くるであろう.

おわりに

EORTC40983では術前・術後FOLFOX4の有効性は明確にならなかった. 切除可能肝転移に対する新しい標準治療の確立はJCOG0603をはじめとする今後の国内外臨床試験の結果に委ねられた. 臨床試験の迅速な推進が望まれる.

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Reduced expression of the *claudin-7* gene correlates with venous invasion and liver metastasis in colorectal cancer

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Received October 15, 2007; Accepted December 5, 2007

Abstract. Claudins, members of a large family of adherent junction proteins, regulate the integrity and function of tight junctions and influence tumorigenesis. Studies have suggested that altered levels of different claudins are related to carcinoma-cell invasion and disease progression. This study examined the relationship between the relative expression of claudin genes and clinicopathological factors, especially invasion and metastasis, in patients with colorectal cancer. We studied surgical specimens of cancer tissue and adjacent normal mucosa from 205 patients with untreated colorectal carcinoma. The relative expression levels of *claudin-1*, *-3*, *-4* and *-7* mRNA in cancer and in normal adjacent mucosa were measured by quantitative real-time, reverse-transcription polymerase chain reaction. The relative expression levels of the *claudin-1*, *-3* and *-4* genes were higher in cancer than in normal adjacent mucosa, whereas the relative expression of the *claudin-7* gene was similar. An analysis of the relationship between the clinicopathological features and gene expression showed that reduced expression of *claudin-7* correlated with venous invasion and liver metastasis. There was also a correlation between *claudin-3* and *-4* gene expression. Our results suggested that a reduced expression of the *claudin-7* gene might lead to venous invasion and liver metastasis in colorectal cancer. Reduced expression of the *claudin-7* gene may thus be a useful predictor of liver metastasis in patients with colorectal cancer.

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Key words: claudin-1, claudin-3, claudin-4, claudin-7, colorectal cancer

Introduction

In simple epithelium, tight junctions are positioned at the boundaries of apical and basolateral plasma membranes. These junctions are thought to play an important role in the paracellular barrier and cell polarity (1-4). Several lines of evidence indicate that the granular cell layer of stratified epithelium of the skin possesses tight junctions that are crucial for barrier function (1,2,5,6). The tight junctions consist of membrane and peripheral proteins. Claudins are membrane proteins composed of four transmembrane domains and two extracellular loops, through which they bind to corresponding claudins in cell-to-cell contact. Claudin-1, -3, -4 and -7 are four representative members of the 24-claudin multigene family (4), associated with cancer. An enhanced expression of claudin-1 has been reported in colorectal cancer (7). Ovarian epithelial cells that express claudin-3 and -4 show increased invasiveness *in vitro* (8). Claudin-4 is a potent inhibitor of the invasiveness and phenotype of pancreatic cancer cells (9). The loss of claudin-7 expression has been observed in ductal carcinoma of the breast and squamous cell carcinoma of the head and neck (10,11). Usami *et al* (12) reported that a reduced expression of claudin-7 correlates with tumor invasion and metastasis in squamous cell carcinoma of the esophagus. However, whether the expression of claudin-1, -3, -4 and -7 is associated with the malignant potential of colorectal cancer remains to be clarified.

In this study, we measured the expression levels of the *claudin-1*, *-3*, *-4* and *-7* genes in 205 pairs of cancer tissue and adjacent normal mucosa obtained from patients with colorectal cancer. To evaluate the clinical significance of the claudins, we examined the correlation between the relative expression of these genes and the clinicopathological features.

Materials and methods

Patients and samples. We studied surgical specimens of cancer tissue and adjacent normal mucosa obtained from 205 patients

Table I. PCR primers and conditions.

Gene	Primer	Temperature (C)	Product size (bp)
Claudin-1	5'-CCAGTTAGAAGAGGTAGTGTG-3' 5'-GAGAGGAAGGCAGTGAATC-3'	60	168
Claudin-3	5'-ACCACCACCACCACCAAC-3' 5'-GGGCTTCCTGGCTTCTGG-3'	65	113
Claudin-4	5'-TGCCTTGCTCACCGAAACCC-3' 5'-CCTCTAAACCCGTCCATCCACTC-3'	64.5	95
Claudin-7	5'-GGAGACGACAAAGTGAAGAAG-3' 5'-GCCATACCAGGAGCAAGC-3'	60	99
ß-actin	5'-AGTTGCGTTACACCCTTTCTTGAC-3' 5'-GCTCGCTCCAACCGACTGC-3'	60	171

with untreated colorectal carcinoma. The patients underwent surgery at the Yokohama City Medical Center, Gastroenterological Center and at the Kanagawa Cancer Center between 2002 and 2006. Informed consent was obtained from each patient and the Ethics Committees of the Yokohama City Medical Center and Kanagawa Cancer Center approved the protocol before initiation of the study. Each tissue sample was embedded in O.C.T. compound (Sakura Finetechnical Co., Ltd., Tokyo) and immediately stored at -80°C until use. No patient had any other malignancies. The histopathological features of specimens stained with hematoxylin and eosin were examined and sections that consisted of >80% carcinoma cells were used to prepare total RNA.

Quantitative real-time, reverse-transcription polymerase chain reaction (PCR). Total RNA isolated from colorectal cancer and adjacent normal mucosa was prepared with the use of Trizol (Gibco, Life Tech, Gaithersburg, MD). Complemetary DNA (cDNA) was synthesized from 2 μg of total RNA with 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 an 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 units/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 (Table I) and a primer extension for 1 min at 72°C followed by 72°C for 10 min. The PCR primer sequences of MMP2, MMP9, MT-MMP, RECK and ß-actin, used as an internal control, are shown in Table I.

Statistical analysis. Gene expression levels of colorectal cancer were compared with those of normal adjacent mucosa with the use of the Wilcoxon test. The relationship between gene expression 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 evaluated with the χ^2 test. Associations between variables were assessed using the Mann-Whitney U test. Correlation coefficients between the different variables were calculated by simple regression analysis. Each statistical analysis was performed using Statview J 5.0 software (Abacus, CA). Two-sided P-values were calculated and a difference was considered significant at P-value <0.05.

Results

Comparison of claudin-1, -3, -4 and -7 mRNA expression between colorectal cancer tissue and adjacent normal mucosa. *Claudin-1*, *-3* and *-4* gene expression levels were higher in cancer than in normal adjacent mucosa (P<0.001, P=0.001 and P<0.001) (Fig. 1A, B and C). The *claudin-7* gene expression level of cancer did not differ significantly from that of normal adjacent mucosa (P=0.524) (Fig. 1D).

Relationship of claudin-1, -3, -4 and -7 gene expression levels to clinicopathological features. Expression levels of the *claudin-1*, *-3*, *-4* and *-7* genes were categorized as low or high according to their median values. The relationship between the expression of these genes and clinicopathological features was then examined. The expression levels of the *claudin-1*, *-3*, *-4* and *-7* genes were unrelated to age, gender, tumor size, lymph node metastasis and lymphatic invasion. There were correlations between *claudin-1* expression and histological type (P=0.047) and between *claudin-4* expression and tumor location (P=0.039). Moreover, a reduced expression of the *claudin-7* gene correlated with venous invasion (P=0.029) and liver metastasis (P=0.022) (Table II).

Associations of claudin-1, -3, -4 and -7 gene expression with lymph node metastasis in patients with colorectal cancer. There was no significant association between the expression level of any gene and the presence or absence of lymph node metastasis (Fig. 2).

Associations of claudin-1, -3, -4 and -7 gene expression with venous invasion in patients with colorectal cancer. *Claudin-3* and *claudin-7* gene expression levels were higher in the absence than in the presence of venous invasion (P=0.043, P=0.001) (Fig. 3).

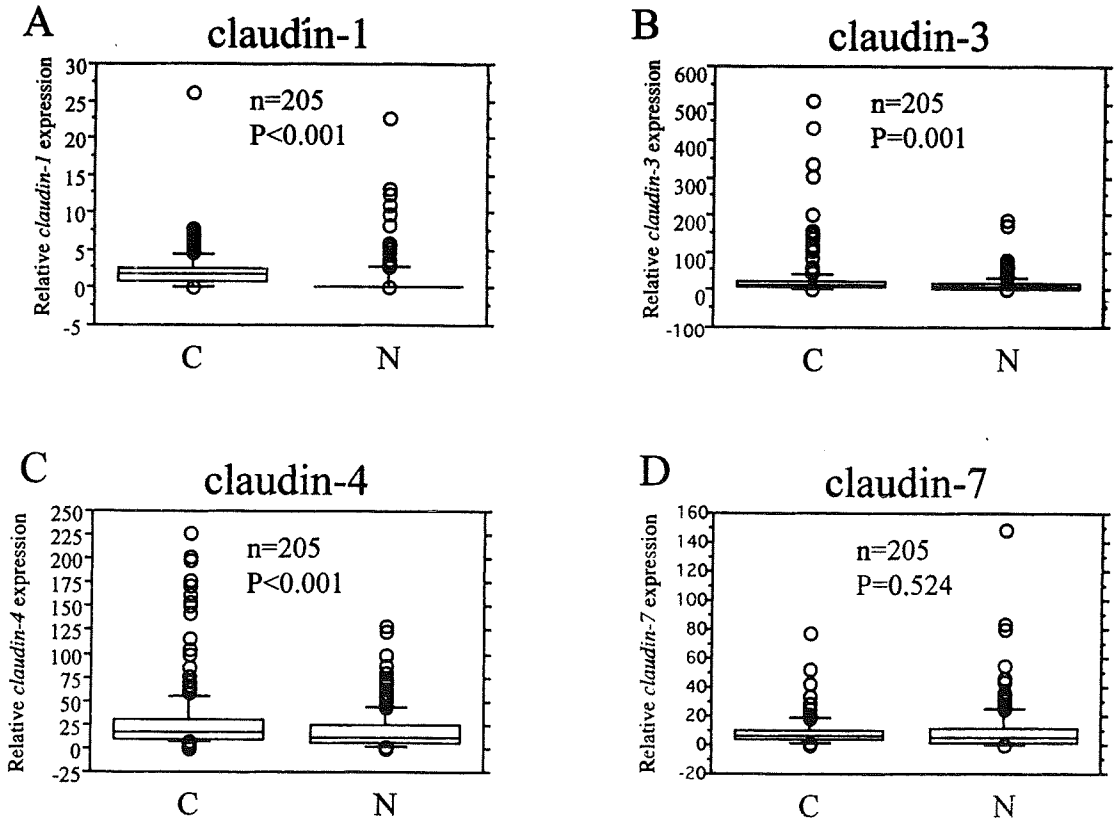

Figure 1. A comparison of *claudin-1*, *-3*, *-4* and *-7* mRNA expression levels between colorectal cancer tissue and adjacent normal mucosa. The *claudin-1*, *-3* and *-4* gene expression levels were higher in cancer than in normal adjacent mucosa (P<0.001, P=0.001, P<0.001). *Claudin-7* gene expression levels did not differ significantly between cancer and normal adjacent mucosa.

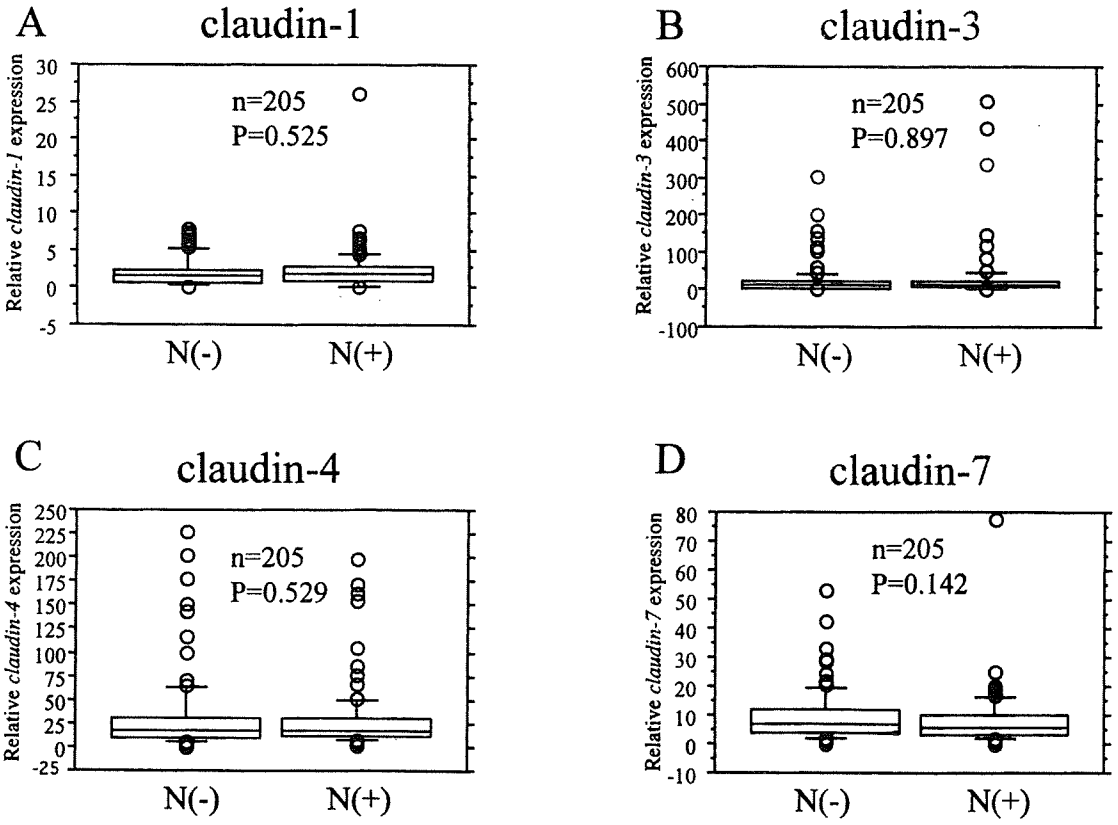

Figure 2. Associations of *claudin-1*, *-3*, *-4* and *-7* gene expression with lymph node metastasis in 205 patients with colorectal cancer. Box boundaries, the 25th and 75th percentiles of the observed values; capped bars, the 10th and 90th percentiles; solid line, median. P-values were calculated by the Mann-Whitney U test. The expression level of none of the genes examined correlated with the presence or absence of lymph node metastasis.

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	

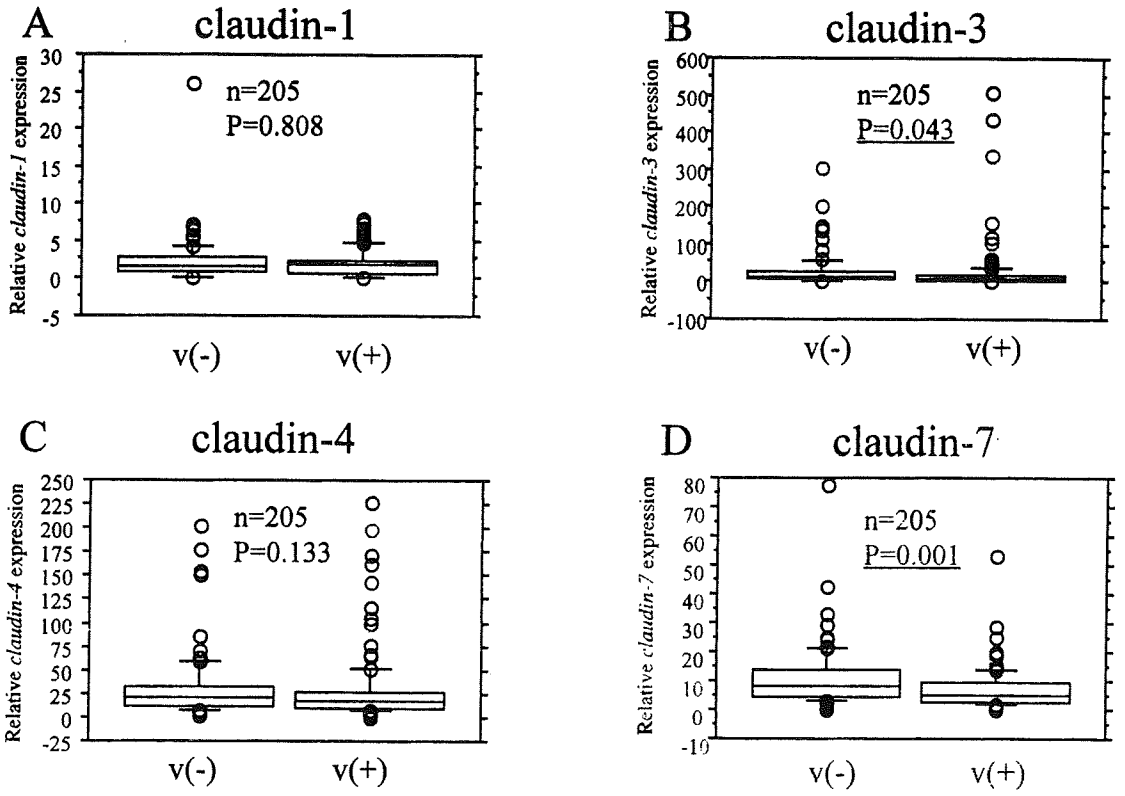

Figure 3. Associations of *claudin-1*, *-3*, *-4* and *-7* gene expression levels with venous invasion in 205 patients with colorectal cancer. Box boundaries, the 25th and 75th percentiles of the observed values; capped bars, the 10th and 90th percentiles; solid line, median. P-values were calculated 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).

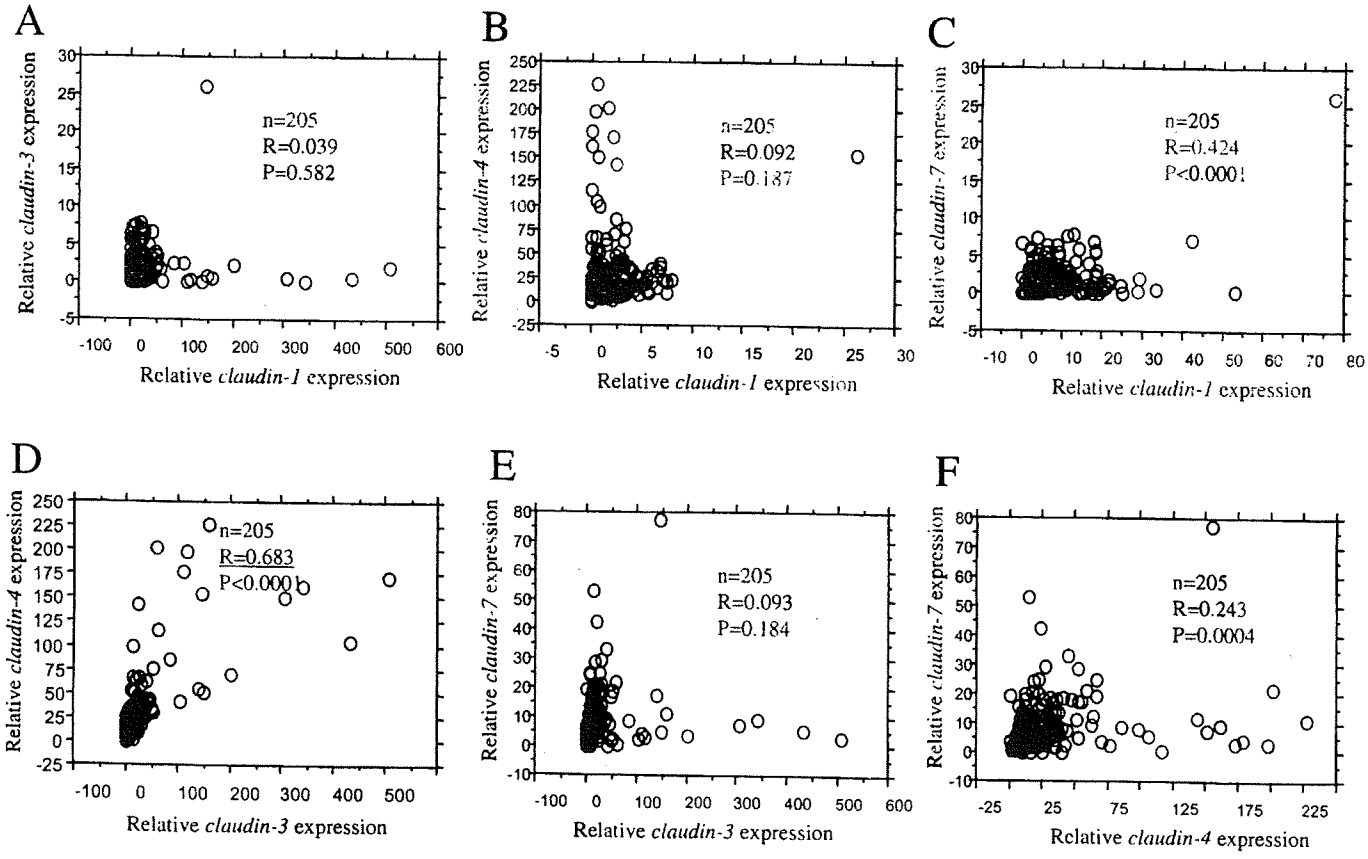

Figure 4. Correlation among *claudin-1*, *-3*, *-4* and *-7* gene expression levels in colorectal cancers. Each gene expression level is relative to that of the *β-actin* gene. The expression of the *claudin-3* gene correlated with that of the *claudin-4* gene (R=0.683).

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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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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Received October 15, 2007; Accepted January 29, 2008

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

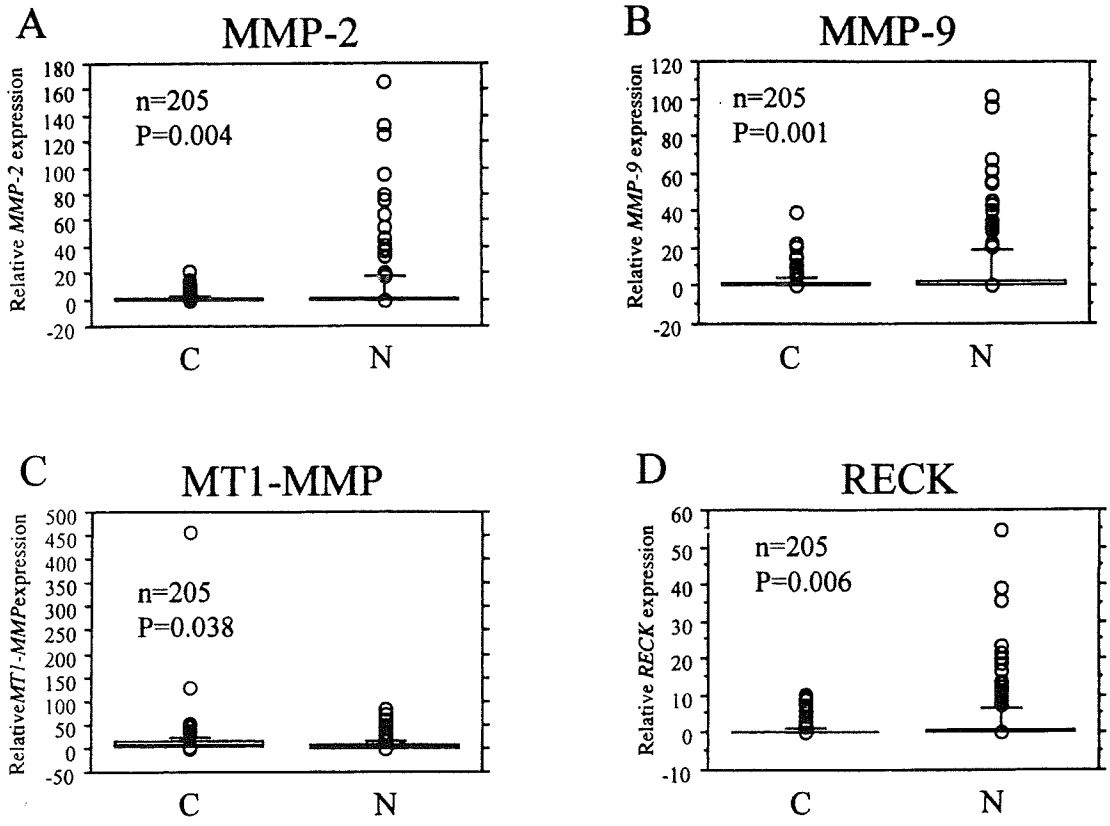

Figure 1. Comparison of *MMP-2*, *MMP-9*, *MT1-MMP* and *RECK* mRNA expression between colorectal cancer tissue and adjacent normal mucosa. *MMP-2*, *MMP-9* and *RECK* gene expression levels were higher in adjacent normal mucosa than in cancer tissue (P=0.0462, 0.0488 and 0.0491). However, the *MT1-MMP*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

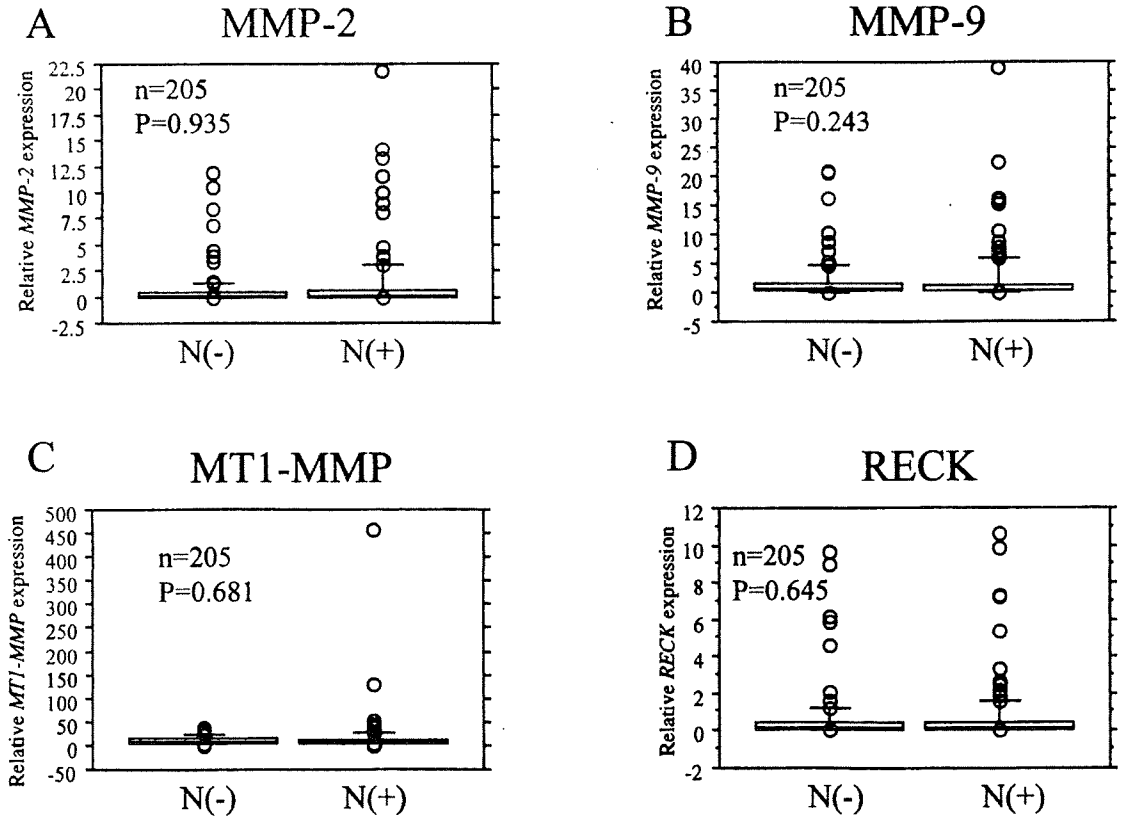

Figure 2. Association of *MMP-2*, *MMP-9*, *MT1-MMP* and *RECK* gene expression with lymph node metastasis in 205 patients with colorectal cancer. Box boundaries, the 25th and 75th percentiles of the observed values; capped bars, the 10th and 90th percentiles; solid line, the median. P-values were assessed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

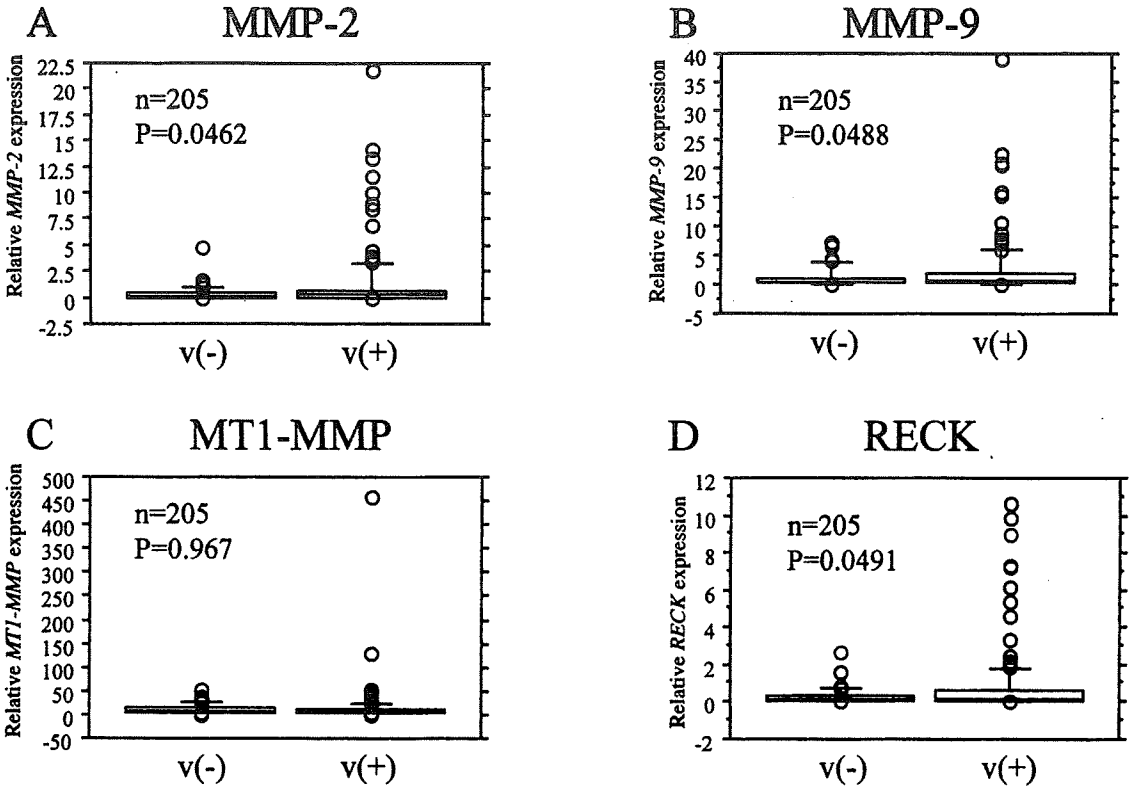

Figure 3. Association of *MMP-2*, *MMP-9*, *MT1-MMP* and *RECK* gene expression with venous invasion in 205 patients with colorectal cancer. Box boundaries, the 25th and 75th percentiles of the observed values; capped bars, the 10th and 90th percentiles; solid line, the median. P-values were assessed 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*.

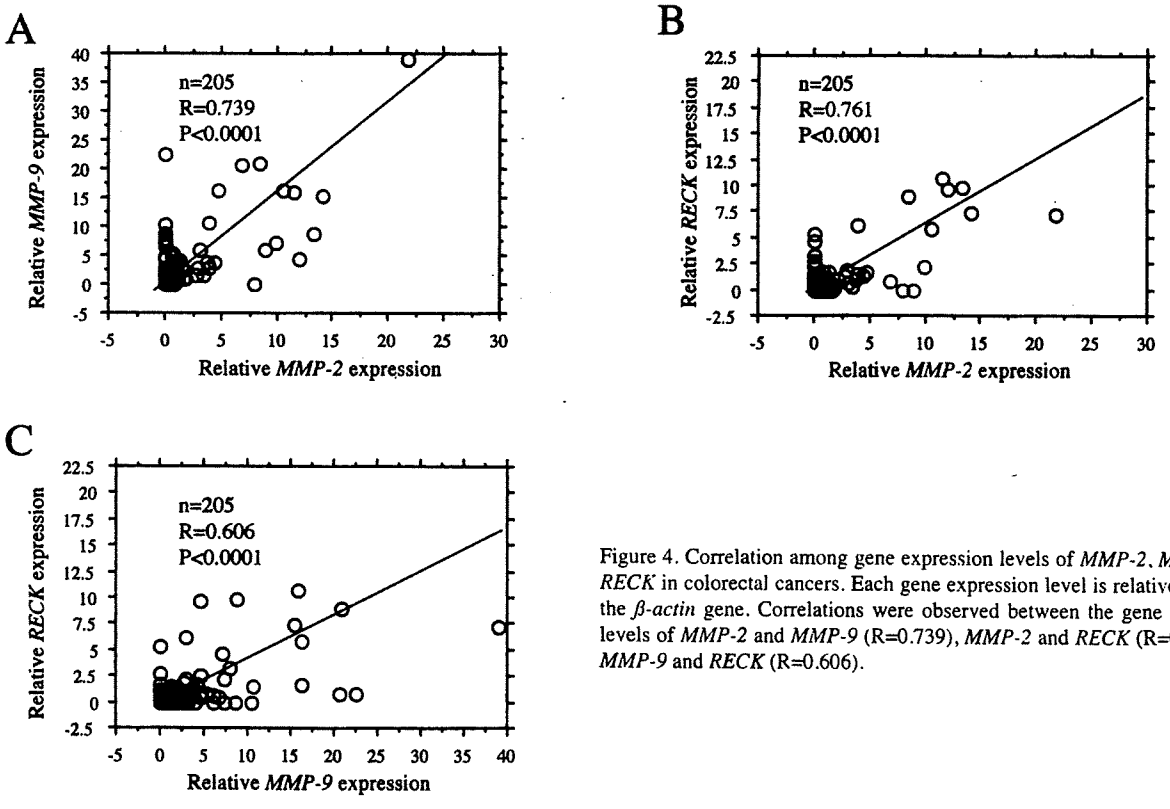

Figure 4. Correlation among gene expression levels of *MMP-2*, *MMP-9* and *RECK* in colorectal cancers. Each gene expression level is relative to that of the *β-actin* gene. Correlations were observed between the gene expression levels of *MMP-2* and *MMP-9* (R=0.739), *MMP-2* and *RECK* (R=0.761) and *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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Amphiregulin is a Promising Prognostic Marker for Liver Metastases of Colorectal Cancer

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Abstract **Purpose:** Aberrant activation of epidermal growth factor receptors (EGFR/HER1) by ligand stimulation or heterodimerization with human epidermal growth factor 2 (HER2) is considered to play an important role in the development of colorectal carcinoma. Amphiregulin (AR) is a ligand of EGFR that might be related to the development and progression of gastrointestinal tumors. The aim of this study was to determine the AR, EGFR, and HER2 protein expression levels and to evaluate their prognostic relevance to the clinical course of colorectal cancer.

Experimental Design: The AR, EGFR, and HER2 protein levels in primary tumors of colorectal cancer ($n = 106$) were examined using immunohistochemistry. Metastatic sites in liver specimens ($n = 16$) were also analyzed in the same manner.

Results: Thirteen (81.6%) metastatic lesions of the liver stained positive for AR. Among the primary lesions of colorectal cancer, 58 (54.7%) stained positive for AR, 13 (12.3%) stained positive for EGFR, and 5 (4.7%) stained positive for HER2. When the relationships between each protein expression level and the clinicopathologic factors were examined, only the AR expression level was significantly related to liver metastasis ($P = 0.0296$). A multivariate analysis of liver metastasis proved that AR expression was an independent prognostic factor of liver metastasis from colorectal cancer ($P = 0.0217$).

Conclusions: AR expression in primary lesions of colorectal cancer is an important predictive marker of liver metastasis.

Epidermal growth factor (EGF) receptors (EGFR) and their various ligands seem to be involved in the progression of gastrointestinal tumors (1). The EGF signal pathway is reportedly activated by several kinds of stimulation. First, ligands like amphiregulin (AR), transforming growth factor-α (TGF-α), and EGF may bind to EGFR. EGFR, a 170-kDa transmembrane glycoprotein (2), is composed of an extracellular ligand-binding domain, a transmembrane region, and an intracellular protein tyrosine kinase domain (3-5). The above-mentioned ligands bind to the extracellular ligand-binding domain of EGFR and stimulate the pathway. Second, the heterodimerization of EGFR and HER2 can reportedly stimulate signaling in the absence of ligands (2). These steps are followed by the stimulation of intrinsic tyrosine kinase activity and tyrosine autophosphorylation (3, 6-8). Receptor activity is modulated by intracellular kinases that mediate negative feedback control via receptor phosphorylation at specific regulatory domains, and receptor inactivation is mediated by receptor internalization and ligand-receptor dissociation. AR has been implicated in the growth and regeneration of intestinal mucosa and might be related to the development and progression of gastrointestinal tumors (9-12). Our microarray analysis in colorectal tumors and liver metastases revealed that AR was down-regulated in adenomatous tumors but was up-regulated in metastatic tumors of the liver (data not shown). These findings suggested that AR might contribute to liver metastasis from colorectal cancer. The aim of this study was to clarify the relationship between AR expression and liver metastasis and to uncover any correlations between the protein levels of AR, EGFR, and HER2 and the proliferation of colorectal cancer.

Materials and Methods

Human tissues. The study population comprised 106 consecutive patients who underwent the resection of colorectal cancer at the Department of Gastroenterologic Surgery, Yokohama City University Hospital. The patient characteristics are described in Table 1. There is another figure, related to patient profile, which shows whether each case has liver metastasis. There were 106 cases. Eighteen cases had synchronous liver metastasis at the surgical treatment of a primary tumor. In 88 cases, there were no liver metastases, but in the remaining 18, liver metastases occurred later. This analysis of the investigation has three parts: The first is concerned with the correlation between the pathologic factors and protein expression and liver metastases; the second about overall survival, or disease-free survival; and the third, about metachronous liver metastases (Fig. 1).

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Received 9/30/07; revised 1/1/08; accepted 1/3/08.

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doi:10.1158/1078-0432.CCR-07-4499

For the immunohistochemical study, formalin-fixed, paraffin-embedded tissue samples from 106 primary colorectal cancers and 16 metastatic tumors of the liver were obtained. The median age of the patients at the time of initial surgery was 67 y (range, 35-84 y). The follow-up period after the initial operation for primary lesions was between 3.5 and 5 y (median, 41.2 mo).

Immunohistochemistry. For AR and HER2 immunostaining with rabbit polyclonal antibodies, tissue sections (3 μm) were deparaffinized in xylene and rehydrated in an ethanol series. The sections were then treated for 30 min with 0.3% hydrogen peroxide to block endogenous peroxidase activity. The sections were subsequently washed with PBS and unmasked in citrate antigen unmasking solution (Mitsubishi Kagaku Iatron, Inc.) in an autoclave for 20 min at 120°C. The sections were incubated with goat serum for 15 min at 37°C and then were incubated with the primary antibody [polyclonal antibody to AR (1/100): Quartett, Inc.; polyclonal rabbit anti-c-erbB-2: Zymed Laboratories, Inc.] for 1 h at 37°C. The bound primary antibodies were detected by adding anti-rabbit secondary antibodies and avidin/biotin/horseradish peroxidase complex (DAKO) for 30 min at room temperature. The sections were visualized using solid diaminobenzine diluted in PBS, counterstained with Mayer's hematoxylin, and finally mounted.

Immunohistochemical staining for EGFR was done using the EGFR pharmDx kit (DakoCytomation), according to the manufacturer's instructions.

Table 1. Patient profile (n = 106)

Pathologic factors	No. patients
Age (y)	
35-84 (Median, 67)	106
Sex	
Male	58
Female	48
Histology	
Well	44
Moderate	49
Poor	5
Mucinous	8
Depth of tumor invasion (tumor penetration of serosa)	
T_1	11
T_2	18
T_3	41
T_4	36
Lymph node metastases	
Negative	53
Positive	53
Vascular invasion	
Negative	41
Positive	65
Lymphatic invasion	
Negative	50
Positive	56
Stage	
0	2
I	21
II	22
II	34
IV	27
Synchronous liver metastases (H)	
Negative	88
Positive	18
Peritoneal metastases (P)	
Negative	99
Positive	7
Extra hepatic metastases (P)	
Negative	49
Positive	57

Evaluation of immunostaining. Two pathologists with no knowledge of the clinical outcome independently examined the stained sections. For AR, the slides were graded according to the staining intensity and the percentage of immunopositive cells, as previously described (13). Specific staining with postimmune serum was semiquantitated by assigning a score of 0 to 3 based on the color intensity of the brown diaminobenzidine precipitate, with 1 representing light brown staining; 2, a moderately brown color; and 3, an intense brown color. The number of positive cells per slide was stratified into three groups based on the percentage of positive cells: group 1, <33%; group 2, 33% to 67%; and group 3, >67%. Semiquantitative scores ranging from 1 to 9 for the specific staining of each specimen were obtained by multiplying the staining intensity by the number of the group that represented the percentage of positive cells within each specimen. A score of zero represents no specific staining. For EGFR and HER2, immunoreactivity was defined in the same manner as the Hercep Test evaluation (ref. 14; Fig. 2).

Statistics. Nonparametric tests were used throughout this study. Two-sided P values <0.05 were considered significant. Correlations were examined using the Fisher's exact, two-tailed Student's t test. Kaplan-Meier survival curves were used to estimate the survival of the patients. All calculations were done using Dr. SPSS II for Windows 11.0.1J software (SPSS, Inc.)

Results

Immunohistochemical analysis. Among the 106 primary lesions, 58 (54.7%) were AR(+), 13 (12.3%) were EGFR(+), and only 5 (4.7%) were HER2(+) (Table 2). The expression of EGFR or HER2 was not significantly related with any of the clinicopathologic factors, whereas AR(+) was significantly correlated with liver metastasis (P = 0.0296). Among the 16 liver metastases obtained by hepatectomy, 13 (81.6%) were AR(+).

A univariate analysis done to explore factors determining the metastasis of colon cancer to the liver using clinicopathologic factors, including AR expression, revealed that vascular invasion (P = 0.0066), AR expression (P = 0.0296), the depth of the tumor (P = 0.068), and lymph node metastasis (P = 0.0804) of the primary lesions were important factors (Table 3). The results of a multivariate logistic regression analysis using these selected factors are summarized in Table 4. AR expression was associated with an increase in the risk of liver metastasis (P = 0.0217; hazard ratio, 3.204; 95% confidence interval, 1.185-8.659). Vascular invasion was the next most strongly associated factor for liver metastasis. Among the 88 patients who had no liver metastasis at the time of the resection of the primary lesions, the disease-free survival period of the 26 patients whose primary lesions were positive for both of these two factors was significantly shorter than that of the other 62 patients (Fig. 3), and hepatic metastasis-free survival of the 26 patients was also shorter than that of the other 62 patients (Fig. 3).

In the coexpression analysis, 10 (10.6%) patients were AR(+) and EGFR(+), 2 (1.8%) were AR(+) and HER2(+), and only 1 (0.9%) was EGFR(+) and HER2(+). The number indicates the number of cases. These coexpressions were not significantly related with any of the clinicopathologic factors.

Discussion

This study shows that AR expression in primary lesions of colorectal cancer is a promising predictive marker of liver metastasis. AR is categorized as belonging to the EGF family;

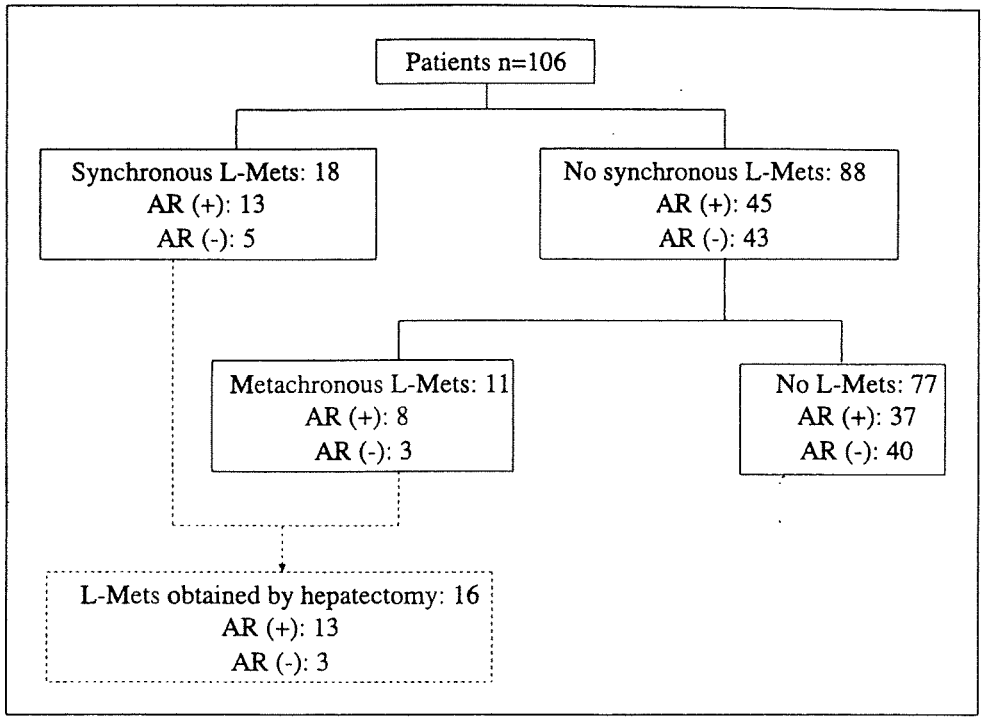

Fig. 1. Diagram of 106 colon cancer patients according to liver metastasis (*L-mets*) and AR staining. Eighteen synchronous L-mets, 88 nonsynchronous L-mets, 11 metachronous L-mets, and 77 no L-mets are the results of AR staining of the primary lesions. Sixteen L-mets obtained by hepatectomy is a result of AR staining of liver metastases.

thus, AR might work in collaboration with EGFR. In this study, however, the immunohistopathologic coexpression of AR with EGFR or HER2 was not correlated with metastasis.

EGFR seems to be involved in regulating the growth of the intestinal mucosa and might be related to the development and progression of gastrointestinal tumors. EGFR, which can be detected in ~60% to 80% of colorectal carcinomas (15, 16), has emerged as a rational target for anticancer therapy for colorectal cancer. Cetuximab, a monoclonal antibody that specifically blocks EGFR, has good clinical activity in ~10% of

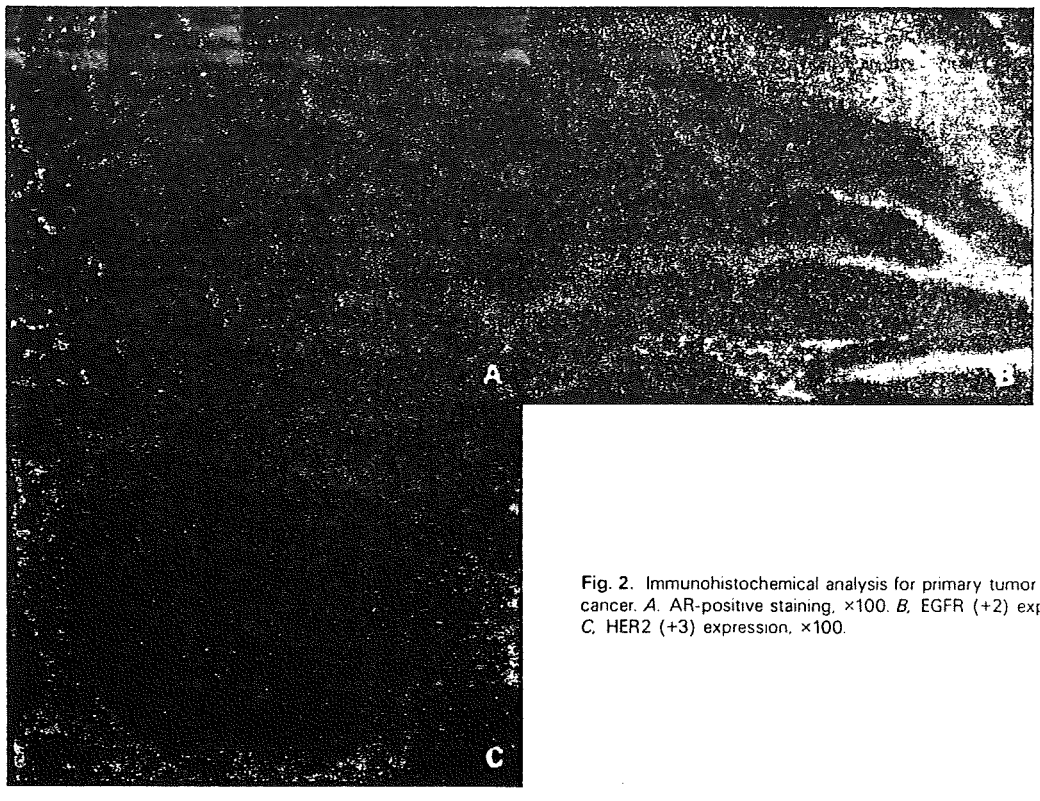

Fig. 2. Immunohistochemical analysis for primary tumor of colorectal cancer. *A*, AR-positive staining, ×100. *B*, EGFR (+2) expression, ×100. *C*, HER2 (+3) expression, ×100.

Table 2. Relationship between AR, EGFR or HER2 expression, and clinicopathologic factor

Clinicopathologic factor	AR			EGFR			HER2		
	Positive (n = 58)	Negative (n = 48)	P	Positive (n = 13)	Negative (n = 93)	P	Positive (n = 5)	Negative (n = 101)	P
Histology									
Well/moderate	50	43	0.7681	11	82	0.6597	4	89	0.4872
Poor/mucinous	8	5		2	11		1	12	
Depth of tumor invasion									
T_1-T_3	38	32	0.9999	9	61	0.9999	4	66	0.6597
T_4	20	16		4	32		1	35	
Lymph node metastases									
Positive	31	22	0.5585	5	48	0.5553	3	50	0.9999
Negative	27	26		8	45		2	51	
Vascular invasion									
Positive	37	28	0.7260	7	58	0.5589	3	62	0.9999
Negative	21	20		6	35		2	39	
Lymphatic invasion									
Positive	29	27	0.5618	4	52	0.1370	2	54	0.6649
Negative	29	21		9	41		3	47	
Liver metastases									
Positive	21	8	0.0296	3	26	0.9999	2	27	0.6127
Negative	37	40		10	67		3	74	
Peritoneal metastases									
Positive	5	2	0.4525	1	6	0.9999	0	7	0.9999
Negative	53	46		12	87		5	94	
Extrahepatic metastases									
Positive	12	11	0.8163	3	20	0.9999	1	22	0.9999
Negative	46	37		10	73		4	79	

patients with chemotherapy-refractory advanced colorectal cancer (17–20). Thus, EGFR clearly plays an important role in the development and progression of colorectal cancer, although the ligand for EGFR remains uncertain.

AR, a ligand of EGFR, is synthesized as a transmembrane precursor that is proteolytically processed to its mature secreted form (10) and is localized in the cytoplasm and nuclei of terminally differentiated, nonproliferative surface columnar and secretory epithelial cells of the mucosa, such as the human ovary, placenta, and colon (21), and has been implicated in the growth and regeneration of intestinal mucosa (9–12). In our study, AR was also detected in the cytoplasm and/or nuclei of cancer cells, and the percentage of AR(+) nuclei in the AR(+) cases was 51.7%.

AR also reportedly contributes to the mitogenic and antiapoptotic growth of human colon malignant cells as well

Table 3. Univariate analyses for liver metastases

Clinicopathologic factor	Liver metastases		
	Positive (n = 29)	Negative (n = 77)	P
Depth of tumor invasion			
T_1-T_3	15	55	0.0680
T_4	14	22	
Lymph node metastases			
Positive	19	34	0.0804
Negative	10	43	
Vascular invasion			
Positive	24	41	0.0068
Negative	5	36	
Lymphatic invasion			
Positive	18	38	0.2800
Negative	11	39	
AR			
Positive	21	37	0.0296
Negative	8	40	
EGFR			
Positive	3	10	0.9999
Negative	26	67	
HER2			
Positive	2	3	0.6127
Negative	27	74	