Matrix metalloproteinase-2 expression in stromal tissues is a consistent prognostic factor in stage II colon cancer

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For patients with stage II colon cancer, the usefulness of adjuvant chemotherapy remains controversial. Therefore, it is important to identify high-risk indicators. The biological prognostic factors for recurrence might allow further insight into the optimal treatment strategy for patients with node-negative disease. Matrix metalloproteinase-2 seems to be one of the essential factors for tumor invasion and lymph node metastasis. In this study, we analyzed the expression of cyclooxygenase-2 and matrix metalloproteinase-2 by immunohistochemical staining in 109 patients with stage II colon cancer. A positive correlation was observed between tumor cyclooxygenase-2 and tumor matrix metalloproteinase-2 expression ($P = 0.0006$) and between tumor cyclooxygenase-2 and stromal matrix metalloproteinase-2 expression ($P < 0.0001$). Stromal matrix metalloproteinase-2 expression was associated with disease-free survival ($P = 0.0095$) and was shown to be an independent risk factor for recurrence by multivariate analysis. In addition, we carried out an invasion assay *in vitro* to investigate whether cyclooxygenase-2 and matrix metalloproteinase-2 affected the tumor-invasive potential of colon cancer cell lines. The invasion assay showed that every cancer cell line acquired invasive potential in coculture with stromal cell lines and the cyclooxygenase-2 inhibitor suppressed this phenomenon by downregulating the matrix metalloproteinase-2 expression of stromal cells. In conclusion, these findings suggest that matrix metalloproteinase-2 expression in stromal cells can be a high-risk indicator for recurrence in patients with stage II colon cancer. (*Cancer Sci* 2009; 100: 852–858)

Colon cancer is among the most common malignancies worldwide and one of the leading causes of cancer-related death. The management of colon cancer has changed dramatically in the last 25 years. The use of adjuvant therapy has become standard practice in locally advanced but curative colon cancer (stage III and selected stage II).[1,2] For patients with stage III colon cancer, the benefit of overall survival by fluorouracil-based chemotherapy has been firmly established, and recent data has suggested further efficacy of the inclusion of oxaliplatin in adjuvant treatment programs.[2,3] For patients with stage II colon cancer, the usefulness of adjuvant chemotherapy remains controversial but might be appropriate in a subset of individuals with a high risk for cancer recurrence. Therefore, it is important to identify high-risk indicators in patients with stage II colon cancer. It has been reported that there might be some risk factors for recurrence, including the presence of budding, β_6 integrin and vascular endothelial growth factor (VEGF).[4,5] Moreover, several studies have proved that there is high expression level and activity of matrix metalloproteinases (MMPs) in many carcinomas, such as esophageal, lung, and stomach cancer.[6–9] Kubben *et al*. reported that MMP-2 was a consistent prognostic factor in gastric cancer.[6] The expression of MMP-2 has been shown to correlate with lymph node and distant metastases as well as shorter survival in breast cancer.[7–9]

MMP-2 activity can be detected in both cancer and stromal tissues.[10] However, there have been few studies about each function of cancer and stromal MMP-2 (s-MMP-2). The interactions between cancer cells and surrounding stromal cells due to the intrinsic properties of cancer cells might influence the mechanism of cancer invasion and metastasis. It has been reported that tumor-associated macrophages are the most important microenvironmental factor in tumor progression.[11–13] It is widely accepted that the existence of tumor-associated macrophages is correlated with poor prognosis, especially in breast cancer.[14]

Cyclooxygenase-2 (COX-2) is associated with elevated production of MMP-2 and plays an important role in tumor invasion and metastasis.[15–17] Sivula *et al*. reported that both COX-2 and MMP-2 in cancer cells were markers of poor prognosis in breast cancer.[18] Therefore, we tried to evaluate the clinical significance of COX-2 and MMP-2 expression in both tumor and stromal tissues of colon cancer. In this study, we evaluated whether the expression of COX-2 and MMP-2 could be a risk factor for recurrence, especially in stage II colon cancer. In addition, we carried out *in vitro* experiments to examine the clinical adequacy. For the purpose of understanding how tumor cells are affected by microenvironmental factors in the process of cancer progression, we examined monoculture (cancer cell controls) and coculture with THP-1, a human acute monocytic leukemia cell line that differentiates into macrophage-like cells after treatment with phorbol ester, using an invasion assay, and confirmed the correlation between invasive activity and MMP-2 expression.

Materials and Methods

Patients and tissue samples. The participants in this study were a total of 109 patients with stage II colon cancer who underwent curative resection from 1996 to 2005 at the First Department of Surgery, Sapporo Medical University Hospital (Sapporo, Japan). We gave those patients information about the planned research, and they decided whether they wished to participate. Informed consent was obtained from each patient in this study. The clinicopathological parameters are shown in Table 1.

Cell lines. Colon cancer cell lines CoCM-1, COLO201, COLO320, DLD-1, and WiDr were supplied by the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). The colon cancer cell line LoVo and human monocytic cell line

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Table 1. Association of positive cyclooxygenase-2 (COX-2) and matrix metalloproteinase-2 (MMP-2) in tumor cells and MMP-2 in stromal cells expression with clinicopathological parameters for patients of colon cancer

Characteristic	No. of patients	COX-2 positive	*P*-value	s-MMP-2 positive	*P*-value	t-MMP-2 positive	*P*-value	s- and t-MMP-2 positive	*P*-value
All cases	109	61		54		32		27	
Age(years)			<0.0001		NS		NS		NS
≤60	33	8		14		10		9	
>60	76	53		40		22		18	
Gender			NS		NS		NS		NS
Female	49	29		27		12		11	
Male	60	32		27		20		16	
Histological type			0.0461		NS		NS		NS
Well. Mod. Por.	103	16		53		31		26	
Muc. Other	6	42		1		1		1	
Tumor size (mm)			NS		NS		NS		NS
<50	70	43		36		24		20	
≥50	37	17		18		8		7	
Unknown	2	1		0		0		0	
Lymphathic invasion			NS		NS		NS		NS
ly0, ly1	105	58		51		30		25	
ly2, ly3	4	3		3		2		2	
Venous invasion			0.0288		<0.0001		0.0472		NS
v0, v1	82	41		30		20		17	
v2, v3	27	20		24		12		10	
Recurrence			NS		0.0037		NS		NS
Positive	14	8		12		5		5	
Negative	95	53		42		27		27	

s-MMP-2, stromal matrix metalloproteinase-2; t-MMP-2, tumor matrix metalloproteinase-2; NS, Not significant; Well., Well differentiated type; Mod., Moderately differentiated type; Por., poorly differentiated type; Muc., Mucinous differentiated type.

THP-1 were supplied by the RIKEN Bioresource Center (Tokyo, Japan). These cell lines were maintained in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Invitrogen) and 1% of an antibiotic-antimycotic solution (Invitrogen).

Immunohistochemistry. Tissue sections were rehydrated and immersed in a 0.3% H_2O_2-methanol solution for 30 min to block endogenous peroxidase activity. To enhance the immunoreactivity of COX-2 and MMP-2, antigens were retrieved by microwaving for 10 min in a target retrieval solution with pH 9.0 (Dako, Tokyo, Japan). Sections were incubated with 10% normal horse or goat serum for 20 min to block non-specific binding of the secondary antibody, then incubated for 18 h with the primary antibodies at room temperature at concentrations of 1:50 for the COX-2 mouse monoclonal antibody (Dako) and the MMP-2 mouse monoclonal antibody (Daiichi Fine Chemical, Toyama, Japan), then incubated for 30 min at room temperature with labeled polymer antimouse horseradish peroxidase (Dako). Antibody binding was detected by AEC substrate system (Dako). Nuclei were counterstained with Mayer's hematoxylin.

COX-2 staining was scored for intensity (0, 1+, 2+, or 3+) (Fig. 1a–c) and the percentage of cytoplasm staining (1, 0–25%; 2, 26–50%; 3, 51–75%; and 4, 76–100%). These scores have been referred to in several published reports.[19–21] The sum of the intensity and percentage counts was used as the final score. Tumor COX-2 expression was analyzed both qualitatively and quantitatively. For qualitative analyses, patients were classified as COX-2-positive (immunostaining score from 3 to 7) or COX-2-negative (immunostaining score from 0 to 2). MMP-2 staining was classified into negative when the staining was <10% or positive when the staining was 10% of the cells. Both lymphatic and venous invasion were classified into four grades according to the Japanese Classification of Colon Carcinoma: v0 and ly0, no invasion; v1 and ly1, slight invasion; v2 and ly2, moderate invasion; and v3 and ly3, extensive invasion.[22]

RNA extraction and complementary DNA synthesis. Total RNA was isolated with TRIzol reagent (Invitrogen) according to the standard acid-guanidium-phenol-chloroform method, followed by DNase (Invitrogen) treatment to avoid contamination of the genomic DNA. cDNA was generated with the Superscript First-strand Synthesis System (Invitrogen) according to the protocol recommended by the manufacturer. For first-strand synthesis, 3 μg total RNA was added to 1 μg oligo(dT) primer (0.5 μg/μL) in a 22 μL mixture. The mixture was incubated at 70°C for 10 min and on ice for 30 s, then 16 μL of a reaction mixture (8 μL 5 × reverse transcriptase buffer, 4 μL 10 mM dNTPs, 4 μL 0.1 M dithiothreitol, 2 μL RNase inhibitor; Invitrogen) was added, followed by incubation at 42°C for 5 min. The mixture was supplemented with 2 μL Superscript II reverse transcriptase (Invitrogen) and incubated at 42°C for 2 h. The reaction was terminated at 70°C for 15 min, followed by addition of 1 μL RNase H and incubation at 37°C for 20 min.

Invasion assay. An invasion assay was carried out in 24-well cell culture chambers using inserts with 8-μm pores (Becton Dickinson Labware, Franklin Lakes, NJ, USA), as described previously.[23] For an invasion assay, the inserts used were coated with Matrigel (Becton Dickinson Labware). Each membrane has a thin layer of Growth Factor Reduced Matrigel Basement Membrane Matrix that serves as a reconstituted basement membrane *in vitro*. Colon cancer cells were suspended in a chemotaxis buffer (RPMI-1640, 10% FBS) at 1.5×10^5/mL and added to inserts that were then transferred to wells containing a buffer with or without THP-1 (1.5×10^5/mL). The invasive activities of CoCM-1, COLO210, COLO320, DLD-1, WiDr, and LoVo were assessed in response to an FBS medium, an FBS medium containing THP-1 (1.5×10^5/mL), and an FBS medium containing THP-1 and a COX-2 inhibitor (COX-2 inhibitor I at 0, 10, or 100 μM) (Calbiochem, San Diego, CA, USA). After incubation for 24 h for the chemoinvasion assay, cells on the lower surface of the membrane were stained with a Diff-Quik kit (International Reagents, Kobe, Japan) and

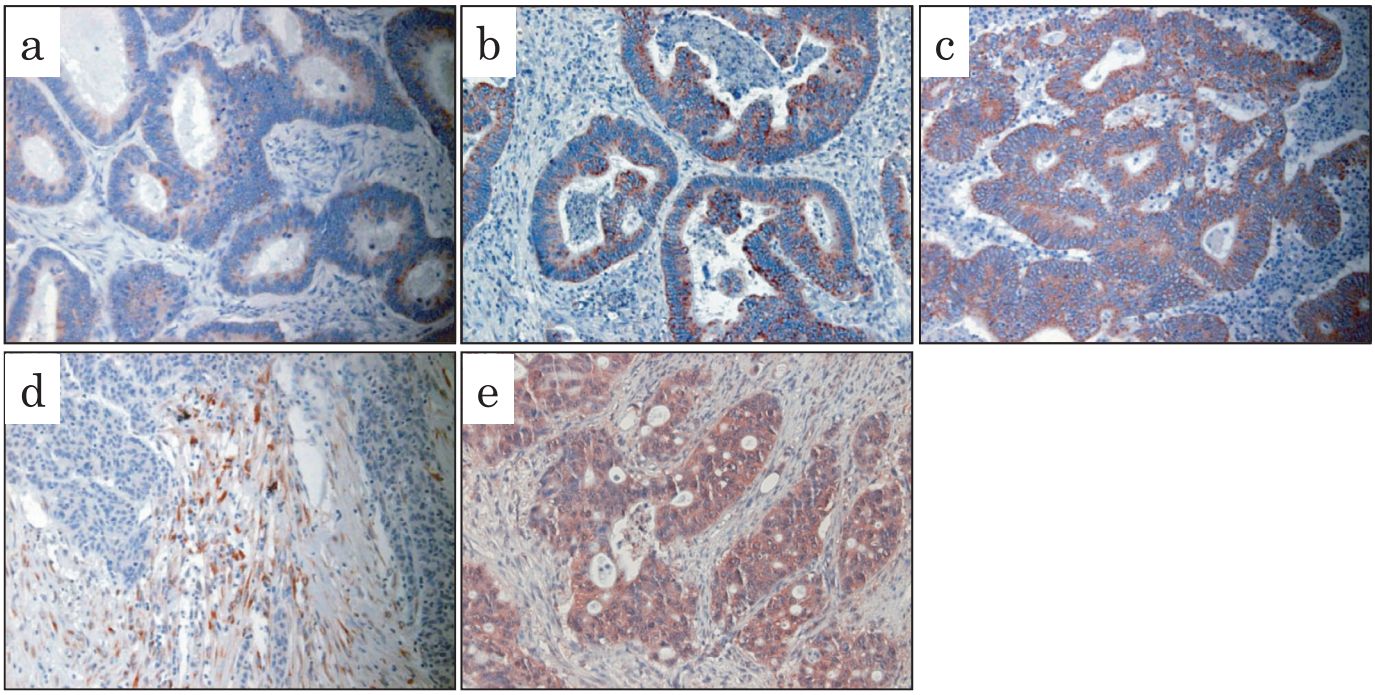

Fig. 1. Immunohistochemical staining for cyclooxygenase-2. Examples of colorectal adenocarcinoma with scoring of (a) 1+, (b) 2+, and (c) 3+ of cyclooxygenase-2 immunostaining intensity (×100). Immunohistochemical staining for (d) stromal matrix metalloproteinase-2 (×100), (e) tumor matrix metalloproteinase-2 (×100).

counted under a light microscope in five different fields (×100).

Polymerase chain reaction (PCR). Polymerase chain reaction was carried out in a Gene Amp PCR system 9700 thermocycler (Biosystems, Foster, CA, USA). The conditions for PCR were 30 s at 95°C for denaturation, 30 s at 60°C for annealing, and 60 s at 72°C for elongation (30 cycles). The cDNA fragments corresponding to MMP-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified with the following sets of primers: MMP-2 sense, AAC CCT CAG AGC CAC CCC TA; MMP-2 antisense, GTG CAT ACA AAG CAA ACT GC; GAPDH sense, GAA GGT GAA GGT CGG AGT; and GAPDH antisense, GAA GAT GGT GAT GGG ATT TC. PCR products were then loaded onto a 1% agarose gel and electrophoretically separated. The gel was then visualized under ultraviolet light after ethidium bromide staining.

Statistical analysis. The primary end point was disease-free survival (DFS) from the time of surgery until the date of recurrence. DFS curves were computed according to the Kaplan–Meier method. The χ^2-test was used to examine the association between the increased expressions of tumor COX-2 (t-COX-2), s-MMP-2, and tumor MMP-2 (t-MMP-2) and the various clinicopathological characteristics. For statistical evaluations, tumor sizes were grouped as small (<50) and large (≥50), lymphathic invasion was grouped as low (scores ly0 and ly1) and high (scores ly2 and ly3), and venous invasion was grouped as low (scores v0 and v1) and high (scores v2 and v3). To identify any independent factors for recurrence, we carried out a logistic analysis considering t-COX-2 positivity, s-MMP-2 positivity, t-MMP-2 positivity, lymphathic invasion, venous invasion, and tumor size as variables. A P-value <0.05 was considered significant.

Results

Expression of COX-2 and MMP-2 in colon cancer. COX-2 expression in tumor cells (t-COX-2) and cancer stromal cells (s-COX-2) was positive in 61 cases (56.0%) and 7 cases (6.4%), respectively. COX-2 expression was predominantly localized in tumor cells ($P < 0.05$). MMP-2 expression in tumor cells (t-MMP-2) and in cancer stromal cells (s-MMP-2) was positive in 32 cases (29.4%) and 54 cases (49.5%), respectively (Fig. 1d,e) (Table 1). The rate of s-MMP-2 expression was higher than that of t-MMP-2 expression ($P < 0.05$).

Associations of COX-2 and MMP-2 expression with clinicopathological factors. We investigated any associations of COX-2 and MMP-2 expression with clinicopathological factors, such as venous invasion, lymph invasion, and tumor histology. t-COX-2 was associated with age ($P < 0.0001$), histological type ($P = 0.0461$), and venous invasion ($P = 0.0288$). t-MMP-2 was associated with venous invasion ($P = 0.0472$). s-MMP-2 expression was associated with recurrence ($P = 0.0037$) and venous invasion ($P < 0.0001$) (Table 1). A positive association was observed between t-COX-2 and s-MMP-2 expression ($P < 0.0001$) and between t-COX-2 and t-MMP-2 expression ($P = 0.0006$) (Fig. 2a,b).

Logistic analysis for recurrence. t-COX-2 positivity, t-MMP-2 positivity, lymphathic invasion, venous invasion and tumor size were not associated with recurrence. However, s-MMP-2 expression was significantly associated with recurrence and was shown to be an independent risk factor for recurrence (Table 2).

Survival analysis. t-COX-2 and t-MMP-2 expression was not associated with worsened survival, whereas the prognosis of s-MMP-2 positive cases was worse than that of negative cases ($P = 0.0095$) (Fig. 2c–e). The five-year DFS of s-MMP-2 negative and positive groups were 92% and 63%, respectively (Fig. 2e).

Invasion assay using colon cancer cell lines. To investigate the effects of stromal cells on the invasive activity of colon cancer cell lines, we carried out an invasion assay using THP-1 as a surrogate. For every cell line, the number of cancer cells that invaded through the Matrigel was significantly higher in coculture with THP-1 than in monoculture of only cancer cells (Fig. 3a–c). We examined MMP-2 mRNA expression in THP-1 cocultured with cancer cell lines to determine the role of

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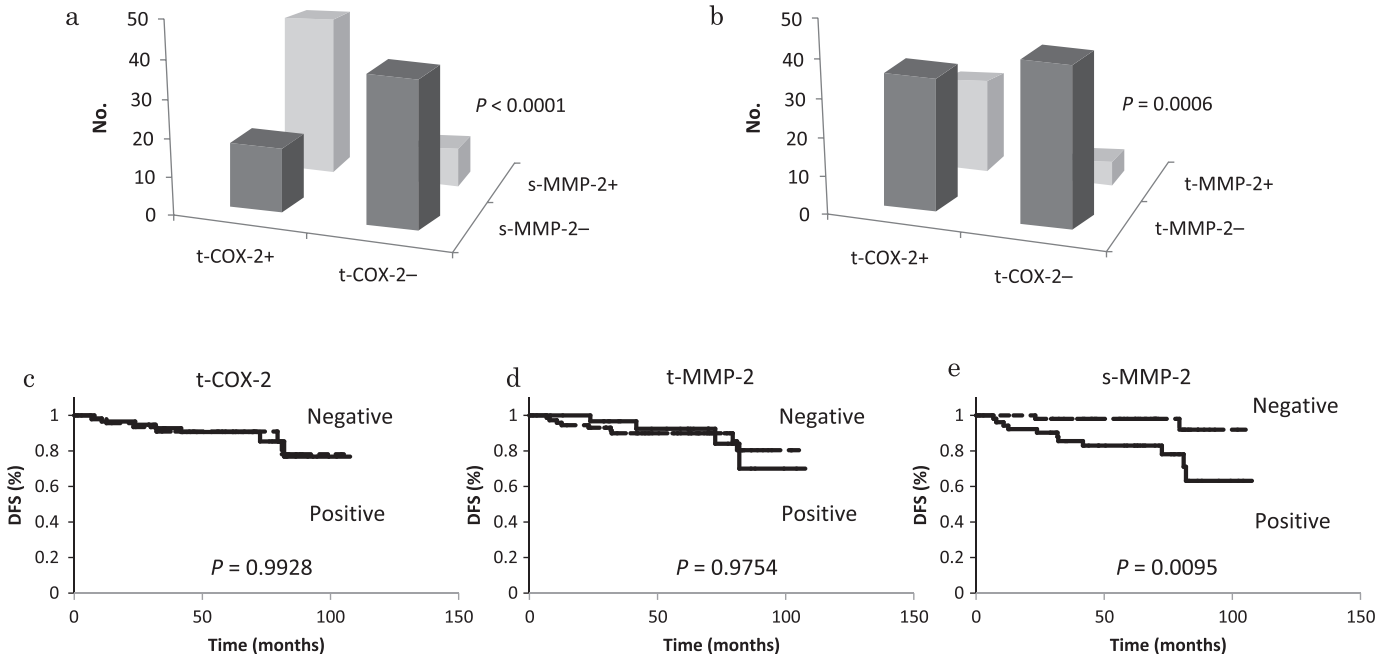

Fig. 2. Correlation between cyclooxygenase-2 (COX-2) and matrix metalloproteinase-2 (MMP-2) expression. (a) Tumor COX-2 (t-COX-2) and stromal MMP-2 (s-MMP-2) expression; (b) t-COX-2 and tumor MMP-2 (t-MMP-2) expression. Survival analysis showing that (c) t-COX-2 and (d) t-MMP-2 expressions were not associated with worsened survival. (e) The prognosis of s-MMP-2 positive cases was worse than that of negative cases ($P = 0.0095$). DFS, disease-free survival.

Table 2. Logistic analysis for recurrence of colon cancer in patients with stage II disease

Factor		No. of patients (No. of patients for recurrence)	Univariate analysis			Multivariate analysis		
			Relative risk	95% CI	*P*-value	Relative risk	95% CI	*P*-value
t-COX-2 positive		61(8)	1.0570	0.340–3.282	NS	0.337	0.079–1.435	NS
s-MMP-2 positive		54(12)	7.5710	1.606–35.700	0.0105	28.380	3.784–212.800	0.0011
t-MMP-2 positive		32(5)	1.3990	0.430–4.557	NS	0.608	0.153–2.419	NS
s- and t-MMP-2 positive		27(5)	1.8430	0.559–6.076	NS	2.065	0.545–7.826	NS
Lymphathic invasion	ly0	45(6)	0.8909	0.391–2.944	NS	0.725	0.238–2.213	NS
	ly1	60(7)						
	ly2	4(1)						
	ly3	0(0)						
Venous invasion	v0	28(3)	1.0640	0.531–2.132	NS	0.562	0.247–1.282	NS
	v1	54(8)						
	v2	22(2)						
	v3	5(1)						
Tumor size (mm)	<30	20(3)	0.8530	0.444–1.640	NS	0.786	0.368–1.676	NS
	≥30<50	50(7)						
	≥50<70	26(3)						
	≥70	11(1)						
	Unknown	2(0)						
Total		109(14)	–	–	–	–	–	–

–, unanalyzable; CI, confidence interval; NS, not significant; s-MMP-2, stromal matrix metalloproteinase-2; t-COX-2, tumor cyclooxygenase-2; t-MMP-2, tumor matrix metalloproteinase-2.

s-MMP-2 in cancer invasion. MMP-2 expression in THP-1 cocultured with cancer cell lines was higher than that in monoculture of THP-1 (Fig. 4a).

Invasion assay in medium including COX-2 inhibitor. For the purpose of downregulation of the MMP-2 expression of THP-1, we added the COX-2 inhibitor to the medium, and its effect was confirmed by reverse transcription–PCR (Fig. 4b). The invasive activity of every cancer cell line was significantly inhibited by the COX-2 inhibitor in a dose-dependent manner (Fig. 5a–d). It was confirmed that these concentrations of the COX-2 inhibitor had no effect on the growth of cancer cells.

Discussion

Gray *et al*. reported that chemotherapy with fluorouracil and folinic acid could improve the survival rate of patients with

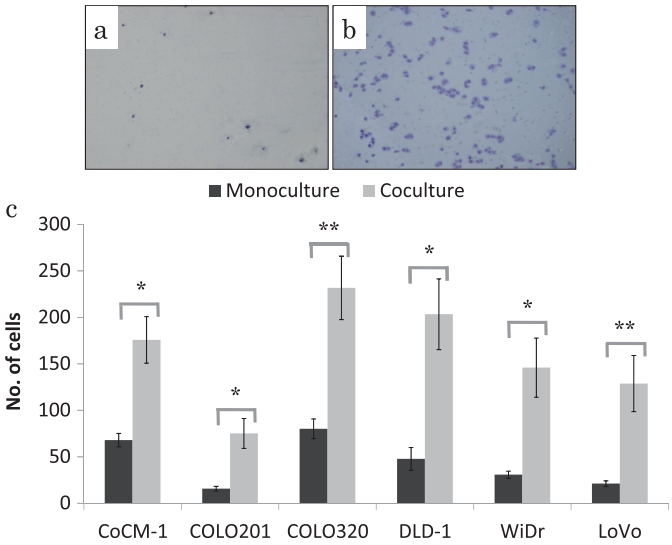

Fig. 3. Invasion assay using colon cancer cell line WiDr. Diff-Quick staining in the cancer cells (×100), with stained cells representing the cancer cells that invaded through the Matrigel. (a) Monocultured WiDr; (b) cocultured WiDr with THP-1. (c) For every cell line, the number of cancer cells that invaded through the Matrigel was significantly higher under the condition of coculture with THP-1 than under that of monoculture. The results are expressed as the mean number of cells that invaded ± standard deviation of five different experiments. $*P < 0.05$; $**P < 0.01$.

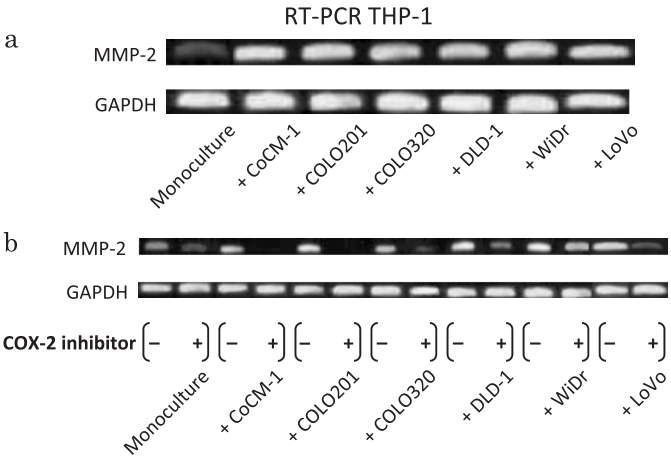

Fig. 4. Electrophoresis of reverse transcription–polymerase chain reaction (RT-PCR) products using a 1% agarose gel. (a) The matrix metalloproteinase-2 (MMP-2) expressions in THP-1 cocultured with cancer cell lines were higher than those in monoculture of THP-1. (b) The MMP-2 expressions in THP-1 cocultured with cancer cell lines were significantly inhibited by the cyclooxygenase-2 (COX-2) inhibitor. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

stage II colon cancer.[24] The five-year survival rate for stage II is 60–85% worldwide,[1,2] and, in Japan, 80–94%.[25–29] These differences may be attributed to differences in race, chemosensitivity, and the grade of malignancy of the cancer itself. The indications for adjuvant chemotherapy for stage II colon cancer remains controversial in Japan.

In this study, we showed that s-MMP-2 expression was associated with DFS and recurrence in colon cancer. Multivariate analysis showed that s-MMP-2 expression was an independent risk factor for recurrence. Malignant tumors express various MMPs, some of which are produced in stromal cells, such as fibroblasts and infiltrating macrophages.[30] Taniwaki *et al*. reported that MMP-2 derived from stromal cells promoted membrane type 1 MMP-dependent tumor growth and that s-MMP-2 might be an important factor for invasion and metastasis.[31]

In a recent study, it was reported that COX-2 was associated with MMP-2 and played an important role in tumor invasion and metastasis. Larkins *et al*. reported that the inhibition of COX-2 decreased breast cancer cell motility, invasion, and MMP expression.[32] To investigate whether a COX-2 inhibitor could affect the invasive activity of colon cancer cell lines, we carried out an invasion assay using a medium including the COX-2 inhibitor. The COX-2 inhibitor downregulated t-COX-2 expression at first, then reduced the amount of COX-2 in the medium. As a result, the COX-2 inhibitor might downregulate stromal (THP-1) MMP-2 expression. Our experiment revealed that the COX-2 inhibitor downregulated the MMP-2 expression of THP-1 and suppressed the invasive potential of all cancer cell lines.

Metastasis is characterized to progress through several steps, most of which are mainly dependent upon proteolysis of the basement membrane and other extracellular barriers. Therefore, the expression of MMPs in tumor cells is thought to be essential for metastasis. Prostaglandins are catalytic products of COX-2 that play important roles in the regulation of the metastatic potential of cancer cells.[33] In particular, prostaglandin E2 has been reported to regulate the metastatic potential of cells through regulation of MMP-2 expression.[32,33] Thus, COX-2 expression has been thought to be associated with MMP-2 expression.

In clinical cases, the suppression of cancer invasion and improvement of the prognosis of colon cancer by giving only a COX-2 inhibitor are considered to be difficult, according to results in a few of clinical trials.[34,35] In the future, a clinical trial could be planned under an adjuvant setting, combined with chemotherapy, if a safe and effective COX-2 inhibitor could be produced.

There are several important prognostic factors of colon cancer. Tanaka *et al*. reported that tumor budding was a useful prognostic finding and its existence could be used to identify patients with stage II colon cancer who have a high risk of recurrence after curative surgery.[36] Furthermore, it has been reported that β_6 integrin is a novel prognostic indicator of aggressive colon cancer,[4] and that VEGF might predict survival in patients with peritoneal surface metastases from mucinous adenocarcinoma.[5]

There are some reports about risk factors for recurrence and survival in stage II colon cancer (Table 3). Emergency presentation and tumor location in the sigmoid or descending colon are considered to be probable prognostic factors.[37] In the current study, lymphovascular invasion was an independent predictor of recurrence. Similar observations were reported by Lennon *et al*. and Resnick *et al*.[38–40] Lennon *et al*. and Morris *et al*. reported that peritoneal involvement discriminated between high- and low-risk Stage II colon cancer.[38,41] The expression of p53, epidermal growth factor receptor, claudin-1 and VEGF can predict event-free survival,[39,40,42] although these have not been confirmed by other studies.

Our study indicated that the communication between cancer and stromal cells might play a critical role in tumor invasion and that s-MMP-2 can be an indicator of the risk of recurrence in patients with stage II colon cancer.

MMP-2 expression in stromal cells can be a high-risk indicator of recurrence in stage II colon cancer. The results obtained from the invasion assay supported our clinical findings with certainty.

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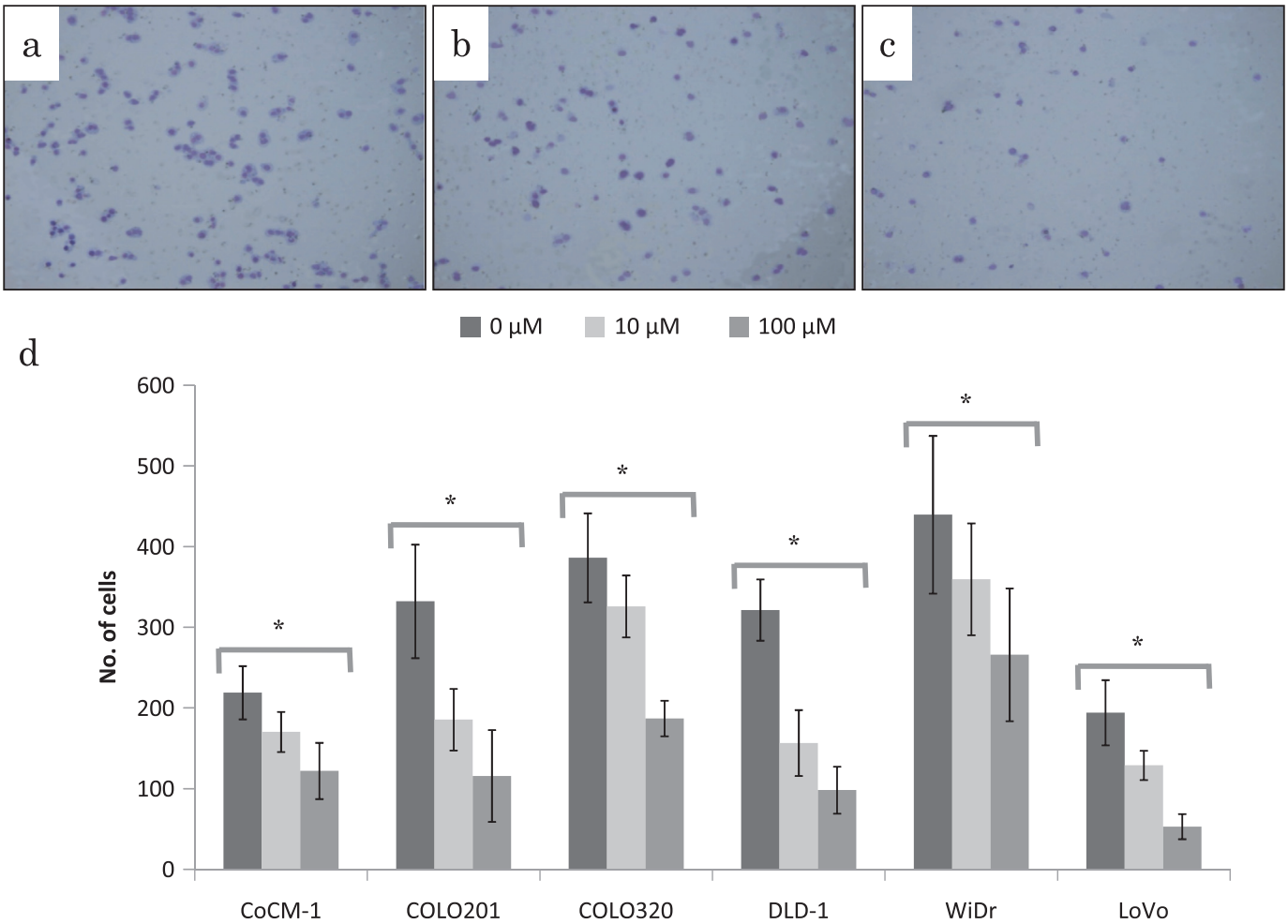

Fig. 5. Invasion assay with the cyclooxygenase-2 (COX-2) inhibitor using DLD-1. Diff-Quick staining in the cancer cells (×100) with stained cells representing the cancer cells that invaded through the Matrigel. DLD-1 cultured with COX-2 inhibitor (a) 0 μM, (b) 10 μM, and (c) 100 μM. (d) The invasion activity of every cancer cell line was significantly inhibited by the COX-2 inhibitor in a dose-dependent manner. The results are expressed as the mean number of cells that invaded ± standard deviation of five different experiments. $*P < 0.05$.

Table 3. Risk factors for cancer recurrence and related death in stage II colon cancer

Author	No. of patients	Risk factor	Endpoint
Merkel[37]	305	Emergency presentation Left colon pT3 >15 mm (invasion >15 mm), pT4	5-year DFS
Lennon[38]	118	Lymphovascular invasion Neural invasion Peritoneal involvement	5-year survival
Resnick[39]	134	P53 EGFR Lymphovascular invasion	Recurrence, survival
Resnick[40]	129	Claudin-1 Lymphovascular invasion	Recurrence, survival Recurrence
Morris[41]	1086	Spread beyond muscularis propria Peritoneal involvement Venous invasion Margin involvement Tumor perforation	Survival
Cascinou[42]	121	VEGF	Recurrence

DFS, disease-free survival; EGFR, epidermal growth factor receptor; VEGF, vascular endothelial growth factor.

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