Clinicopathological significance of the gene expression of *matrix metalloproteinase-7*, *insulin-like growth factor-1*, *insulin-like growth factor-2* and *insulin-like growth factor-1 receptor* in patients with colorectal cancer: *Insulin-like growth factor-1 receptor* gene expression is a useful predictor of liver metastasis from colorectal cancer

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Abstract. Matrix metalloproteinase-7 (MMP-7), secreted by cancer cells, has been implicated classically in the basement membrane destruction associated with tumor cell invasion and metastasis. Epidemiological studies have established a correlation between high levels of circulating insulin-like growth factor-1 (IGF-1) and the relative risk of colorectal cancer, which is known to produce MMP-7. We examined the clinicopathological significance of the relative expression of *MMP-7*, *IGF-1*, *IGF-2* and *IGF-1 receptor* 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-7*, *IGF-1*, *IGF-2*, *IGF-1R* and ß-actin mRNA in cancer tissue and adjacent normal mucosa were measured by quantitative real-time reverse-transcriptase polymerase chain reaction. *MMP-7* and *IGF-1R* gene expression levels were higher in cancer tissue than in adjacent normal mucosa. In contrast, *IGF-1* gene expression was lower in cancer tissue than in adjacent normal mucosa. As for the relationship of gene expression to clinicopathological factors, *IGF-1R* expression correlated with venous invasion and liver metastasis. *IGF-1R* gene expression is thus considered a useful predictor of liver metastasis from colorectal cancer.

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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, the excessive degradation of matrix is one of the hallmarks (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, outcomes, or both (4). MMP-7 is a member of the MMP family and, when activated, displays broad proteolytic activity against a variety of extracellular matrix substrates, including collagens, proteoglycans, elastin, laminin, fibronectin and casein (5-7). Unlike MMPs, which are synthesized by stromal cells, MMP-7 is produced exclusively by cancer cells. Miyamoto *et al* (8) reported that MMP-7, produced by cancer cells, regulates the bioavailability of insulin-like growth factors (IGFs) in the surrounding tissue.

IGFs have been studied extensively for possible roles in cancer growth (9-12). They are expressed ubiquitously and act as endocrine, paracrine and autocrine growth factors. Insulin-like growth factor-1 (IGF-1) is associated with an increased risk of cancer (13). Functionally, IGF-1 not only stimulates cell proliferation, but also inhibits apoptosis. The combination of these mitogenic and antiapoptotic effects is

now recognized to have a profound impact on tumor growth (14). Previous studies have reported that IGF-2 is related to tumor progression and patient survival and that it has been suggested that IGF-2 acts as an autocrine growth factor in colorectal carcinoma (15). Insulin-like growth factor-1 receptor (IGF-1R) is the receptor of IGF-1 and IGF-2. IGF-1R overexpression promotes tumor growth, progression, invasion and metastasis (16).

In this study, we examined the clinicopathological significance of the relative expression of the *MMP-7*, *IGF-1*, *IGF-2* and *IGF-1 receptor* 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 from 2002 through to 2006. Informed consent was obtained from each patient and the Yokohama City Medical Center Committee and Kanagawa Cancer Center Committee approved the study. Each tissue sample was embedded in an O.C.T. compound (Sakura Finetechnical Co., Ltd., Tokyo) and stored at -80˚C immediately before use. No patient had any other malignancy. After examining the histopathological features of specimens stained with hematoxylin and eosin, sections consisting of >80% of carcinoma cells were used to prepare total RNA.

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 with the use of an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). After synthesis, the cDNA was diluted at 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 concentrations 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 10 min at 72˚C. The PCR primer sequences of MMP-7, IGF-1, IGF-2, IGF-1R 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 of gene expression levels to potential explanatory variables, including age, gender, tumor size, histological type, depth of invasion, lymph node metastasis, tumor 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 of <0.05 were considered to indicate a statistical significance.

Results

Comparison of MMP-7, IGF-1, IGF-2 and IGF-1R mRNA expression between colorectal cancer tissue and adjacent normal mucosa. *MMP-7* and *IGF-1R* gene expression levels were higher in cancer tissue than in adjacent normal mucosa ($P<0.001$, $P<0.001$; Fig. 1A and D). In contrast, *IGF-1* gene expression was lower in cancer tissue than in adjacent normal mucosa ($P<0.001$; Fig. 1B). There was no significant difference between *IGF-2* gene expression in cancer tissue and that in adjacent normal mucosa ($P=0.546$; Fig. 1C).

Relationship of clinicopathological features to MMP-7, IGF-1, IGF-2 and IGF-1R gene expression levels. After categorizing the expression levels of *MMP-7*, *IGF-1*, *IGF-2* and *IGF-1R* 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-7*, *IGF-1*, *IGF-2* and *IGF-1R* gene expression levels were unrelated to age, tumor size, histological type, lymph node metastasis, tumor location and lymphatic invasion. *IGF-1R* gene expression levels were significantly related to venous invasion ($P=0.027$). *IGF-1R* gene expression was significantly related to liver metastasis ($P=0.033$) (Table II).

Comparison of MMP-7, IGF-1, IGF-2 and IGF-1R gene expression levels between the presence and absence of venous invasion. *IGF-1R* gene expression levels differed significantly between the presence and absence of venous invasion ($P=0.048$) (Fig. 2).

Correlation among MMP-7, IGF-1, IGF-2 and IGF-1R expression. The results of the correlation analysis are shown in Fig. 3. No significant correlations were observed among the expression of these genes.

Discussion

Unlike other MMPs, which are produced by stromal cells, MMP-7 is produced by cancer cells and is implicated in the basement membrane destruction associated with cancer cell invasion and metastasis (17). IGF-1, IGF-2 and their receptor IGF-1R, participate in the development and progression of cancer (18-20). Previous studies have reported that MMP-7 produced by cancer cells regulates the bioavailability of IGFs in surrounding tissue (8).

In the present study, we examined *MMP-7*, *IGF-1*, *IGF-2* and *IGF-1R* mRNA expression in colorectal cancer tissue and adjacent normal mucosa. We studied the relationship of these gene expression levels to clinicopathological features, as well as correlations among the expression of these genes.

Table I. PCR primers and conditions.

Gene	Primer	Temperature (°C)	Product size (bp)
MMP-7	5'-CACTGTTCCTCCACTCCATTTAG-3' 5'-CATTTATTGACATCTACCCACTGC-3'	62.6	151
IGF-1	5'-GTGGATGAGTGCTGCTTC-3' 5'-ACTTCCTTCTGGGTCTTGG-3'	58	134
IGF-2	5'-TACCGCCATCTCCCTTCTC-3' 5'-TCCCTCTGACTGCTCTGTG-3'	60	122
IGF-1R	5'-TGCCTTGGTCTCCTTGTC-3' 5'-TTTCCCTGCTTTGATGGTC-3'	58	154
β-actin	5'-AGTTGCGTTACACCCTTTCTTGAC-3' 5'-GCTCGCTCCAACCGACTGC-3'	60	171

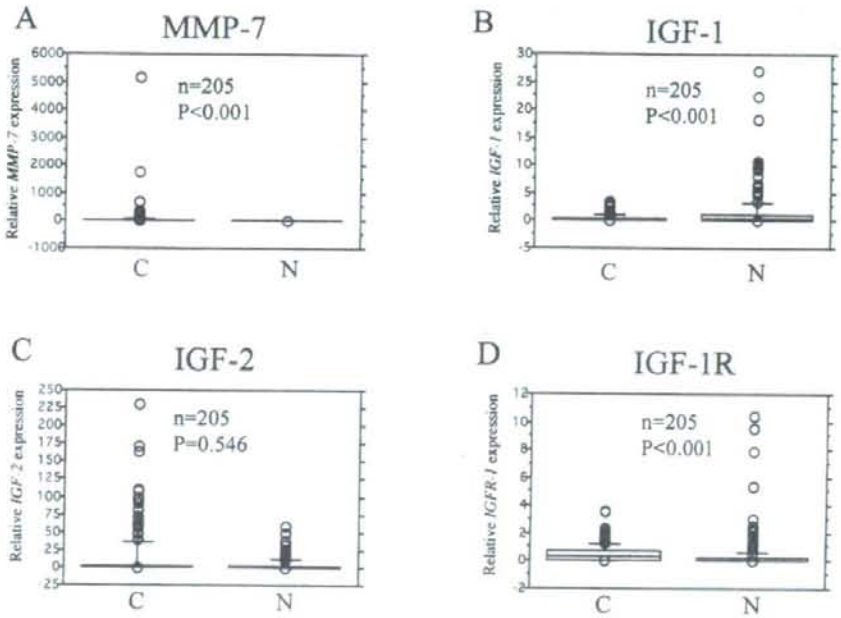

Figure 1. Comparison of *MMP-7*, *IGF-1*, *IGF-2* and *IGF-1R* mRNA expression levels between colorectal cancer tissue and adjacent normal mucosa. *MMP-7* and *IGF-1* gene expression levels were higher in cancer tissue than in adjacent normal mucosa (P<0.001, P<0.001). In contrast, *IGF-1* gene expression levels were lower in cancer tissue than in adjacent normal mucosa (P<0.001). *IGF-2* gene expression did not differ significantly between cancer tissue and adjacent normal mucosa.

Several previous studies have compared *MMP-7*, *IGF-1*, *IGF-2* and *IGF-1R* mRNA expression levels between colorectal cancer tissue and adjacent normal mucosa. Miyata *et al* (17) reported that the expression of MMP-7 in tumor cells was significantly higher than that in normal cells. Freier *et al* (21) found that IGF-1R gene expression was higher in colorectal cancer than in adjacent normal mucosa. Nosho *et al* (22) showed that IGF-IR mRNA expression was detected ~40% of colorectal tissues, though was undetectable in adjacent nontumor tissue. IGF-1 gene expression in colorectal cancer was reported to be higher than that in adjacent normal mucosa (21). Li *et al* (23) reported that the expression level of the IGF-2 gene was significantly increased in colorectal cancer as compared with that in adjacent normal mucosa. In our study, *MMP-7* and *IGF-1R* gene expression levels were higher in cancer tissue than in adjacent normal mucosa. Conversely, *IGF-1* gene expression was lower in cancer tissue than in adjacent normal mucosa. *IGF-2* gene expression did not differ significantly between cancer tissue and adjacent normal mucosa.

In a study of the relationship of clinicopathological features to gene expression levels, Nosho *et al* (22) found that *MMP-7* gene expression correlates with tumor size, location and histopathology in early colorectal carcinoma. Miyata *et al* (17) reported that MMP-7 expression in cancer cells correlates with an advanced pathological tumor stage. In our study, *MMP-7*

Table II. Relationship between the expression of MMP-7, IGF-1, IGF-2, or IGF-1R genes and clinicopathological features.

Variables/categories	MMP-7 expression		P-value	IGF-1 expression		P-value	IGF-2 expression		P-value	IGF-1R 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	66.8±10.6	64.8±10.9	0.187	66.0±11.1	65.7±10.5	0.837	66.4±10.4	65.2±11.2	0.387	65.3±11.1	66.3±10.5	0.484
Gender												
Male	58	62	0.628	53	59	0.318	58	62	0.628	51	61	0.185
Female	44	41		50	42		44	41		51	42	
Size												
≤5 cm	56	59	0.731	60	55	0.434	64	51	0.056	61	54	0.287
>5 cm	46	44		42	48		38	52		41	49	
Histological type												
Well differentiated	32	29	0.700	31	30	0.926	31	30	0.864	29	32	0.457
Moderately differentiated	58	58		58	58		56	60		56	60	
Poorly differentiated	12	16		13	15		15	13		17	11	
Depth of invasion												
T1	7	9	0.888	11	8	0.837	10	9	0.178	11	8	0.559
T2	49	48		47	47		54	40		42	52	
T3	41	39		39	41		33	47		42	38	
T4	5	7		5	7		5	7		7	5	
Lymph node metastasis												
Absent	45	50	0.525	50	45	0.444	50	45	0.444	45	50	0.485
Present	57	53		52	58		52	58		58	53	
Location												
Colon	58	54	0.524	61	51	0.139	60	52	0.231	56	56	0.939
Rectum	44	49		41	52		42	51		46	47	
Lymphatic invasion												
Absent	67	67	0.924	64	70	0.824	67	67	0.924	63	71	0.281
Present	35	36		38	39		35	36		39	32	
Venous invasion												
Absent	38	39	0.928	43	34	0.176	45	32	0.054	46	31	0.027
Present	64	64		59	69		57	71		56	72	
Liver metastasis												
Absent	69	70	0.962	70	69	0.802	71	68	0.582	79	60	0.033
Present	33	33		32	34		31	35		23	43	

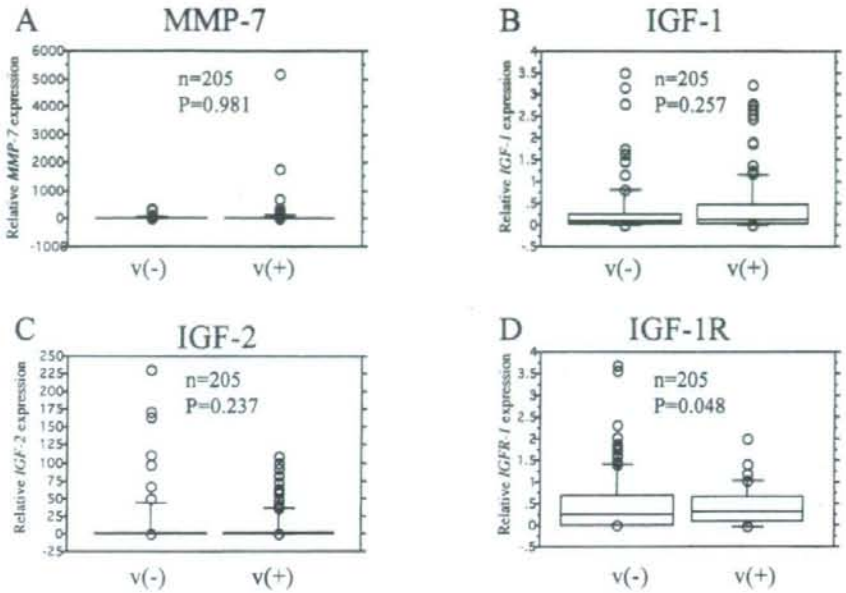

Figure 2. The association of *MMP-7*, *IGF-1*, *IGF-2* and *IGF-1R* gene expression with venous invasion in 205 patients with colorectal cancer. Box boundaries, the 25th and 75th percentiles of the observed values; capped bars, the 10th and 90th percentiles; solid line, the median. P-values were assessed by the Mann-Whitney U test. The presence or absence of venous invasion was significantly related to the gene expression levels of *IGF-1R*.

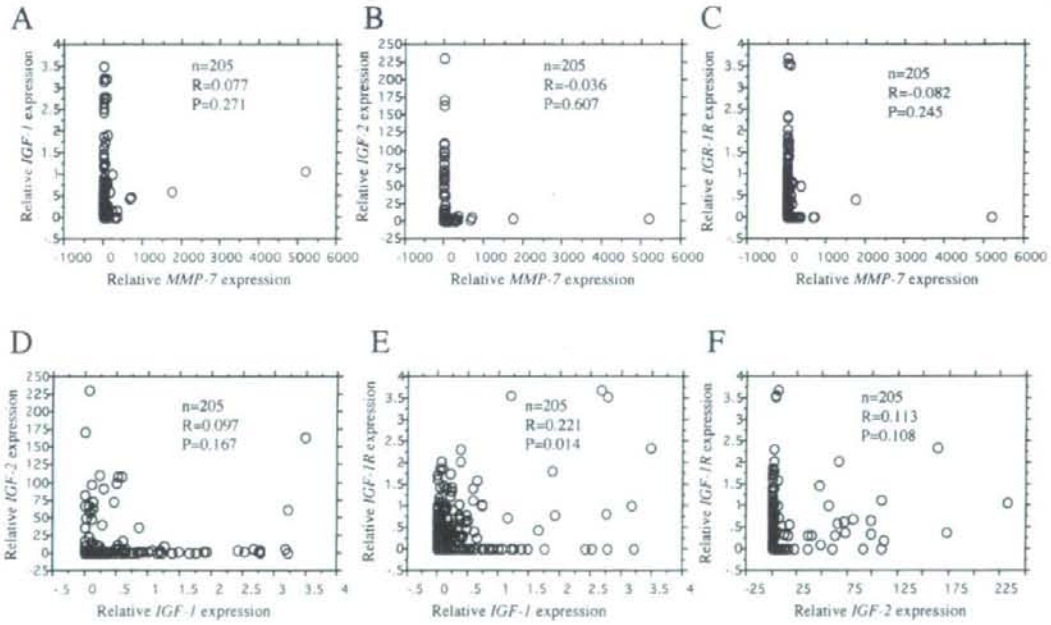

Figure 3. Correlations among gene expression levels of *MMP-7*, *IGF-1*, *IGF-2* and *IGF-1R* in colorectal cancers. No significant correlations were observed among the expression levels of these genes.

gene expression levels significantly correlated with gender. As for IGFs, Peters *et al* (24) showed that *IGF-1* gene expression does not correlate with any clinicopathological characteristic. Nosho *et al* (22) reported that *IGF-2* gene expression correlates with age and tumor size, whereas *IGF-1R* gene expression does not correlate with any clinicopathological characteristic in patients with early colorectal carcinoma. Mita *et al* (25) reported that *IGF-1R* gene expression does not

correlate with any clinicopathological characteristic in prostate cancer. However, Furukawa *et al* (26) reported that increased postoperative tumor growth and the presence of liver metastasis were associated with significantly higher IGF-1R mRNA expression in gastrinomas. Our study found no significant correlation between *IGF-1* or *IGF-2* gene expression and any clinicopathological characteristic, whereas *IGF-1R* gene expression was significantly related to venous invasion and liver metastasis.

In a study examining interrelations among MMP-7, IGF-1, IGF-2 and IGF-1R, Miyamoto *et al* (8) showed that MMP-7 regulates IGF-1. Furukawa *et al* (26) reported a significant correlation ($r=0.66$, $P<0.0001$) between the expression levels of the *IGF-1* and *IGF-1R* genes. In our study, there were no significant correlations among these genes.

In conclusion, our study showed that *IGF-1R* gene expression levels were higher in adjacent normal mucosa than in cancer tissue and were significantly related to venous invasion and liver metastasis. *IGF-1R* gene expression is thus 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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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 = 0.0068), 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,

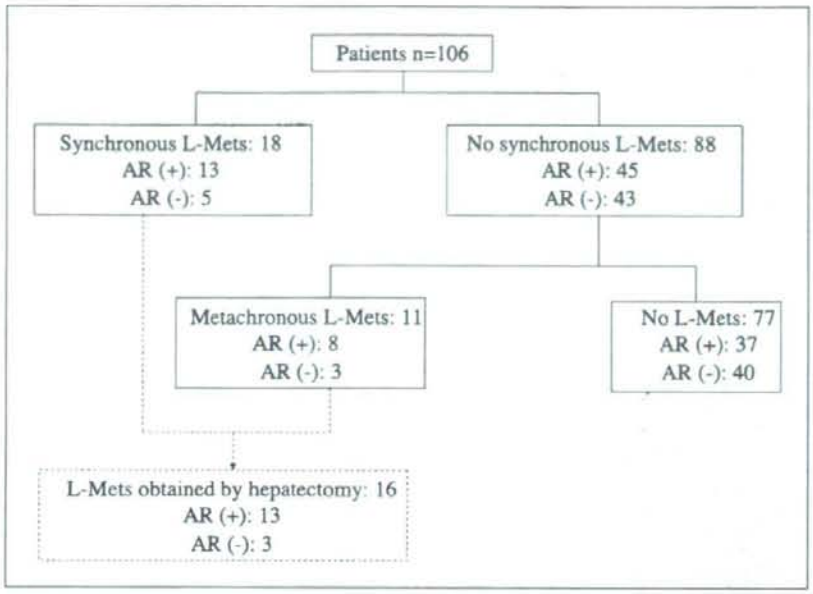

Fig. 1. Diagram of 106 colon cancer patients according to liver metastasis (*L-mets*) and AR staining. Eighteen synchronous L-mets, 88 nonsynchronous L-mets, 11 metachronous L-mets, and 77 no L-mets are the results of AR staining of the primary lesions. Sixteen L-mets obtained by hepatectomy is a result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

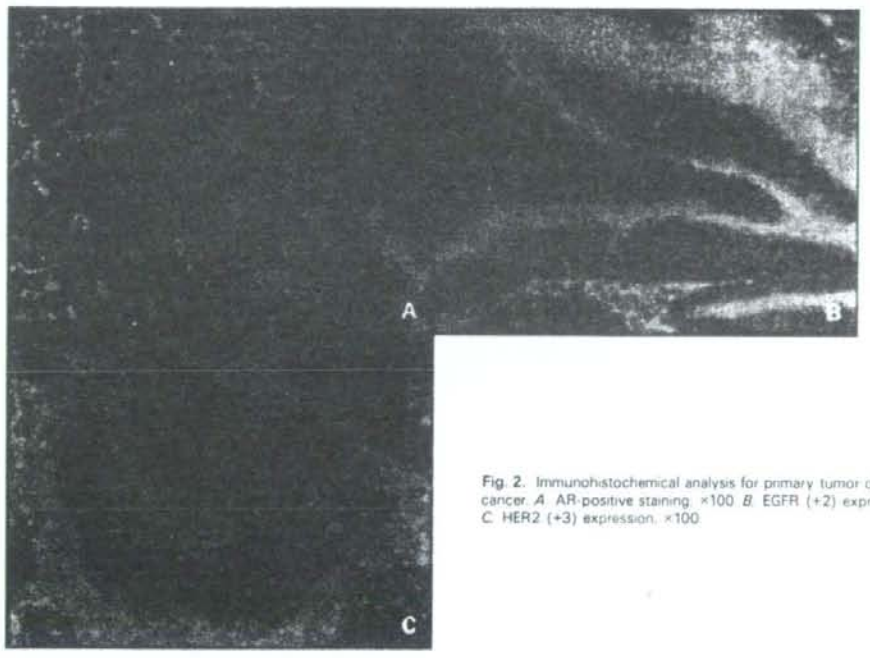

Fig. 2. Immunohistochemical analysis for primary tumor of colorectal cancer. *A*, AR-positive staining, ×100. *B*, EGFR (+2) expression, ×100. *C*, HER2 (+3) expression, ×100.

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	

Table 4. Multivariate analyses for liver metastases (logistic regression analysis)

	Hazard ratio (95% confidence interval)	P
Vascular invasion; positive (vs negative)	3.122 (0.892-10.928)	0.0748
AR; positive (vs negative)	3.204 (1.185-8.659)	0.0217

as breast, prostate, cervix, and liver cancer cells (22, 23). Interference with AR production by specific antisense small interfering RNAs or neutralizing antibodies reduced cell proliferation (24) and reversed many of the neoplastic phenotypic traits of cancer cells *in vitro*, although the expressions of other ligands of the EGFR were preserved in these cells (21, 25, 26). In ~50% of human primary colon carcinomas, AR was overexpressed (27). These reports suggest that AR is an important ligand for EGFR in colon cancer cell transformation.

Zvibel et al. (28) showed that site-specific metastasis was determined by the extracellular matrix of the colonized organ, whereas AR at the secondary colonization site was induced by typical liver-matrix components and stimulated cancer cell proliferation. Under certain conditions, hepatocyte-derived extracellular matrix stimulated the proliferation of colon cancer cells via the induction of AR. Thus, we supposed that AR-positive cells had a strong affinity with the liver, explaining why AR expression was related to liver metastasis and why AR-positive cancer cells were more frequently observed in metastatic lesion of the liver than in the primary lesion. We also indicated that disease-free survival and hepatic metastasis-free survival were related to both venous invasion and AR expression in the primary lesion (Fig. 3). These results might depend on the malignant behavior of AR, as mentioned above.

Previous reports showed that the coexpression of EGFR and c-erbB-2 protein may be related to the distant metastasis of colon cancer (29-32). In the present study, a relationship between malignant behavior and the coexpression of EGFR, HER2, and/or AR in colorectal cancer could not be shown. The low immunoreactivity for EGFR (12.3%) and HER2 (4.7%) in this study might explain the above result. Generally, immunoreactivity depends on the fixation time or the storage time of the archived tissue sections, especially when testing colorectal adenocarcinomas for EGFR expression using the DakoCytomation EGFRpharmDX or breast cancer using the Herceptest. The evaluation of EGFR expression is also dependent on the storage time of archived tissue sections, especially with colorectal adenocarcinomas. The tissue sections should be tested within 9 months to avoid false-negative results (1, 33, 34).

This study is the first report revealing that AR expression in primary lesions of colorectal cancer is significantly correlated with liver metastasis. We conclude that AR expression in colorectal cancer is an important predictive marker for liver metastases.

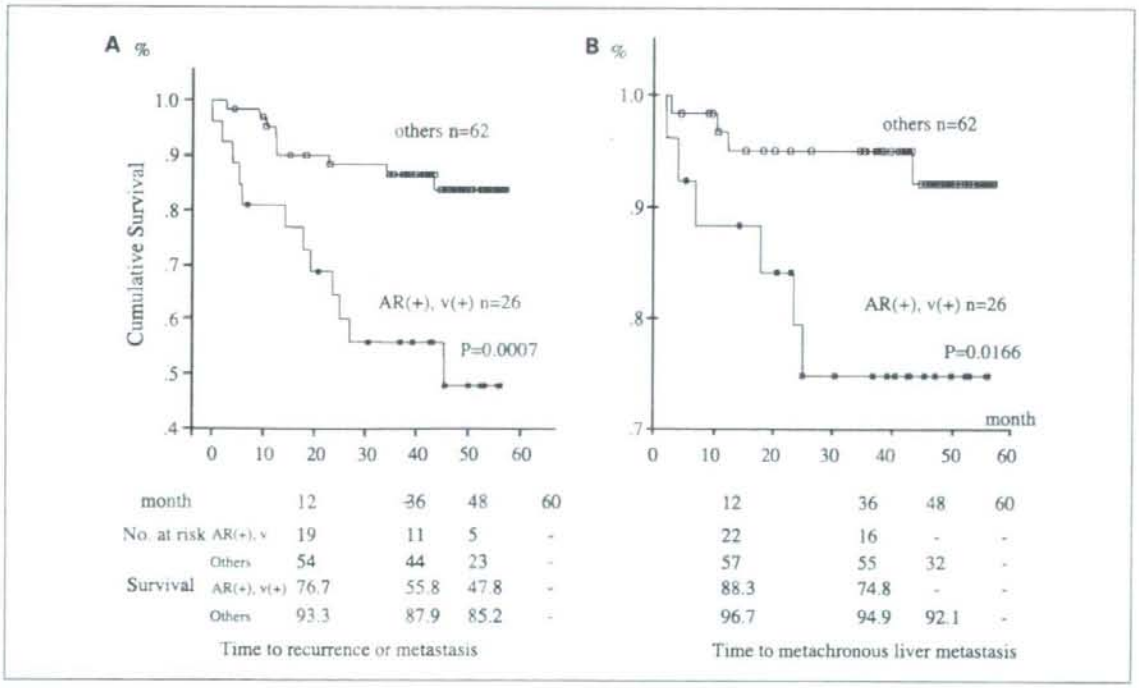

Fig. 3. Disease-free survival (*A*) and hepatic metastasis-free survival (*B*) after curative colectomy for colorectal cancer without synchronous metastases ($n = 88$).

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Overexpression of *EphA4* gene and reduced expression of *EphB2* gene correlates with liver metastasis in colorectal cancer

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Abstract. The Eph receptors, members of a large family of transmembrane receptor tyrosine kinases, play important roles in a variety of biological functions. Recent studies have suggested that EphA4 and EphB2 participate in the growth and development of various carcinomas. This study examined the relationship of *EphA4* and *EphB2* gene expression to clinicopathological factors, especially metastasis, in patients with colorectal cancer. We studied surgical specimens of cancer tissue and adjacent normal mucosa obtained from 205 patients with untreated colorectal cancer. The relative expression levels of *EphA4* and *EphB2* mRNA in the specimens were measured by quantitative real-time, reverse-transcription polymerase chain reaction. The relative expression level of *EphA4* mRNA was higher in the presence than in the absence of liver metastasis, whereas the relative expression levels of *EphB2* mRNA were similar. Analysis of the relationship between clinicopathological features and gene expression showed that high expression of the *EphA4* gene and low expression of the *EphB2* gene correlated with liver metastasis. There was no correlation between *EphA4* and *EphB2* gene expression. Our results suggest that overexpression of the *EphA4* gene and reduced expression of the *EphB2* gene might promote liver metastasis in colorectal cancer. Overexpression of the *EphA4* gene and reduced expression of the *EphB2* gene may thus be a useful predictor of liver metastasis in patients with colorectal cancer.

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Key words: *EphA4*, *EphB2*, colorectal cancer

Introduction

The Eph receptor family constitutes one of the largest groups of transmembrane receptor tyrosine kinases (1). They are activated by a second family of cell surface-anchored ligands, the ephrins, which are attached to the plasma membrane via either a glycosylphosphatidylinositol (GPI) linkage (type A) or a transmembrane sequence (type B). The Eph receptors are also divided into type A or type B according to their ligand-binding specificities. In general, type A receptors bind type A ephrin ligands, and type B ephrin ligands stimulate type B receptors. One molecule that shows an exception to this rule is EphA4, which can bind and respond to type B as well as type A ephrin ligands (2). These Eph receptors and their ligands have been implicated in a variety of biological functions, including axon guidance and migration of neural crest cells in the nervous system, establishment of segmental boundaries, and formation of angiogenic capillary plexi (3-7). Among Eph receptor family members, EphA4 and EphB2 are frequently overexpressed or functionally altered in many types of cancers, suggesting a role in tumor progression or angiogenesis (8-14).

In this study, we measured expression levels of the *EphA4* and *EphB2* genes in 205 pairs of cancer tissue and adjacent normal mucosa obtained from patients with colorectal cancer. To evaluate the clinical significance of EphA4 and EphB2, we examined correlations between the relative expression of these genes and clinicopathological features.

Materials and methods

Patients and samples. We studied surgical specimens of cancer tissue and adjacent normal mucosa obtained from 205 patients with untreated colorectal cancer. The patients underwent surgery at Yokohama City Medical Center, Gastroenterological Center and at Kanagawa Cancer Center between 2002 and 2006. Informed consent was obtained from each patient and the ethics committees of Yokohama City Medical Center and Kanagawa Cancer Center approved the protocol before

Table I. PCR primers and conditions.

Gene	Primer	Temperature (°C)	Product size (bp)
EphA4	5'-AGTCCTTCTGGTCTCTGTCTC-3' 5'-CTTCATCCGCTTCTTGTTTGG-3'	60	116
EphB2	5'-GCTTTCTGCTTACTGACTTAGG-3' 5'-GGTGGGAGGAGGGAAGAG-'3	60	105
ß-actin	5'-AGTTGCGTTACACCCTTTCTTGAC-3' 5'-GCTCGCTCCAACCGACTGC-3'	60	171

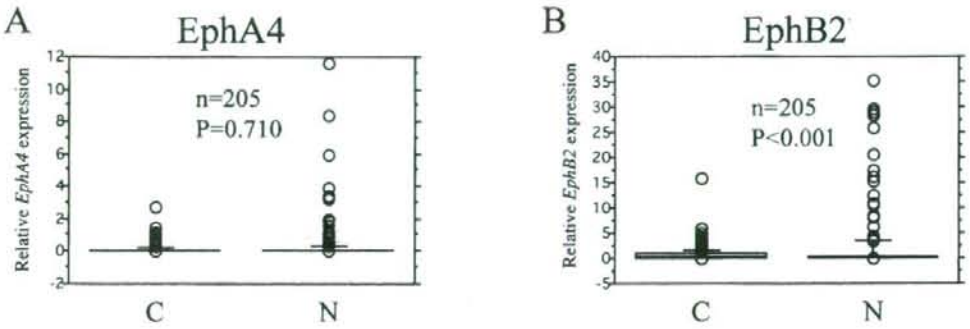

Figure 1. Comparison of *EphA4* and *EphB2* mRNA expression levels between colorectal cancer tissue and adjacent normal mucosa. P-values were calculated by the Wilcoxon test. *EphB2* gene expression levels were higher in adjacent normal mucosa than in cancer (P<0.001). *EphA4* gene expression levels did not differ significantly between cancer and adjacent normal mucosa.

initiation of the study. All tissue samples were 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% cancer 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 at 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 mRNA, 0.27 μM of each primer, 7.5 μl of iQ SYBR-Green Supermix containing dATP, dCTP, dGTP, and dTTP at concentrations 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 (Table I) and a primer extension for 1 min at 72°C followed by 10 min at 72°C. The PCR primer sequences of *EphA4*, *EphB2* and ß-actin, used as internal controls, are shown in Table I.

Statistical analysis. Gene expression levels of colorectal cancer were compared with those of adjacent normal mucosa by 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 different variables were calculated by simple regression analysis. All statistical analyses were performed using Statview J 5.0 software (Abacus, CA). Two-sided P-values were calculated and a difference was considered significant if the P-value was <0.05.

Results

Comparison of EphA4 and EphB2 mRNA expression between colorectal cancer tissue and adjacent normal mucosa. *EphB2* gene expression levels were higher in adjacent normal mucosa than in cancer (P<0.001) (Fig. 1B). *EphA4* gene expression levels were similar in cancer and adjacent normal mucosa (P=0.710) (Fig. 1A).

Relationship of EphA4 and EphB2 gene expression levels to clinicopathological features. Expression levels of the *EphA4* and *EphB2* genes were categorized as low or high according to their median values. The relationship between the expression of these genes and clinicopathological features were then examined. Expression levels of the *EphA4* and *EphB2* genes

Table II. Relationship between expression of the *EphA4*, *EphB2* genes and clinicopathological features.

Variables/categories	*EphA4* expression		P-value	*EphB2* expression		P-value
	low (n=103)	high (n=102)		low (n=103)	high (n=102)	
Age	65.4±10.5	66.3±11.1	0.534	66.0±10.9	65.6±10.7	0.912
Gender						
Male	60	52	0.296	55	57	0.721
Female	43	50		48	45	
Size						
≤5 cm	52	63	0.104	62	53	0.235
>5 cm	51	39		41	49	
Histological type						
Well differentiated	26	35	0.258	30	31	0.864
Moderately differentiated	64	52		60	56	
Poorly differentiated	13	15		13	15	
Depth of invasion						
T1	10	9	0.071	6	13	0.059
T2	38	56		52	42	
T3	48	32		36	44	
T4	7	5		9	3	
Lymph node metastasis						
Absent	49	46	0.722	47	48	0.838
Present	54	56		56	54	
Location						
Colon	54	58	0.524	63	49	0.059
Rectum	49	44		40	53	
Lymphatic invasion						
Absent	71	63	0.281	70	64	0.376
Present	32	39		32	38	
Venous invasion						
Absent	34	43	0.176	37	40	0.626
Present	69	59		66	62	
Liver metastasis						
Absent	78	63	0.031	64	77	0.039
Present	25	39		39	25	

were unrelated to age, gender, tumor size, lymph node metastasis, lymphatic invasion and venous invasion. High expression of the *EphA4* gene and low expression of the *EphB2* gene correlated with liver metastasis (P=0.031, 0.039) (Table II).

Relationship of EphA4 and EphB2 gene expression levels to liver metastasis. The highest rate of liver metastasis was associated with high expression of the *EphA4* gene and low expression of the *EphB2* gene (Fig. 2).

Associations of EphA4 and EphB2 gene expression with lymph node metastasis in patients with colorectal cancer. There was no significant association between the expression level of either gene and the presence or absence of lymph node metastasis (Fig. 3).

Associations of EphA4 and EphB2 gene expression with liver metastasis in patients with colorectal cancer. *EphA4*

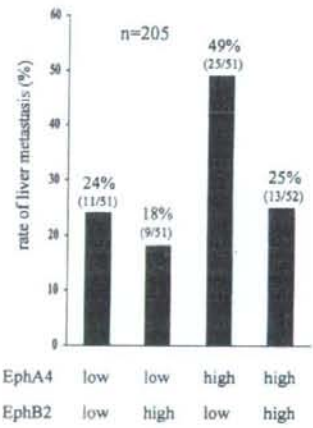

Figure 2. Relationship of *EphA4* and *EphB2* gene expression levels to liver metastasis. The highest rate of liver metastasis was associated with high expression of the *EphA4* gene and low expression of the *EphB2* gene.

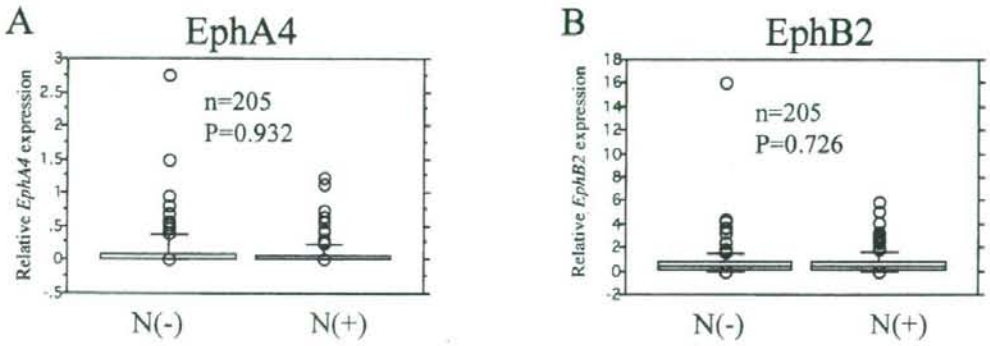

Figure 3. Associations of *EphA4* and *EphB2* gene expression with lymph node metastasis in 205 patients with colorectal cancer. Box boundaries, the 25th and 75th percentiles of the observed values; capped bars, the 10th and 90th percentiles; solid line, median. P-values were calculated by the Mann-Whitney U test. There was no correlation between the expression level of either gene and the presence or absence of lymph node metastasis.

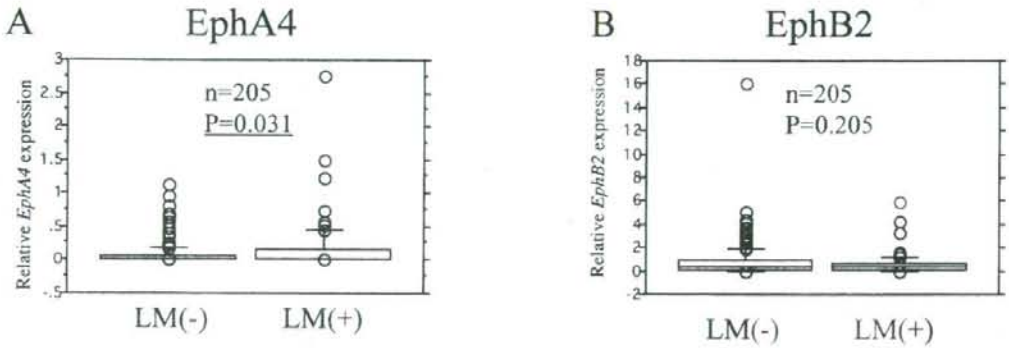

Figure 4. Associations of *EphA4* and *EphB2* gene expression levels with liver metastasis in 205 patients with colorectal cancer. Box boundaries, the 25th and 75 th percentiles of the observed values; capped bars, the 10th and 90th percentiles; solid line, median. P-values were calculated by the Mann-Whitney U test. *EphA4* gene expression levels were higher in the presence than in the absence of liver metastasis (P=0.031).

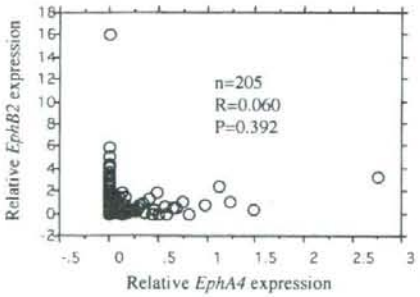

Figure 5. Correlation between *EphA4* and *EphB2* gene expression levels in colorectal cancers. Each gene expression level is relative to that of the B-actin gene. There was no correlation between *EphA4* and *EphB2* expression levels.

gene expression levels were higher in the absence than in the presence of liver metastasis (P=0.031) (Fig. 4A).

Correlation between EphA4 and EphB2 gene expression. The correlation between *EphA4* and *EphB2* gene expression levels is shown in Fig. 5. There was no correlation between *EphA4* expression and *EphB2* expression.

Discussion

Receptor tyrosine kinases and their ligands play critical roles in the regulation of a variety of cell activities, including cellular survival, proliferation, differentiation and tissue organization (15). Eph receptors and their ligands, ephrins, are indeed involved in several cell processes during embryonic development, such as pattern formation, cell aggregation and migration, segmentation, neural development, angiogenesis, and vascular hierarchical remodeling (3-7). The overexpression of some Eph receptor family members has an important role in the development and progression of various cancers. In particular, EphA4 and EphB2 overexpression is frequently associated with human invasive cancers (10-12,16,17).

In this study, we examined expression levels of the *EphA4* and *EphB2* genes in colorectal cancer and in adjacent normal mucosa. We also studied the relationship of these gene expression levels to clinicopathological features, as well as correlations among the expression of these genes.

Several previous studies have compared *EphA4* and *EphB2* mRNA expression levels between colorectal cancer tissue and adjacent normal mucosa. Ashida *et al* (17) found that the expression of *EphA4* is significantly higher in human prostatic cancer than in adjacent normal prostatic epithelium. Liu *et al*

(18) reported that the expression of EphB2 mRNA is lower in nonmalignant cell lines than in the colon cancer cell lines *in vitro*. Mao *et al* (19) showed that the expression of *EphB2* is higher in colorectal cancer tissue than in normal colorectal tissue (n=11). However, Guo *et al* (20) found that the expression of EphB2 protein is significantly higher in normal colorectal mucosa than in colorectal cancer. Our study (n=205) demonstrated that *EphB2* gene expression levels were higher in adjacent normal mucosa than in colorectal cancer tissue. This finding is consistent with the results of a previous study showing that EphB2 suppresses carcinogenesis, including the transition from colorectal adenoma to carcinoma (20). In contrast, *EphA4* gene expression levels did not differ significantly between cancer and adjacent normal mucosa.

A previous study examining the relationship between clinicopathological features and gene expression levels, found no significant correlation between EphA4 expression and the histological type of pancreatic ductal adenocarcinoma (16). This result was unexpected because the expression of exogenous EphA4 promotes the growth of pancreatic ductal adenocarcinoma cells (16). In our study, there was no significant correlation of EphA4 expression with tumor size, histological type, invasion, or lymph node metastasis in colorectal cancer. However, high *EphA4* gene expression correlated with liver metastasis.

As for EphB2, Wu *et al* (21) reported that *EphB2* gene expression does not correlate with clinical stage or histological grade in breast cancers. Guo *et al* (20) found that low expression of EphB2 correlates with invasion and metastasis in colorectal cancers. In our study, reduced *EphB2* gene expression correlated with liver metastasis in colorectal cancer. This result is considered reasonable, because EphB2 receptor activity suppresses colorectal cancer progression and metastasis (22,23). Thus, overexpression of the *EphA4* gene and reduced expression of the *EphB2* gene is associated with liver metastasis in colorectal cancer.

When expression levels of the *EphA4* and *EphB2* genes were contrasted with the presence or absence of lymph node metastasis, no correlation was noted for either gene. We also examined potential correlations of gene expression levels with the presence or absence of liver metastasis. Iiizumi *et al* (16) reported that EphA4 contributes to properties such as invasiveness or metastasis in a wide range of malignancies. Thorstensen *et al* (22) found that loss of heterozygosity at the *EphB2* locus was frequently associated with liver metastasis. In our study, *EphA4* gene expression levels were higher in the presence than in the absence of liver metastasis. This finding suggested that overexpression of *EphA4* mRNA might contribute to liver metastasis in colorectal cancer.

We then examined correlations between EphA4 and EphB2 gene expression in colorectal cancers. There was no significant correlation between the expression levels of these genes.

In conclusion, our results show that overexpression of the *EphA4* gene and reduced expression of the *EphB2* gene correlates with liver metastasis in colorectal cancer. Overexpression of the *EphA4* gene and reduced expression of the *EphB2* gene may thus be a novel marker or predictor of liver metastasis.

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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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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
β-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).

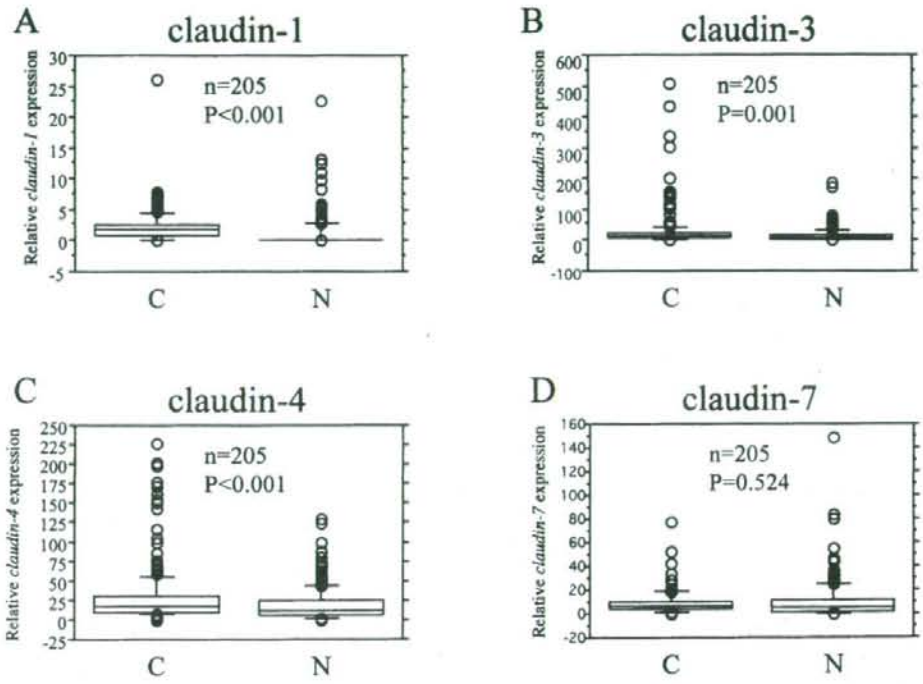

Figure 1. A comparison of *claudin-1*, *-3*, *-4* and *-7* mRNA expression levels between colorectal cancer tissue and adjacent normal mucosa. The *claudin-1*, *-3* and *-4* gene expression levels were higher in cancer than in normal adjacent mucosa (P<0.001, P=0.001, P<0.001). *Claudin-7* gene expression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

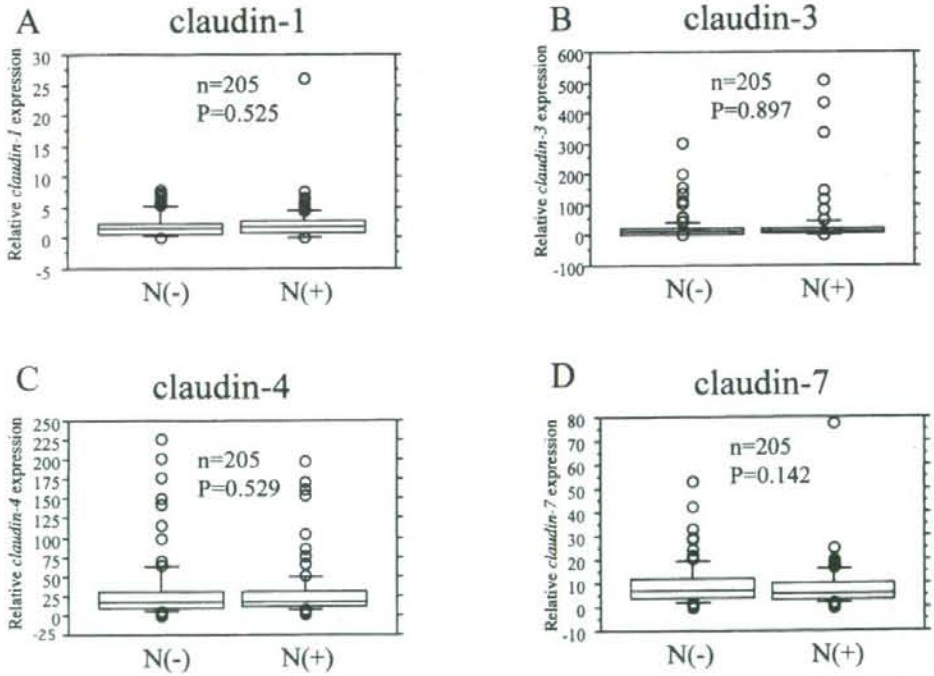

Figure 2. Associations of *claudin-1*, *-3*, *-4* and *-7* gene expression with lymph node metastasis in 205 patients with colorectal cancer. Box boundaries, the 25th and 75th percentiles of the observed values; capped bars, the 10th and 90th percentiles; solid line, median. P-values were calculated 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.