded in O.C.T. compound (Sakura Finetechnical Co., Ltd., Tokyo) and stored at -80˚C, immediately before use. The patients had no other form of malignancy. After examining the histopathological features of specimens stained with hematoxylin and eosin, sections including >80% carcinoma cells were used for total RNA preparation.

Quantitative real-time reverse-transcriptase polymerase chain reaction (PCR). Total RNA from colorectal cancer tissue and adjacent normal mucosa was prepared with the use of Trizol (Gibco, Life Tech, Gaithersburg, MD). cDNA was synthesized from 2 μg of total RNA using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). After synthesis, the cDNA was diluted 1:4 with water and stored at -20˚C until use. Quantitative real-time PCR was performed with iQ SYBR-Green Supermix (Bio-Rad Laboratories). PCR reactions were carried out in a total volume of 15 μl, containing cDNA derived from 75 ng of RNA, 0.27 μM of each primer, 7.5 μl of iQ SYBR-Green Supermix containing dATP, dCTP, dGTP and dTTP at a concentration of 400 μM each and 50 U/ml of iTag DNA polymerase. The PCR consisted of 10 min at 94˚C followed by 50 cycles of denaturation of the cDNA for 30 sec at 94˚C, annealing for 30 sec at an appropriate temperature according to Table I and a primer extension for 1 min at 72˚C, followed by 72˚C for 10 min. The PCR primer sequences of MMP-2, MMP-9, MT1-MMP, RECK and ß-actin, used as an internal control are shown in Table I.

Statistical analysis. Associations of the gene expression levels of colorectal cancer with those of adjacent normal mucosa were evaluated by the Wilcoxon test. The relationship between the gene expression levels and potential explanatory variables, including age, gender, tumor size, histological type, depth of invasion, lymph node metastasis, location, lymphatic invasion, venous invasion and liver metastasis, were assessed with the χ^2 test. Associations among variables were evaluated with the Mann-Whitney U test. Correlation coefficients between different variables were determined by a simple regression analysis. Statistical analyses were performed using Statview J 5.0 software (Abacus, CA). Two-sided P-values were calculated and P-values <0.05 were considered to indicate statistical significance.

Results

Comparison of MMP-2, MMP-9, MT1-MMP and RECK mRNA expression between colorectal cancer tissue and adjacent normal mucosa. *MMP-2*, *MMP-9* and *RECK* gene expression levels were lower in cancer tissue than in adjacent normal mucosa (P=0.004, 0.001 and 0.006; Fig. 1A, B and D). In contrast, *MT1-MMP* gene expression in cancer tissue was higher than that in adjacent normal mucosa (P=0.038; Fig. 1C).

Relationship between clinicopathological features to MMP-2, MMP-9, MT1-MMP and RECK gene expression levels. After

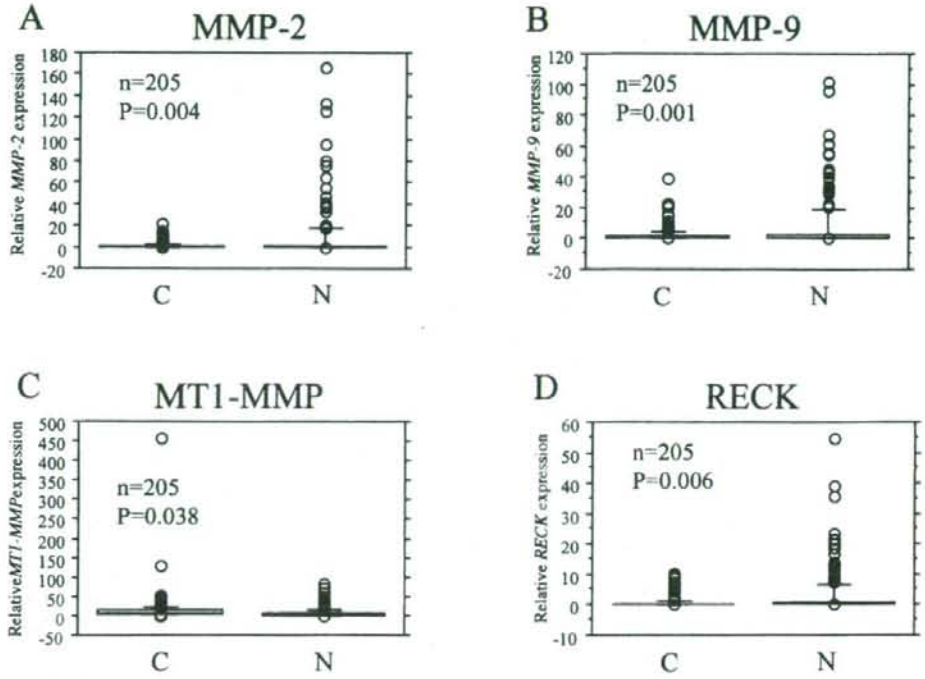

Figure 1. Comparison of *MMP-2*, *MMP-9*, *MT1-MMP* and *RECK* mRNA expression between colorectal cancer tissue and adjacent normal mucosa. *MMP-2*, *MMP-9* and *RECK* gene expression levels were higher in adjacent normal mucosa than in cancer tissue (P=0.0462, 0.0488 and 0.0491). However, the *MT1-MMP* gene expression level did not differ significantly between cancer tissue and adjacent normal mucosa.

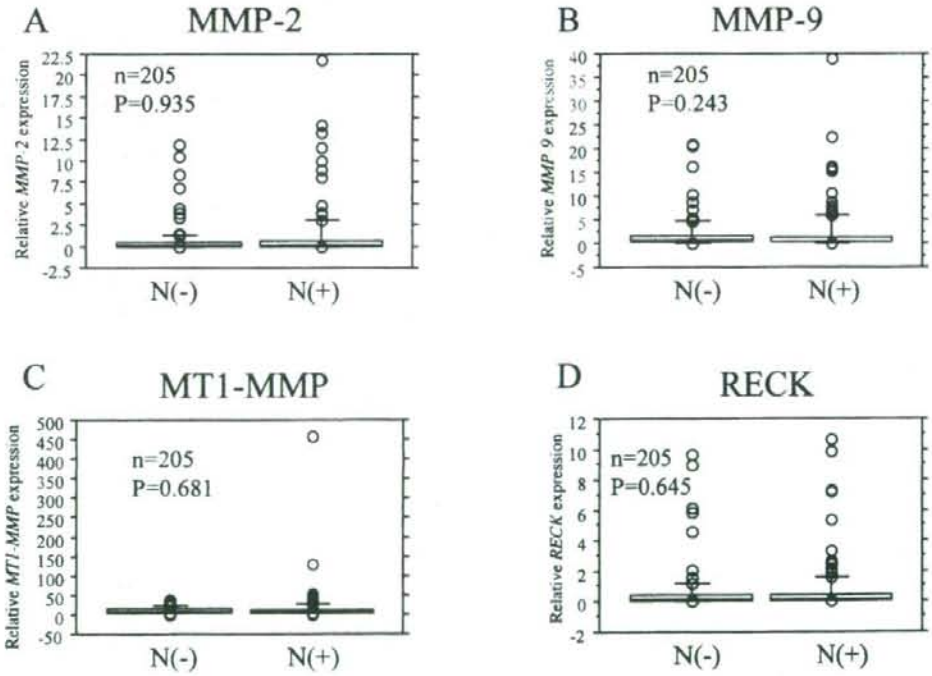

Figure 2. Association of *MMP-2*, *MMP-9*, *MT1-MMP* and *RECK* gene expression with lymph node metastasis in 205 patients with colorectal cancer. Box boundaries, the 25th and 75th percentiles of the observed values; capped bars, the 10th and 90th percentiles; solid line, the median. P-values were assessed by the Mann-Whitney U test. The presence or absence of lymph node metastasis was unrelated to the expression level of any gene.

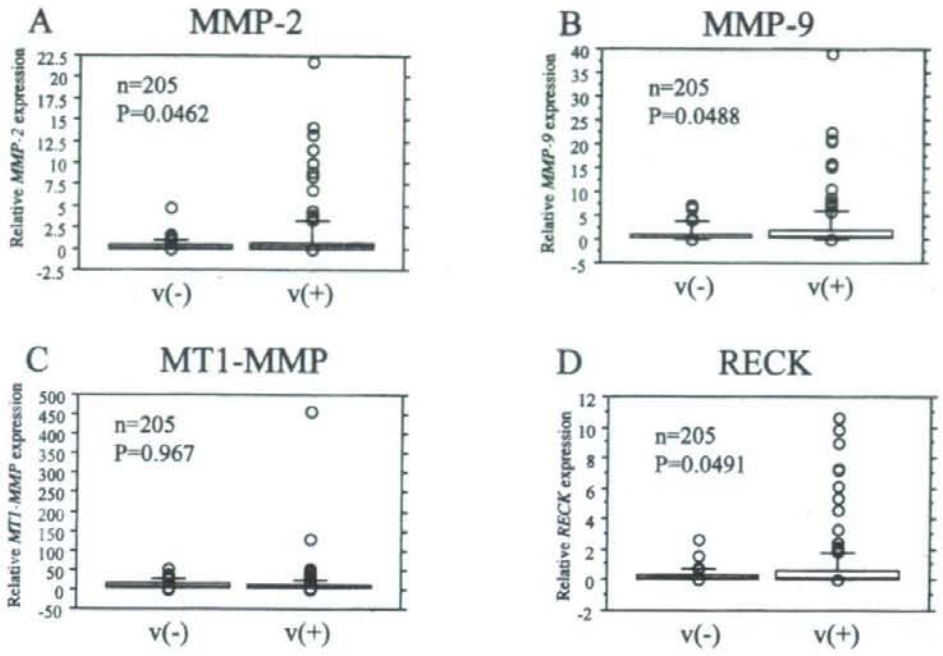

Figure 3. Association of *MMP-2*, *MMP-9*, *MT1-MMP* and *RECK* gene expression with venous invasion in 205 patients with colorectal cancer. Box boundaries, the 25th and 75th percentiles of the observed values; capped bars, the 10th and 90th percentiles; solid line, the median. P-values were assessed by the Mann-Whitney U test. The presence and absence of venous invasion was significantly related to the gene expression levels of *MMP-2*, *MMP-9* and *RECK*.

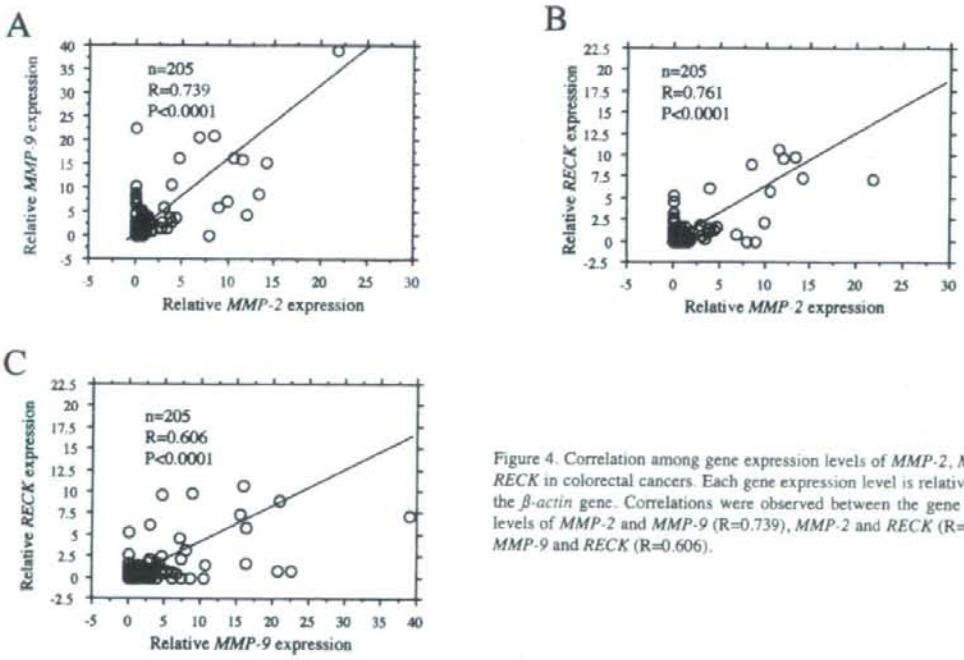

Figure 4. Correlation among gene expression levels of *MMP-2*, *MMP-9* and *RECK* in colorectal cancers. Each gene expression level is relative to that of the *β-actin* gene. Correlations were observed between the gene expression levels of *MMP-2* and *MMP-9* (R=0.739), *MMP-2* and *RECK* (R=0.761) and *MMP-9* and *RECK* (R=0.606).

categorizing expression levels of *MMP-2*, *MMP-9*, *MT1-MMP* and *RECK* genes as low or high according to their respective median values, we examined the relationship between the expression levels of each gene and clinicopathological features. *MMP-2*, *MMP-9*, *MT1-MMP* and *RECK* gene expression levels were unrelated to age, gender, tumor size, histological type, lymph node metastasis, tumor location and lymphatic invasion. *MMP-2* expression was significantly

Table II. The relationship between the expression of MMP-2, MMP-9, MT-MMP or RECK genes and clinicopathological features.

Variables/categories	MMP-2 expression			MMP-9 expression			MT1-MMP expression			RECK-7 expression		
	low (n=103)	high (n=102)	P-value	low (n=103)	high (n=102)	P-value	low (n=102)	high (n=103)	P-value	low (n=103)	high (n=102)	P-value
Age	66.6±10.2	65.0±11.3	0.294	66.2±10.6	65.4±10.9	0.586	65.9±11.3	65.2±10.2	0.929	64.9±11.9	66.7±9.5	0.229
Gender												
Male	52	60	0.231	57	55	0.838	53	59	0.444	54	58	0.523
Female	51	42		46	47		49	44		49	44	
Size												
≤5 cm	59	56	0.731	60	55	0.532	61	54	0.287	60	55	0.532
>5cm	44	46		43	47		41	49		43	47	
Histological type												
Well differentiated	32	31	0.995	31	32	0.395	31	31	0.495	28	33	0.492
Moderately differentiated	57	57		61	53		59	55		62	53	
Poorly differentiated	14	14		11	17		11	17		13	16	
Depth of invasion												
T1	16	3	0.018	11	8	0.272	12	7	0.455	10	9	0.337
T2	46	48		50	44		49	45		53	41	
T3	36	44		39	41		36	44		34	46	
T4	5	7		3	9		5	7		6	6	
Lymph node metastasis												
Absent	51	44	0.360	43	52	0.185	47	48	0.940	49	46	0.722
Present	52	58		60	50		55	55		54	56	
Location												
Colon	61	51	0.185	58	54	0.628	59	53	0.401	60	52	0.296
Rectum	42	51		45	48		44	50		43	50	
Lymphatic invasion												
Absent	70	64	0.490	70	64	0.490	72	63	0.155	67	68	0.807
Present	33	37		33	37		30	40		36	34	
Venous invasion												
Absent	48	30	0.011	47	31	0.025	43	35	0.228	47	31	0.025
Present	55	72		56	71		56	68		56	71	
Liver metastasis												
Absent	77	62	0.032	69	70	0.802	72	67	0.396	70	69	0.962
Present	26	40		34	32		30	36		33	33	

related to the depth of invasion (P=0.018). *MMP-2*, *MMP-9*, and *RECK* gene expression levels were significantly related to venous invasion (P=0.011, 0.025 and 0.035). *MMP-2* expression was also significantly related to liver metastasis (P=0.032) (Table II).

Comparison of MMP-2, MMP-9, MT1-MMP and RECK gene expression levels according to the presence and absence of lymph node metastasis. There were no significant differences in *MMP-2*, *MMP-9*, *MT1-MMP* and *RECK* gene expression levels according to the presence or absence of lymph node metastasis (Fig. 2).

Comparison of MMP-2, MMP-9, MT1-MMP and RECK gene expression levels according to the presence or absence of venous invasion. *MMP-2*, *MMP-9* and *RECK* gene expression levels differ significantly according to the presence or absence of venous invasion (P=0.0462, 0.0488 and 0.0491) (Fig. 3).

Correlation among MMP-2, MMP-9 and RECK expression. The results of a correlation analysis are shown in Fig. 4. Correlations were observed between the gene expression levels of *MMP-2* and *MMP-9* (R=0.739), *MMP-2* and *RECK* (R=0.761) and *MMP-9* and *RECK* (R=0.606) (Fig. 4).

Discussion

MMP-2 and MMP-9 play key roles in the development and progression of human malignancies (13-15). These matrix metalloproteinases mediate the destruction of extracellular matrix and are considered an important early step in tumor invasion and metastasis. MMP-2 and MMP-9 also have angiogenic activity and participate in early tumorigenesis and tumor growth, including metastasis (16,17). The over-expression of MT1-MMP in tumor cells promotes growth (18). The RECK gene is believed to regulate multiple MMP family members, such as MMP-2, MMP-9 and MT1-MMP (12).

Several previous studies have compared *MMP-2*, *MMP-9*, *MT1-MMP* and *RECK* mRNA expression levels between colorectal cancer tissue and adjacent normal mucosa. Kim *et al* (19) reported that *MMP-2* and *MMP-9* gene expression levels (n=24) are higher in colorectal cancer than in adjacent normal mucosa. Lubbe *et al* (20) found that the *MMP-9* gene expression level in colorectal cancer (n=28) is higher than that in adjacent normal mucosa. However, in our study (n=205), *MMP-2*, *MMP-9* gene expression levels were higher in adjacent normal mucosa than in cancer tissue. We believe that this result was related to the higher expression of MMP-2 and MMP-9 in interstitial tissues than in cancer cells. Atkinson *et al* (21) showed that the *MT1-MMP* gene expression level is higher in cancer tissue than in adjacent normal mucosa, while Takeuchi *et al* (22) reported that the *RECK* gene expression level is higher in adjacent normal mucosa than in colorectal cancer. In our study, *RECK* gene expression levels were higher in adjacent normal mucosa than in cancer tissue. Conversely, the *MT1-MMP* gene expression level was higher in cancer tissue than in adjacent normal mucosa.

Zheng *et al* (23) studied the relationship between the clinicopathological features and gene expression levels of MMPs. The expression levels of MMP-2 and MMP-9 were found to be closely linked to venous and lymph node invasion. Ogata *et al* (24) reported that MMP-9 expression is related to lymph node metastasis and severe venous invasion. Takeuchi *et al* (22) reported that RECK expression is significantly associated with lymph node metastasis, Dukes' stage and venous invasion. In our study, *MMP-2*, *MMP-9* and *RECK* expression levels were significantly related to venous invasion. *MMP-2* expression was also significantly related to tumor depth and liver metastasis. MT1-MMP has been reported to specifically activate MMP-2 (25). The association of *MMP-2* expression with tumor depth, venous invasion and liver metastasis may be related to the finding that the *MT1-MMP* gene expression level was higher in cancer tissue than in adjacent normal mucosa in our study.

In a study examining interrelations among RECK, MMP-2, and MMP-9, van der Jagt *et al* found that RECK expression levels strongly correlate with the inhibition of MMP-2 enzyme activity, though not with the inhibition of MMP-9 activity (26). Masui *et al* reported a significant negative correlation between RECK activation and MMP-2 activation (27). In our study, correlations were observed between gene expression levels of *RECK* and *MMP-2*, *RECK* and *MMP-9* and *MMP-2* and *MMP-9*. These results demonstrated a positive correlation between the expression of *RECK* and *MMP-2* at the mRNA level, although RECK inhibited MMP-2 activity at the enzyme level.

In conclusion, our study showed that *MMP-2*, *MMP-9* and *RECK* gene expression levels were higher in adjacent normal mucosa than in cancer tissue and correlated with each other. Expression levels of these genes were significantly related to venous invasion. *MMP-2* gene expression is considered a useful predictor of liver metastasis from colorectal cancer.

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Surgical usefulness of indocyanine green as an alternative to India ink for endoscopic marking

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Abstract
Background India ink has been commonly used for preoperative colonic tattooing, but various complications have been reported. This study aimed to evaluate the usefulness of indocyanine green (ICG) marking as a replacement for India ink.
Methods This study enrolled 40 patients who between January 2005 and February 2006 underwent laparoscopic or open surgery for colorectal lesions considered difficult to locate intraoperatively. Because one patient had a history of allergy to iodinated contrast material, metal clipping was used instead of ICG to mark the lesion. Endoscopists injected 5 ml of ICG suspension and saline solution adjacent to the lesion at duplicate locations to evaluate the visibility, duration, and adverse effects of the dye. For 39 patients, the date of the preoperative colonoscopy was not set for examination of the appropriate interval between endoscopic marking and the surgical operation.
Results The median interval between ICG marking and surgery was 4 days (range, 1–73 days). All 29 patients who underwent surgery within 8 days after marking had positive green ICG staining at the time of surgery. After 9 days or more, however, positive staining was seen clearly in only two of the remaining 10 patients. The staining tended to grow weaker and fainter over the time course, eventually dissipating. No perioperative adverse reactions to the dye were observed.
Conclusion This study supports the use of ICG as a safe technique that can be identified reliably during operations performed within 8 days after endoscopic injection.

Keywords Colorectum · Endoscopic marking · India ink · Indocyanine green · Laparoscopic surgery

Tattooing with India ink, first described in 1975 by Ponsky and King [1], has been used commonly before surgery by endoscopists to mark small lesions and those not palpable in the colon and rectum. Clinically relevant complications of the tattooing were considered to be rare [2], but many have been reported, including focal peritonitis, inflammatory pseudotumor, abscess, and postoperative adhesion ileus [3–9], despite sterilization and dilution of the India ink before injection. Moreover, no proof exists to date for the long-term safety of this practice [10]. Several specialists have described new methods to prevent improper injection of India ink into the muscularis propria or peritoneal cavity [11, 12], thus indicating the difficulty that general endoscopists face when injecting the dye properly into the submucosa.

Sterilized, commercially available India ink has been used for colonic tattooing. During 6 months, between July and December 2004, however, three patients presented with severe adhesions and bulky granulomas (Fig. 1A). For these patients, the operation was difficult because the black ink restricted the surgical field of view. Moreover, it was difficult to collect all the ink spread throughout the

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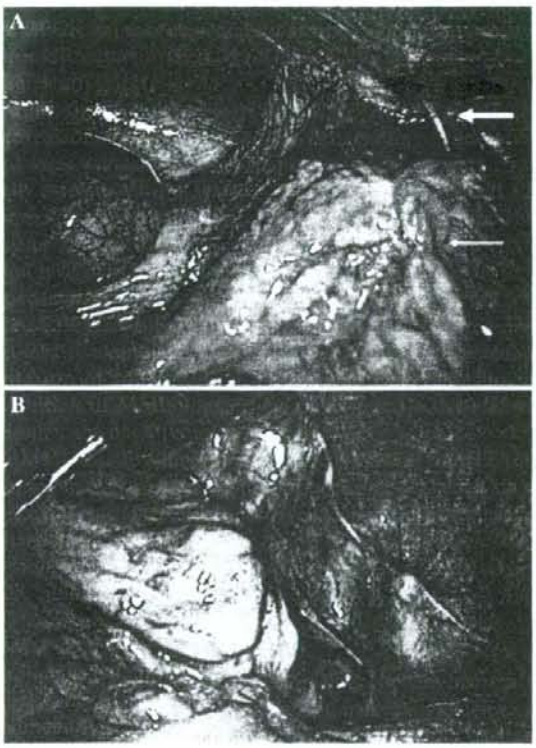

Fig. 1 Subumbilical laparoscopic view. **(A)** Severe adhesion (*thick arrow*) of the ascending colon to the abdominal wall, forming a bulky granuloma with the great omentum (*thin arrow*) 5 days after endoscopic India ink injection. The patient had moderate abdominal pain immediately after the India ink injection without a significant fever. **(B)** Green stain on the rectosigmoid junction with no adhesion 3 days after endoscopic indocyanine green injection

abdominal cavity. Consequently, the India ink will remain in the abdominal cavity of these patients for the rest of their lives. These experiences prompted this clinical study to investigate some other tattooing agents.

Tattooing agents, such as methylene blue, indigo carmine, lympazurine, and indocyanine green (ICG), are reported to dissipate within days and therefore are of limited use clinically [13]. Of these, ICG has been described in some reports of animal models as an alternative candidate to India ink [14–16]. In terms of human cases, one exploratory study explored the surgical usefulness of ICG for colonic marking of 12 patients in 1993 [17].

Although adverse reactions to ICG are known to occur [18], it has been used for more than 40 years in tests of cardiac and hepatic function and recently for sentinel node detection in cancer surgery, with a high level of safety [19, 20]. Therefore, ICG was selected as a marking dye, and this study was conducted, starting in January 2005, to evaluate the visibility, duration, and adverse effects of ICG in a relatively large number of patients.

Patients and methods

This study included 40 consecutive patients who underwent either laparoscopic or open colorectal surgery in the Department of Surgery at the Osaka Medical Center for Cancer and Cardiovascular Diseases between January 2005 and February 2006 for any lesions that may have been difficult to locate intraoperatively. Because one patient had a history of allergy to iodinated contrast material, metal clipping was used instead of ICG to mark the lesion. Written informed consent was obtained from the remaining 39 patients.

The day before surgery, all the patients received mechanical preparations because of the potential need for intraoperative colonoscopy to detect any small lesions. For the 39 patients, the date of the preoperative colonoscopy was not preset. The timing for the colonoscopy depended on the decision of the medical endoscopist and the patient to examine the staining intensity of ICG and to determine the interval between endoscopic marking and the operation. The choice of either laparoscopic or open surgery depended on the discretion of the two colorectal surgeons.

During a preoperative colonoscopy, ICG (Dai-Ichi Pharm Co. Ltd., Tokyo, Japan) was injected adjacent to the lesion by the endoscopist in duplicate on opposite walls using a standard 23-gauge sclerotherapy needle (Top Co. Ltd., Tokyo, Japan) passed through the biopsy channel. The stock solution of ICG was prepared by dissolving 25 mg of powdered ICG in 2 ml of the solvent (sterilized water) provided by the manufacturer. For each injection, 1 ml of the suspension was used.

Endoscopic marking was performed according to a modification of previously reported procedures [11, 12]. First, 2 ml of normal saline solution (Otsuka Pharmaceutical Co., Ltd., Japan) was injected tangentially into the submucosal layer to form a submucosal elevation. Then the syringe used for the saline solution was replaced by another syringe containing ICG, and 1 ml was injected. Subsequently, the ICG syringe was replaced by the first syringe of saline, and about 2 ml of the saline solution was added to push out the ICG remaining in the needle device.

During surgery, the visibility or staining intensity of ICG on the serosa of the colon or the anterior wall of the rectum was evaluated and classified into strong, weak, or none (Fig. 2). Probable complications associated with ICG marking such as focal peritonitis, inflammatory pseudotumor, abscess, or intraoperative adhesions all were recorded.