Overexpression of *MMP-13* Gene in Colorectal Cancer with Liver Metastasis

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Abstract. *Matrix metalloproteinase-7 (MMP-7), MMP-9, MMP-13, and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) are considered to have important roles in the invasiveness and outcomes of colorectal cancer (CRC). This study examined the clinicopathological significance of the relative expression of these genes in patients with colorectal cancer, especially as related to liver metastasis. The study analysed surgical specimens of cancer tissue and adjacent normal mucosa obtained from 202 patients with untreated colorectal cancer. MMP-7, MMP-9, MMP-13, TIMP-1, and β-actin mRNA of cancer tissue and adjacent normal mucosa were measured by quantitative real-time, reverse-transcriptase polymerase chain reaction. Expression levels of MMP-7, MMP-9, MMP-13 and TIMP-1 were higher in cancer tissue than in adjacent normal mucosa. On analysis of the relations between gene expression and clinicopathological factors, MMP-13 expression was found to correlate with liver metastasis. Moreover, MMP-13 expression levels were higher in tumour tissue with liver metastasis than in that without liver metastasis. It is concluded that MMP-13 gene expression is a useful predictor of liver metastasis in patients with CRC.*

Colorectal cancer (CRC), one of the most prevalent cancers worldwide (1), is the second leading cause of cancer-related mortality in developed countries (2). Tumour invasion and metastasis involve degradation of different components of the extracellular matrix, catalysed by proteolytic enzymes such as matrix metalloproteinases (MMPs) (3). Several MMPs have been shown to be associated with tumour stage and outcomes in CRC (4). Degradation of type IV collagen correlates with metastatic potential (5, 6). Type IV collagen is particularly abundant in basement membranes and is degraded by not only MMP-2 and MMP-9, but also by MMP-3, MMP-7, MMP-10, MMP-11, MMP-13, MMP-26.

The MMP family can cleave several extracellular matrix components during tumour invasion (7, 8). Expression of MMP-7 is elevated in several types of malignancy (9-11). MMP-9 can degrade denatured collagen, as well as types IV, V, VII, IX, and X collagen. *MMP-9* has been implicated in the progression, invasion, and metastasis of colorectal cancer in animal models and patients (12). Human collagenase-3 (MMP-13), which represents the third member of the collagenase subfamily, has been identified in human breast carcinomas and osteoarthritic cartilage (13, 14). *MMP-13* is detected in several types of malignancy (15-21). *MMP-13* has been shown to correlate with poor outcomes in different types of cancer (15, 22-28). In addition to acting as an MMP, MMP-13 also has a central role in the MMP activation cascade (15). MMP-13 is activated by MMP-2, MMP-3 and MT-MMP and then activates MMP-2 and MMP-9 (29-32). The activities of MMPs are regulated not only at the gene expression level, but also at the protein level by inhibitors, such as the family of tissue inhibitors of metalloproteinases (TIMPs). Recently, TIMP-1 has been demonstrated to have various functions such as inhibition of apoptosis, stimulation of growth and promotion of angiogenesis (33-39).

This study evaluated the relations of expression levels of *MMP-7, MMP-9, MMP-13* and *TIMP-1* to clinicopathological variables, especially liver metastasis, in patients with CRC.

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Key Words: MMP-13, colorectal cancer, liver metastasis.

Table I. *PCR primers and conditions*.

Gene	Primer	Temperature (˚C)	Product size (bp)
MMP-7	5'-CACTGTTCCTCCACTCCATTTAG-3' 5'-CATTTATTGACATCTACCCACTGC-3'	62.6	151
MMP-9	5'-TGGTCCTGGTGCTCCTGGTG-3' 5'-GCTGCCTGTCGGTGAGATTGG-3'	61.2	111
MMP-13	5'-CACTTTATGCTTCCTGATGACG-3' 5'-TCCTCGGAGACTGGTAATGG-3'	60.0	144
TIMP-1	5'-CTGTTGTTGCTGTGGCTGATAG-3' 5'-CGCTGGTATAAGGTGGTCTGG-3'	58.9	146
β-Actin	5'-AGTTGCGTTACACCCTTTCTTGAC-3' 5'-GCTCGCTCCAACCGACTGC-3'	60.0	171

Materials and Methods

Patients and samples. Analysis was performed on surgical specimens of cancer tissue and adjacent normal mucosa obtained from 202 patients with untreated CRC. The patients underwent surgery at Yokohama City University Medical Center, Gastroenterological Center and at Kanagawa Cancer Center, Department of Gastrointestinal Surgery between 2002 and 2006. Informed consent was obtained from all patients. The ethics committees of Yokohama City University Medical Center and Kanagawa Cancer Center approved the protocol before initiation of the study. All tissue samples were embedded in OCT 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 CRC and adjacent normal mucosa was prepared with the use of Trizol (Gibco, Life Tech, Gaithersburg, MD, USA). Complementary DNA (cDNA) was synthesised from 2 μg of total RNA with an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). 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 iQSYBR-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 50U/ml of iTag DNA polymerase. The PCR consisted of 10 min 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 *MMP-7, MMP-9, MMP-13, TIMP-1* and *β-actin*, used as an internal control, are shown in Table I.

Statistical analysis. Gene expression levels of CRC were compared with those of adjacent normal mucosa by the Wilcoxon test. Relations between gene expression and potential explanatory variables, including age, gender, tumour size, histological type, depth of invasion, location, lymphatic invasion, lymph node metastasis, venous invasion and liver metastasis, were evaluated with the χ^2 test. Associations between variables were assessed using the Mann-Whitney *U* test. All statistical analyses were performed using the SPSS II program, version 11.0.1J for Windows (SPSS Inc., Chicago, IL, USA). Two-sided *p* values were calculated, and a difference was considered statistically significant at $p<0.05$. Data are expressed as median±SD.

Results

Comparison of MMP-7, MMP-9, MMP-13 and TIMP-1 gene expression between CRC tissue and adjacent normal mucosa. Gene expression levels of *MMP-7, MMP-9, MMP-13* and *TIMP-1* were significantly higher in cancer tissue than in adjacent normal mucosa (*MMP-7*: 4.10±392.80 *versus* 0.05±4.65, $p<0.001$; *MMP-9*: 0.59±4.54 *versus* 0.11±5.78, $p<0.001$; *MMP-13*: 7.27±34.02 *versus* 2.20±11.23, $p<0.001$; and *TIMP-1*: 6.12±12.60 *versus* 0.66±2.53, $p<0.001$) (Figure 1).

Relations of clinicopathological features to MMP-7, MMP-9, MMP-13 and TIMP-1 gene expression levels in CRC tissue. Expression levels of each gene were categorised as low or high according to their respective median values. Relations between the expression levels of each gene and clinicopathological features were then examined. *MMP-7, MMP-9* and *TIMP-1* gene expression levels were unrelated to age, gender, tumour size, histological type, depth of invasion, tumour location, lymphatic invasion, lymph node metastasis, venous invasion and liver metastasis. High expression of the *MMP-13* gene was significantly related to liver metastasis (40/101 *versus* 22/101, $p=0.009$), but was unrelated to age, gender, tumour size, histological type, depth of invasion, tumour location, lymphatic invasion, lymph node metastasis and venous invasion (Table II).

Comparison of MMP-7, MMP-9, MMP-13 and TIMP-1 gene expression levels in CRC tissue according to the presence or absence of liver metastasis. Only *MMP-13* gene expression differed significantly according to the presence or absence of liver metastasis (8.89±59.16 *versus* 6.67±9.70, $p=0.015$).

Table II. *Relations between the expression of MMP-7, MMP-9, MMP-13 and TIMP-1 genes and clinicopathological features.*

Variable/category	MMP-7 Expression			MMP-9 Expression			MMP-13 Expression			TIMP-1 Expression		
	Low (n=101)	High (n=101)	*p*-Value	Low (n=101)	High (n=101)	*p*-Value	Low (n=101)	High (n=101)	*p*-Value	Low (n=101)	High (n=101)	*p*-Value
Age	66.8±10.7	64.6±10.9	0.155	66.1±10.7	65.3±11.0	0.564	65.3±11.1	66.1±10.5	0.572	66.1±11.4	65.3±10.2	0.627
Gender												
Male	62	48	0.066	56	54	0.888	50	60	0.203	58	52	0.480
Female	39	53		45	47		51	41		43	49	
Size												
<5 cm	55	57	0.887	58	54	0.671	62	50	0.119	60	52	0.322
≥5 cm	46	44		43	47		39	51		41	49	
Histological type												
Well differentiated	32	27	0.605	28	31	0.563	31	28	0.693	27	32	0.428
Moderately differentiated	57	58		61	54		58	57		62	53	
Poorly differentiated	12	16		12	16		12	16		12	16	
Depth of invasion												
T1/T2	55	55	1.000	59	51	0.323	58	51	0.323	52	58	0.480
T3/T4	46	46		42	50		43	50		49	43	
Location												
Colon	57	52	0.572	59	50	0.259	58	51	0.397	53	56	0.778
Rectum	44	49		42	51		43	50		48	45	
Lymphatic invasion												
Absent	66	66	1.000	68	64	0.658	71	61	0.183	64	68	0.658
Present	35	35		33	37		30	40		37	33	
Lymph node metastasis												
Absent	44	49	0.572	42	51	0.259	48	45	0.778	43	50	0.397
Present	57	52		59	50		53	56		58	51	
Venous invasion												
Absent	37	38	1.000	40	35	0.560	42	33	0.244	34	41	0.382
Present	64	63		61	66		59	68		67	60	
Liver metastasis												
Absent	68	72	0.647	70	70	1.000	79	61	0.009	73	67	0.446
Present	33	29		31	31		22	40		28	34	

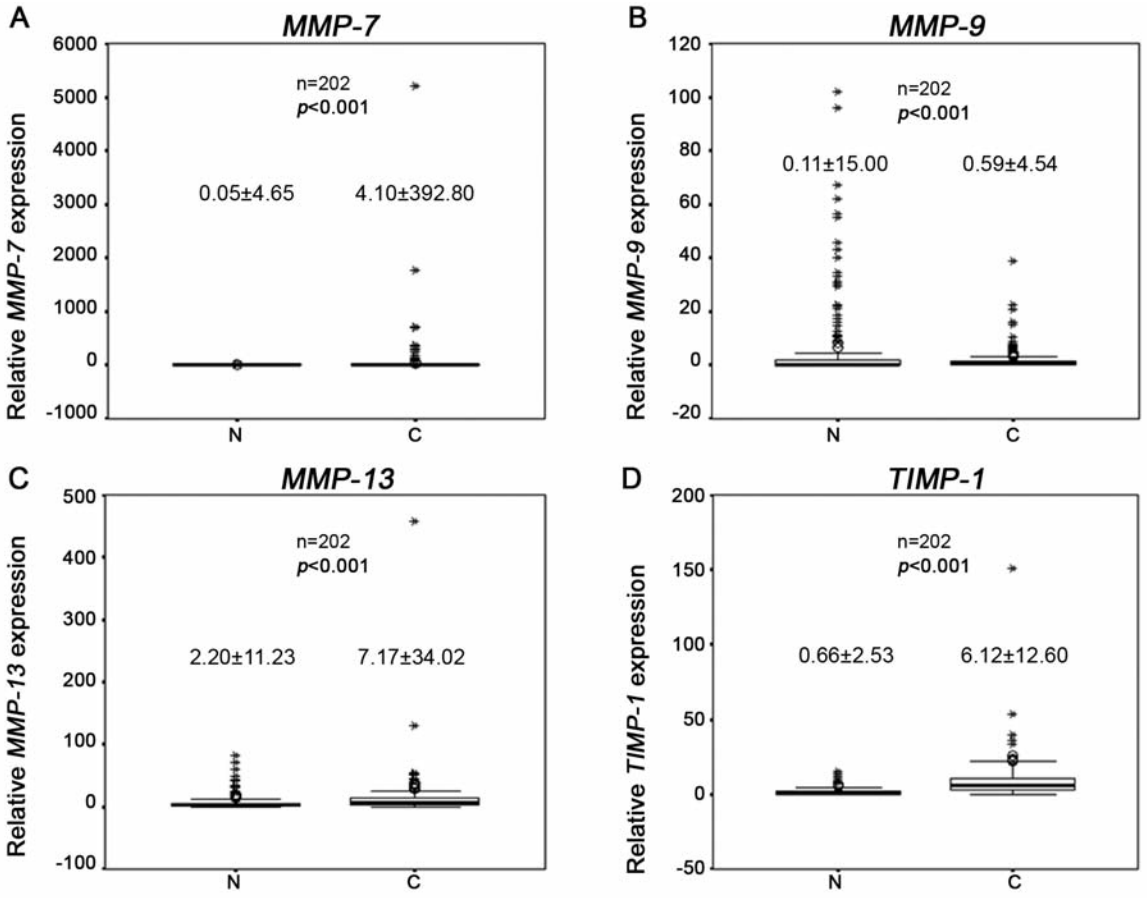

Figure 1. *Comparison of expression levels of MMP-7, MMP-9, MMP-13 and TIMP-1 genes between colorectal cancer tissue (C) and adjacent normal mucosa (N). Expression levels of MMP-7, MMP-9, MMP-13 and TIMP-1 genes were higher in cancer tissue than in normal adjacent mucosa (p<0.001 for all genes). p-Values were calculated by the Mann-Whitney U test.*

The expression levels of the other genes did not differ significantly according to the presence or absence of liver metastasis (Figure 2).

Discussion

MMPs play a key role in the development and progression of human malignancies (3, 40, 41). MMP-7, MMP-9 and MMP-13 correlate with tumour invasion, angiogenesis, metastasis and progression in CRC (15, 23, 42-47). Several previous studies have compared *MMP-7, MMP-9, MMP-13* and *TIMP-1* mRNA expression levels between CRC tissue and adjacent normal mucosa. Miyata *et al*. (48) reported that the expression of MMP-7 in tumour cells is significantly higher than that in normal cells. Pesta *et al*. (49) showed that the levels of *MMP-7* and *TIMP-1* mRNA differ significantly between normal colorectal tissue and tumour tissue. Kim *et al*. (50) and Lubbe *et al*. (51) found that *MMP-9* gene expression levels are higher in CRC than in adjacent normal mucosa. Leeman *et al*. (15) reported that MMP-13 activity is significantly higher in tumour tissue than in normal colonic mucosa and that such activity is localised to the cytoplasm of tumour cells. Offenberg *et al*. (52) demonstrated that *TIMP-1* is upregulated in CRC tissue as compared with normal tissue. Asano *et al*. (53) reported that the expression of *MMP-7* and *MMP-9* differs significantly between cancer tissue and normal mucosa, whereas the expression of *MMP-13* and *TIMP-1* does not. Asano *et al*. (53) compared the expression levels of these genes between 112 specimens of CRC and 20 specimens of normal mucosa. In the present study, gene expression levels were compared between 202 specimens of cancerous mucosa and 202 specimens of adjacent normal mucosa. The study showed that the gene expression levels of *MMP-7, MMP-9, MMP-13* and *TIMP-1* are higher in CRC tissue than in adjacent normal mucosa.

The present study examined whether expression levels of the *MMP-7, MMP-9, MMP-13* and *TIMP-1* genes are related to clinicopathological features. Nosho *et al*. (54) found that *MMP-7* gene expression correlates with tumour size, location and histopathological type in early CRC. Miyata *et al*. (48)

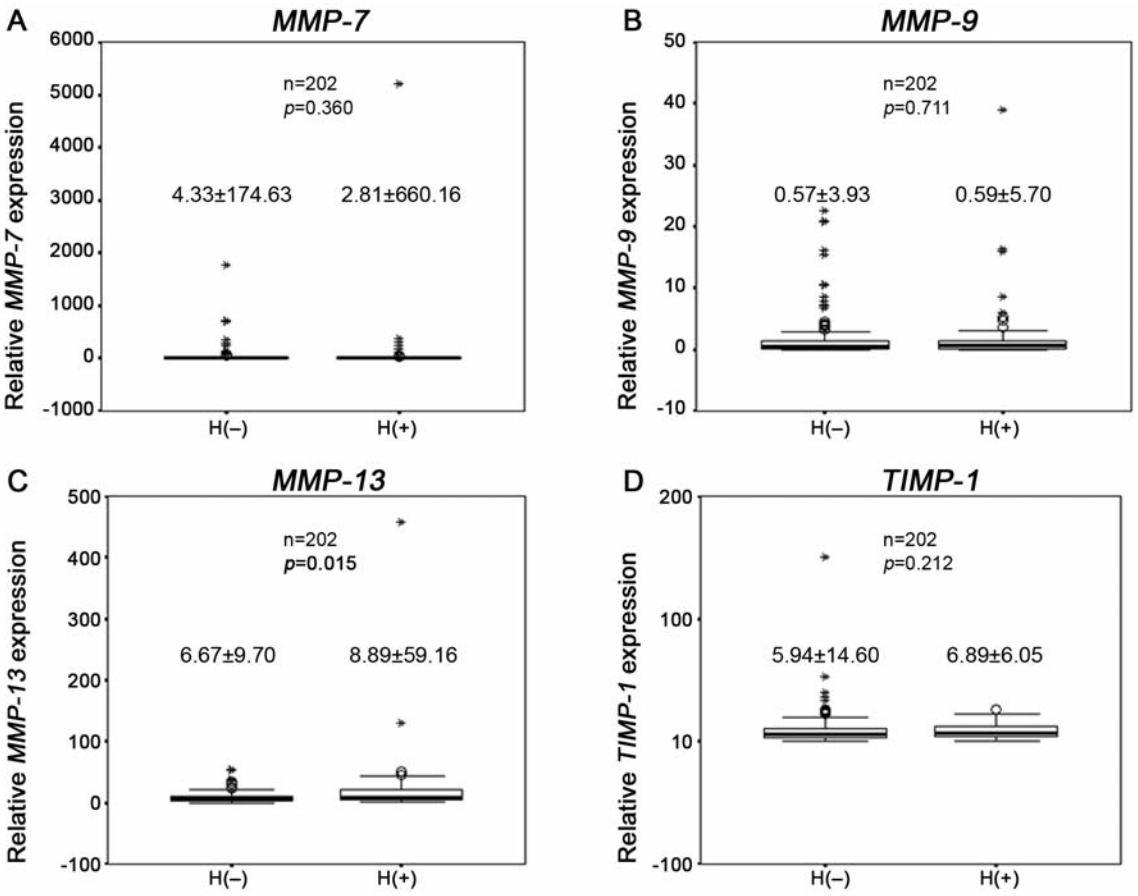

Figure 2. *Comparison of expression levels of MMP-7, MMP-9, MMP-13 and TIMP-1 genes in CRC tissue according to the presence (H+) or absence (H–) of liver metastasis. The expression level of MMP-13 gene was higher in the presence than in the absence of liver metastasis (p=0.015). p-Values were calculated by the Mann-Whitney U test.*

reported that MMP-7 expression in cancer cells correlates with an advanced pathological tumour stage. Ogata *et al*. (55) showed that MMP-9 expression is related to lymph node metastasis and severe venous invasion. Leeman *et al*. (15) demonstrated that MMP-13 activity is associated with poor survival in CRC. Pesta *et al*. (49) reported no significant correlation between *TIMP-1* expression and clinicopathological features. In the present study, *MMP-7, MMP-9* and *TIMP-1* expression, were unrelated to clinicopathological features. *MMP-13* expression was significantly associated with liver metastasis, but not with any other clinicopathological feature.

A number of previous studies have examined whether enzymatic expression levels of MMP-7, MMP-9, MMP-13 in CRC tissue correlate with the presence or absence of liver metastasis. Ogawa *et al*. (56) showed that the expression of MMP-7 is associated liver metastasis. Previous studies reported that MMP-7 plays an important role in the development of liver metastasis from human CRC (45-47). Zeng and Guillem (57) suggested that activation of MMP-9 may be a pivotal event in the formation of colorectal liver metastasis. Matsuyama *et al*. (58) reported that active-form MMP-9 is more strongly expressed in colon carcinomas from patients with liver metastasis than in those from patients without liver metastasis. Kopitz *et al*. (59) showed that *TIMP-1* promotes liver metastasis. The present study examined the relations of gene expression levels of *MMP-7, MMP-9, MMP-13* and *TIMP-1* to clinicopathological factors, including liver metastasis in patients with CRC. It was found that *MMP-7, MMP-9* and *TIMP-1* expressions were not related to liver metastasis in CRC. No previous study has examined whether *MMP-13* expression is related to liver metastasis in CRC. In this study, only *MMP-13* gene expression differed significantly according to the presence or absence of liver metastasis. These results showed that a higher *MMP-13* expression level is associated with a higher rate of liver metastasis. This finding suggests that *MMP-13* plays an important role in liver metastasis in patients with CRC. It is concluded that *MMP-13* gene expression may be a useful predictor of liver metastasis from CRC.

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