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

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Abstract. Matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9) and membrane-type matrix metalloproteinase 1 (MT1-MMP) are involved in colorectal cancer invasion and metastasis. Reversion-inducing cysteine-rich protein with Kazal motifs (RECK) inhibits MMP-2, MMP-9 and MT1-MMP. We examined the clinicopathological significance of the relative expression of these genes in patients with colorectal cancer, especially with regard to metastasis. We studied surgical specimens of cancer tissue and adjacent normal mucosa obtained from 205 patients with untreated colorectal carcinoma. *MMP-2*, *MMP-9*, *MT1-MMP*, *RECK* and *ß-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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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

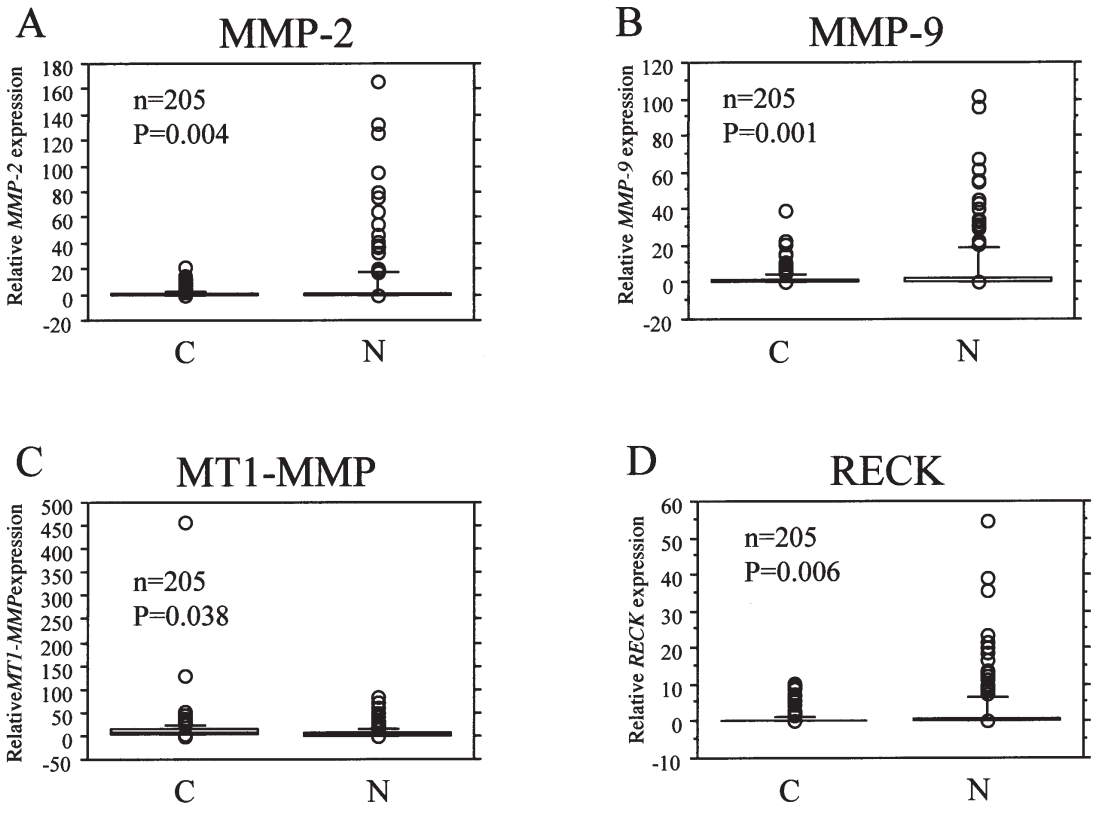

Figure 1. Comparison of *MMP-2*, *MMP-9*, *MT1-MMP* and *RECK* mRNA expression between colorectal cancer tissue and adjacent normal mucosa. *MMP-2*, *MMP-9* and *RECK* gene expression levels were higher in adjacent normal mucosa than in cancer tissue (P=0.0462, 0.0488 and 0.0491). However, the *MT1-MMP*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

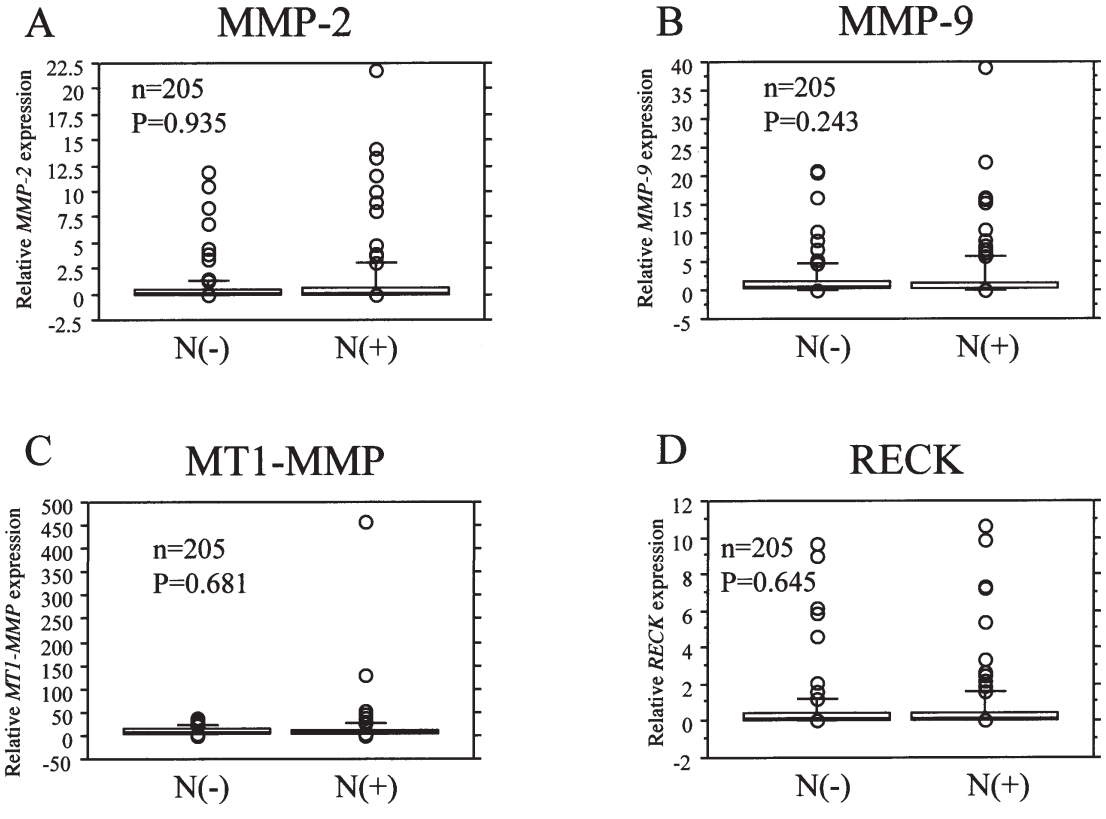

Figure 2. Association of *MMP-2*, *MMP-9*, *MT1-MMP* and *RECK* gene expression with lymph node metastasis in 205 patients with colorectal cancer. Box boundaries, the 25th and 75th percentiles of the observed values; capped bars, the 10th and 90th percentiles; solid line, the median. P-values were assessed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

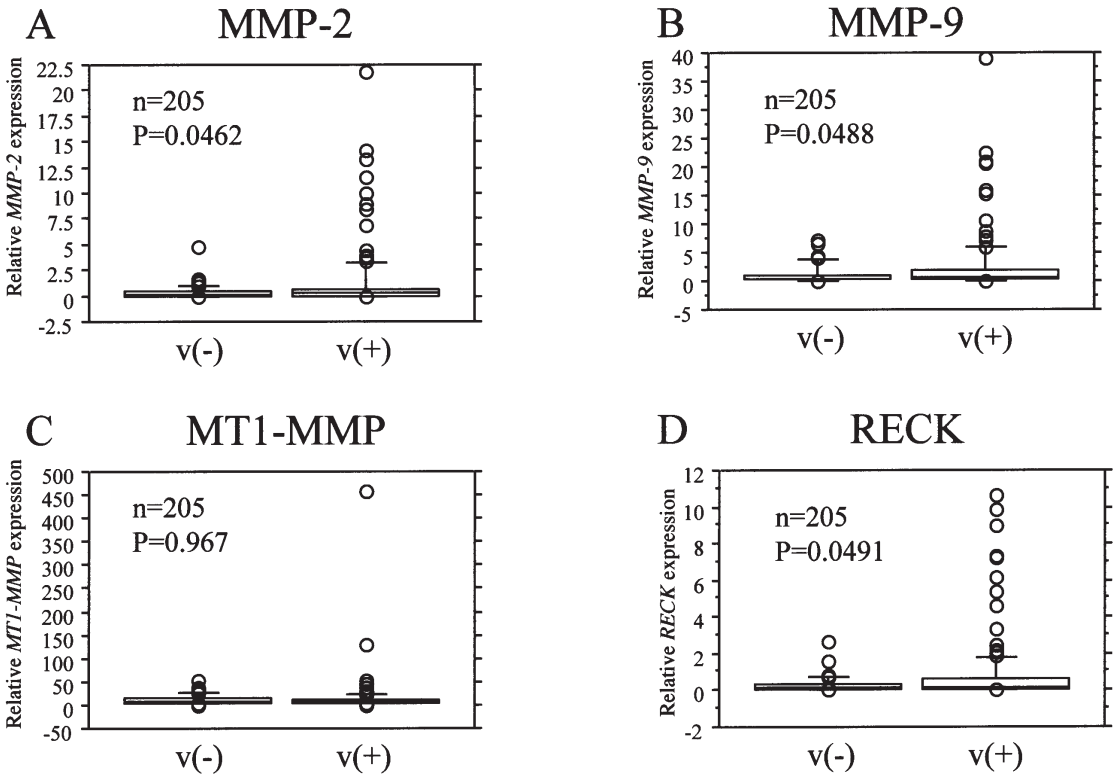

Figure 3. Association of *MMP-2*, *MMP-9*, *MT1-MMP* and *RECK* gene expression with venous invasion in 205 patients with colorectal cancer. Box boundaries, the 25th and 75th percentiles of the observed values; capped bars, the 10th and 90th percentiles; solid line, the median. P-values were assessed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

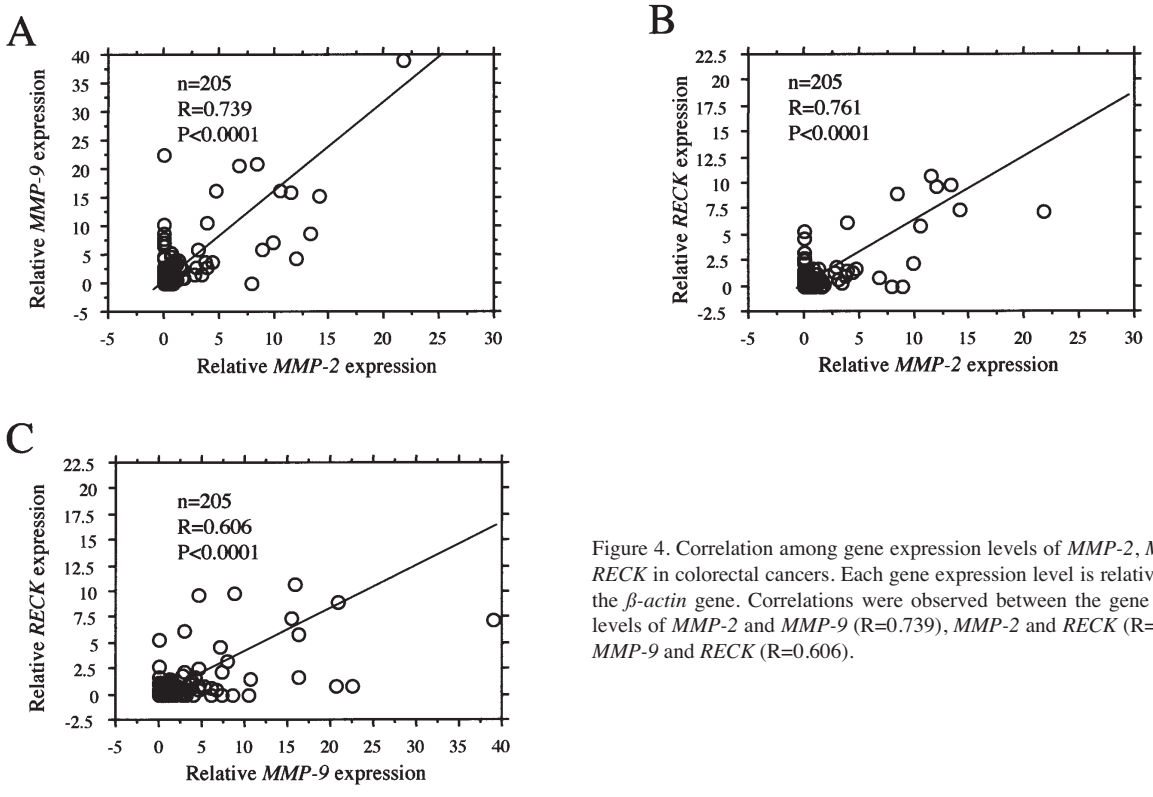

Figure 4. Correlation among gene expression levels of *MMP-2*, *MMP-9* and *RECK* in colorectal cancers. Each gene expression level is relative to that of the *β-actin* gene. Correlations were observed between the gene expression levels of *MMP-2* and *MMP-9* (R=0.739), *MMP-2* and *RECK* (R=0.761) and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