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Correlation between Expression of Matrix Metalloproteinase-2 (MMP-2), and Matrix Metalloproteinase-9 (MMP-9) and Angiogenesis in Colorectal Adenocarcinoma

Matrix metalloproteinases-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9), which degrade extracellular matrix, are believed to play a crucial role in tumor invasion and metastasis. Angiogenesis is also perceived as an important step in tumor growth and metastasis. To investigate the expression of MMPs and the correlation between the expression of MMPs and angiogenesis in colorectal adenocarcinoma, we studied 72 cases of colorectal adenocarcinoma in Inha University Hospital from 1996 to 1997. We evaluated the expression of MMPs by immunohistochemistry and angiogenesis by counting the microvessels. The expression of MMP-2 was increased according to the Astler-Coller stage ($p<0.05$). Angiogenesis in the metastatic group was higher than that of the localized one ($p<0.05$). The expression of MMP-2 positively correlated with angiogenesis ($p<0.05$), and marked expression of MMP-9 positively correlated with angiogenesis ($p<0.05$). The present results suggest that the expression of MMP-2 provides clues for tumor progression and angiogenesis provides significant information to predict whether metastasis is present in colorectal adenocarcinoma.

Key Words: *Metalloproteinases; Angiogenesis factor; Adenocarcinoma; Colorectal neoplasms*

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INTRODUCTION

Matrix metalloproteinases (MMPs) are perceived to play an essential role in tissue remodeling during morphogenesis, wound healing, angiogenesis and pathological processes, such as tumor invasion and metastasis (1). MMP family consists of structurally related enzymes, which are collectively capable of degrading various extracellular matrices that are important not only as structural support but also as a biological regulator of cell growth and differentiation (2, 3). Two interstitial collagenases, three stromelysins, matrilysin, and macrophage metalloproteinase belong to the MMP family. Recently, a group of epithelial cell membrane bound MMPs (membrane type-MMP-1, -2, and -3) has been described which activates pro-MMP-2 (4).

The 72 kDa MMP-2 and 92 kDa MMP-9 are closely related in their structures and show similar substrate specificites, like type IV collagen which is the major component of basement membrane (5, 6). In colorectal adenocarcinomas, MMP-2 and MMP-9 play the most important role in degrading the basement membrane (7-9). Recently, several studies proved that the expression of MMP-2 and MMP-9 are directly related to the metastasis of colorectal adenocarcinomas and quantitatively by zymography (10, 11). However, they were performed by zymography, so they could not prove the localization of MMPs. In previous studies, the pattern of cellular localization of MMPs in colorectal adenocarcinomas was reported as predominantly located in the stromal cells including fibroblasts or infiltrating inflammatory cells (12-14), showing an importance of tumor-stromal cell interaction in the production of MMPs. However, several studies using immunohistochemistry showed that the expression of MMP-2 or MMP-9 was localized to colorectal adenocarcinomas with polyclonal antibodies to these enzymes, and could be regarded as a prognostic indicator by comparison to Astler-Coller (A-C) stage (8, 15).

It has been proven that angiogenesis plays a major role in tumor growth and highly contributes to tumor metastasis (16). Intratumoral microvessel density (MVD) correlates with tumor size and depth, local or distant meta-

stasis, and patient's survival (17). Several previous studies showed an association between MVD and metastasis in colorectal adenocarcinomas (18, 19). Frank et al. (20) showed MVD is an important independent prognostic predictor especially in node-negative colorectal adenocarcinomas [Astler-Coller stage A/B].

In the present study, we found that the expression of MMP-2 and MMP-9 was significantly higher in metastatic colorectal adenocarcinomas than in localized ones semiquantitatively, and also demonstrated correlations between the expression of MMPs and angiogenesis in each metastatic and localized colorectal adenocarcinoma.

MATERIALS AND METHODS

Patients

Seventy-two potentially curable and operable colorectal adenocarcinoma patients (43 colon, 13 sigmoid colon, 16 rectum), whose cancer has not spread to distant organs except liver, were operated on between January 1996 and November 1997. No patients received adjuvant chemotherapy, while 30 patients with colorectal adenocarcinomas, separated by modified Astler-Coller classification system B2, received postoperative radiotherapy of 25 Gy for five days. The patients were 38 women and 34 men of ages ranging from 15 to 87 yr (60.4±13.1 yr). Seventy-two patients were potentially cured with a radically excised tumor in modified Astler-Coller classification A, B, C (6, 34, and 28 patients, respectively) and D (four patients).

Immunohistochemical staining

Serial 4 μm sections were performed with deparaffination and hydration. Then the sections were stained, using the avidin-biotin staining technique (ABC Elite, Vector, Burlingame, CA, U.S.A.). Anti-CD34 monoclonal antibody, anti-MMP-2 monoclonal antibody, and anti-MMP-9 monoclonal antibody were obtained from Novocastra (Newcastle, Tyne, U.K.) and Oncogene Science (U.S.A.). MMP-2 was raised against the C-terminus of the proenzyme of human MMP-2 (21), and MMP-9 was raised against intact human MMP-9, recognizing both the latent and active forms of the enzyme (22). Deparaffinized tissue sections were immersed in methanol containing 0.03% H_2O_2 for 30 min to block endogenous peroxidase activity. All of the sections were treated with 0.1 N citrate buffer to retrieve the unmasked antigens for three five min (23). Then the sections were incubated in normal horse serum (diluted 1:20) for 30 min to block the nonspecific antibody binding sites. The monoclonal antibodies for CD34, MMP-2, and MMP-9 were diluted in phosphate-buffered saline supplemented with 5% normal horse serum and 1% bovine serum albumin at dilution ratio 1:200, 1:200, and 1:300, respectively. The sections were treated consecutively at 4℃ with each monoclonal antibody for 18 hr. Sections were treated with biotinylated anti-mouse or rabbit IgG horse serum (diluted 1:100, Vector) for 30 min, and avidin DH-biotinylated horseradish peroxidase complex (Vector) for 30 min. Afterwards, sections were colored with 3,3'-diaminobenzidine tetrahydrochloride (Sigma, U.K.) in 50 mmol/L Tris-HCl (pH 7.5) for 15 min, and then counterstained with 1% Mayer's hematoxylin.

Histological evaluation

Tumor stage & differentiation

Tumor differentiation was assessed according to WHO recommendations (24) and tumor staging according to modified Astler-Coller classification system (25).

Immunohistochemistry of MMP-2, MMP-9, CD34

All the sections were stained by immunohistochemistry for all three molecules and they were scored separately by two authors to exclude the interobserver variability. Only cytoplasmic staining was regarded as positive for all three molecules.

The sections were scanned at low magnification (×100) using light microscopy. Areas with the predominant staining pattern were chosen for further evaluation at higher magnification (×200). Immunoreactivity was graded as (−) to (++) as follows for MMP-2: (−) almost no positive cells, (+) 10-40% of tumor cells showed weak to moderate immunoreactivity, (++) 40-100% of tumor cells showed moderate or strong immunoreactivity.

The stained sections formed endothelial cells which were scanned at ×100 magnification. Areas with the predominant staining pattern were chosen for further evaluation. Staining for CD34 showed that there was an increase in the number of MVD in intratumoral stroma rather than in normal mucosa. The endothelial cells immunoreactive for CD34 were found in a majority of these areas. In the surrounding tumor stroma, a few spindle cells, scattered in the connective tissue stroma, expressed CD34. In these areas, individual MVD was made on a microscopic field (×20 objective and ×10 ocular, Olympus BH-2 microscope, 0.74 mm^2 per field).

Statistical evaluation

Statistical analysis was performed using the Kruskal-Wallis test for differentiating the means between the groups, Pearson's chi-squared test for trend, and Fisher'

exact test for differentiating the expression of MMPs between the two groups, including metastatic and localized colorectal adenocarcinomas. *P*-value less than 0.05 was considered as statistically significant.

RESULTS

MMP-2

Positive staining for MMP-2 was restricted to cytoplasm of colorectal adenocarcinomas at the invasive edge of the tumor, with no staining of interstitial stroma or basement membrane and showing intense stainability (Fig. 1). The intensity and distribution of the staining for MMP-2 significantly correlated with modified Astler-Coller classification ($p<0.05$) (Fig. 2). However, the intensity and distribution of the staining for MMP-2 had tendency to increase according to the progression of metastasis and depth of invasion. There was a tendency for stainability to be more intense and more frequent in MMP-2 in patients with well-differentiated colorectal adenocarcinoma than in patients with a poorly-differentiated one ($Q_{MH}=27.0$, $p<0.05$, χ^2 test). MMP-2 expression was independent to MMP-9 expression.

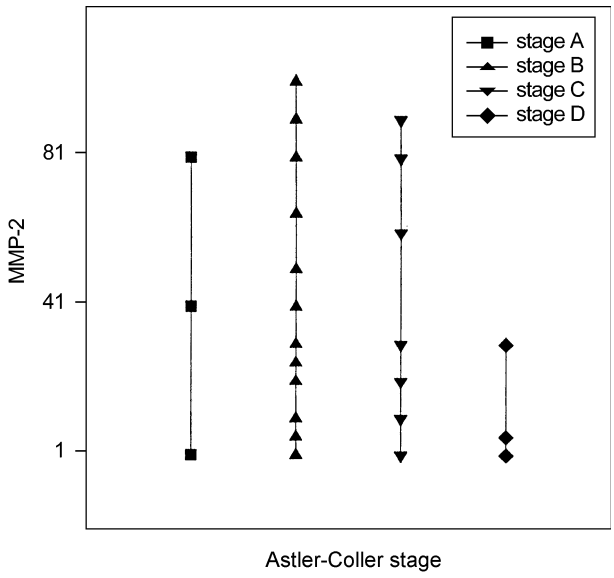

Fig. 2. The expression of matrix metalloproteinase-2 showed a tendency to increase according to the progression of the Astler-Coller stage in colorectal adenocarcinomas.

MMP-9

Positive staining for MMP-9 was restricted to the cytoplasm of colorectal adenocarcinomas and scarcely in activated fibroblasts. The intensity and distribution of MMP-9 staining were correlated with modified Astler-Coller classification, regardless of the metastasis to the lymph node was present or not, and depth of invasion, but intensity and distribution were not significant. There was a tendency for stainability to more intense and more frequent for MMP-9 in patients with well-differentiated colorectal adenocarcinoma than in patients with a poorly-differentiated one ($Q_{MH}=35.0$, $p<0.05$, χ^2 test) (Fig. 3).

CD34

MVD was not correlated with patient's age or gender. Mean MVD showed an increasing tendency according to the progression of Astler-Coller stage in colorectal adenocarcinoma, but it was not significant. Mean MVD in

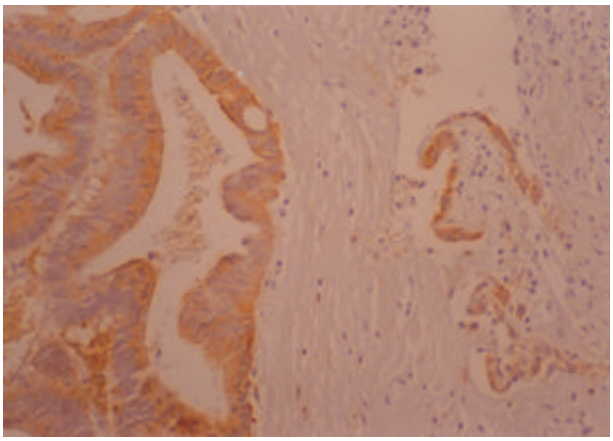

Fig. 1. Immunohistochemistry for matrix metalloproteinase-2 shows positive staining in the invasive edge of well-differentiated colorectal adenocarcinoma, and no stainability in the infiltrating mononuclear inflammatory cells (×200).

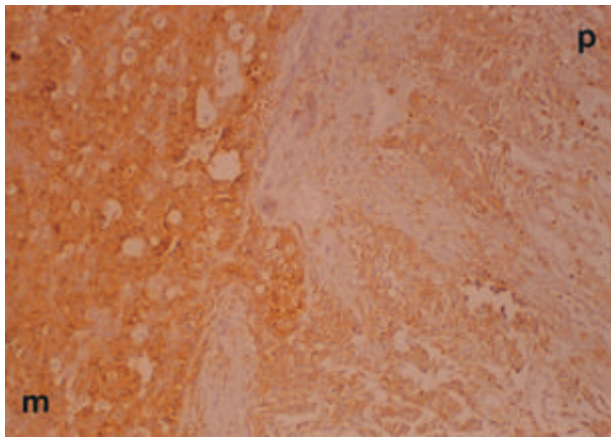

Fig. 3. Immunohistochemistry for matrix metalloproteinase-9 shows marked positve staining in the moderatedly differentiated foci (m), otherwise adjacent poorly differentiated area (p) shows faint stainability. Scarcely activated fibroblasts in the stroma also shows positive staining in their cytoplasms (×100).

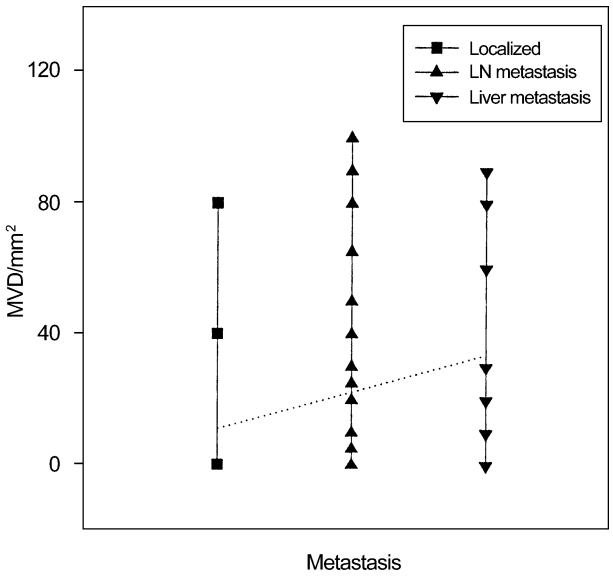

Fig. 4. Mean microvessel density increased according to the progression of colorectal adenocarcinoma from localized to distant metastatic one ($p<0.05$).

patients with metastatic colorectal adenocarcinoma (A-C stage C1, C2, D) was larger than that in the patients with a localized one (A-C stage A, B1, B2) ($p<0.05$) (Fig. 4). Mean MVD in deeply invasive colorectal adenocarcinoma (A-C stage B2, C2, D) was larger than that in patients with superficially invasive colorectal adenocarcinoma (A-C stage A, B1, C1), but it was not significant. Also the mean MVD in patients with well-differentiated colorectal adenocarcinoma was larger than that in patients with a poorly-differentiated one ($p<0.05$) (Fig. 5). Mean MVD was larger in patients with colorectal adenocarcinoma expressing MMP-2, and smaller in patients with colorectal adenocarcinoma without expressing MMP-9 ($p<0.05$) (Table 1).

DISCUSSION

It has been proven that overexpression of MMP-2 and MMP-9 in colorectal adenocarcinoma were considered crucial for invasion and metastasis (1, 26) by augmented immunohistochemistry. In the present study, we found that MMP-2 and MMP-9 were overexpressed in colorectal adenocarcinomas otherwise negligible in normal colonic mucosa. Evidently, normal or benign proliferative lesions in colorectal mucosa showed negligible immunoreactivity for MMP-2, in contrast, almost all of the invasive colorectal adenocarcinomas were positive (8, 15). These results suggests that MMP-2 and MMP-9 might be a component expressed in normal mucosa which is

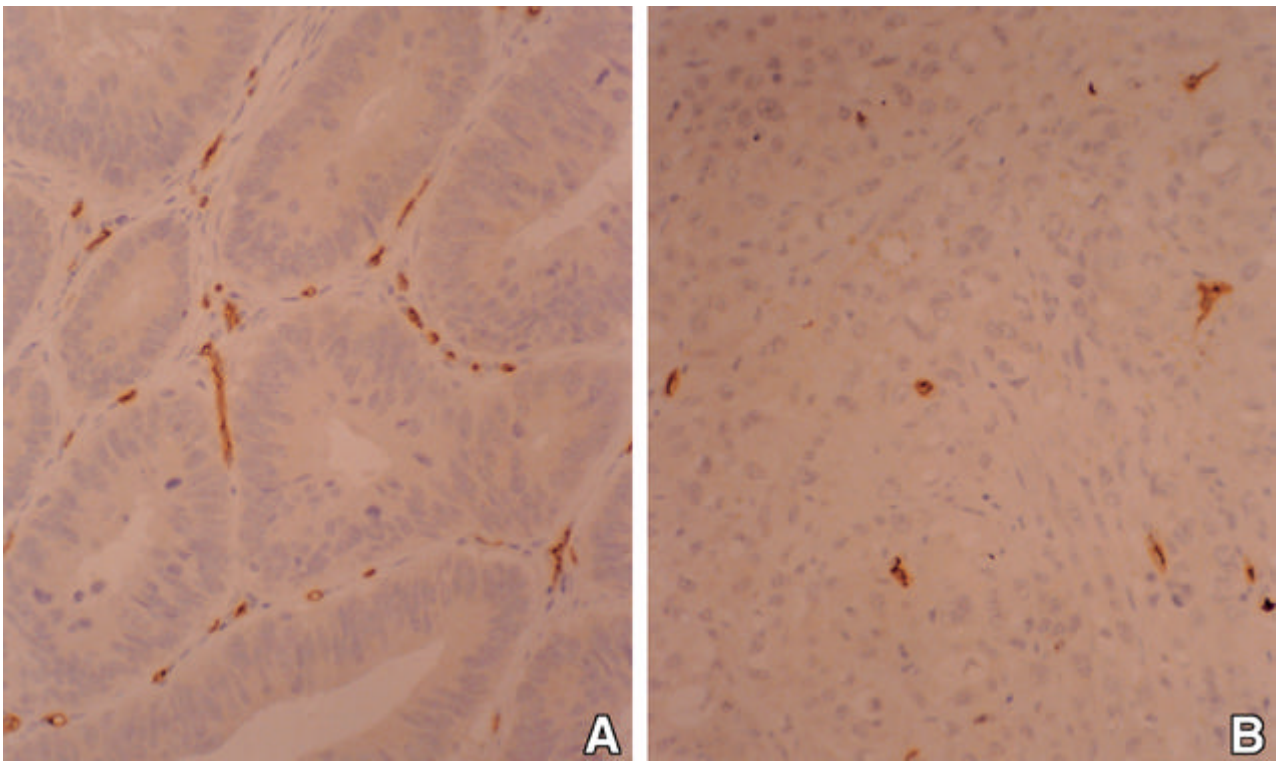

Fig. 5. Immunohistochemistry for CD34 shows marked differences in microvessel density between well-differentiated colorectal adenocarcinomas and poorly differentiated ones (A: well-differentiated, B: poorly differentiated colorectal adenocarcinoma) (×200).

Table 1. Clinical and pathological characteristics of patients with 72 colorectal adenocarcinomas

	No.	MVD (mean±SD)	*p*-value
Age			
15-87 years	72	60.4±13.1	
Gender			NS
M	34	82.1±16.6	
F	38	82.4±38.1	
Depth of invasion			NS
A	6	69.7±9.3	
B1C1	33	70.2±37.8	
B2C2D	46	79.1±20.4	
Metastasis			
A B1B2	30	66.9±22.0	<0.05
C1C2	19	87.7±41.0	NS
D	4	86.4±17.4	
Histologic grade			<0.05
Well differentiated	21	77.8±27.8	
Moderately differentiated	15	68.4±39.0	
Poorly differentiated	6	52.7±16.0	
Astler-Coller stage			NS
A	3	83.9±28.8	
B	33	72.3±26.2	
C	27	71.6±36.0	
D	3	79.1±20.3	
MMP 2			
2	15	52.6±6.6	<0.05
1	15	71.6±29.5	.
0	32	101.3±31.5	.
MMP 9			
2	42	71.5±24.9	.
1	9	69.5±25.9	.
0	9	61.1±8.1	<0.05

Data are expressed as mean±standard deviation
Statistical significance is valued at $p<0.05$ with Kruskal-Wallis test
NS, not significant; No, number; M, male; F, female; MVD, microvessel density

dramatically overexpressed in many invasive and metastatic colorectal adenocarcinomas (9). Also we found that the expression of MMP-2 and MMP-9 showed an increasing tendency to raise the Astler-Coller stage of colorectal adenocarcinomas. Previous studies, investigating the correlation between the expression of MMPs and the stage in colorectal adenocarcinomas, showed their positive relationships (27), even though other reports suggested that the expression of MMPs were independent on the stage (28).

More recently, Emmert-Buck et al. (29) showed quantitatively by microdissective study that proMMP-9 and proMMP-2 increased 12-fold and 3-fold in levels of activity in invasive colonic adenocarcinomas rather than in normal colonic mucosa. In our semiquantitative study, the incidence of MMP-9 expression was higher than that of MMP-2 in colorectal adenocarcinomas as previously described. However the expression of MMP-2 was significantly increased according to A-C stage in colorectal adenocarcinomas though the increase of MMP-9 expression was not significant according to A-C stage in colorectal adenocarcinomas (Fig. 2).

Levy et al. (8) demonstrated the elevation of the expression of mRNA for MMP-2 in colorectal adenocarcinomas and they showed concomitant expression at the protein level using immunohistochemistry. However, overexpression does not mean immediate use of MMPs for ECM degradation since they are produced or secreted as zymogen (pro-MMPs) (34, 5). MMPs are activated by typical two-step mechanism involving a partially activated intermediate, resulting from an upstream cleavage of propeptide (30). The activation of secreted MMP-9 is influenced by activation of MMP-2 localized in cellular membrane (31) and furthermore the secretion and activation of MMP-2 are stimulated by the activation of membrane type-MMPs which are unique in their transmembrane domains (32, 33). In this study, our investigation showed that not only the expression of MMP-9 was independent of MMP-2 in colorectal adenocarcinomas but also that the expression of MMP-9 is higher than that of MMP-2 (Table 1). These results suggest that results from in vitro study (31) would not be directly applicable to humans considering the many factors involved such as tissue inhibitor of metalloproteinases (TIMPs) or various stimulating factors for MMPs, including interleukin-6 and tumor necrosis factor-α.

The expression of MMPs, especially, was increased at the invasive edge of well or moderately differentiated colorectal adenocarcinomas (7). Also the expression of MMPs was exclusively increased in well or moderately differentiated colorectal adenocarcinomas compared with poorly differentiated ones, and this is the focus of our results which is different from previous studies (34). In addition, MMP expressions showed higher in well-differentiated foci within poorly differentiated colorectal adenocarcinomas rather than in adjacent poorly differentiated areas, showing negligible expression of MMPs. All cases showed markedly reduced or negligible expression in fibroblast-like cells or infiltrating macrophages than in colorectal adenocarcinoma ahead of the invasive edge. However, regional differences also exist between cases (34).

We found that the expressions of MMP-2 and MMP-9 were localized overwhelmingly to colorectal adenocarcinomas, and we were only able to demonstrate MMPs in a minority of peritumoral stromal cells or macrophages in this study (Fig. 1). There are many arguments which can be proposed against the hypothesis that stromal cells contribute significantly basement membrane remodeling

in colorectal adenocarcinomas. We selected the areas containing both tumor and normal mucosa without peritumoral desmoplasia and infiltration of macrophages as well as possible and normal mucosa. Our results showed that peritumoral stromal cells have the ability to synthesize MMPs and they may participate actively together with tumor cells in breaching the basement membrane integrity, although some studies indicated that stromal fibroblasts and macrophages in peritumoral desmoplastic stroma have a potential to degrade basement membranes through synthesizing MMPs (1, 12-14). Recently, many in vitro studies showed the origin of mRNA expression for MMP-2 is of epithelial cell origin by Northern blot analysis (35, 36), and in vivo study also demonstrated that the expression of mRNA for MMP-2 is higher in colorectal adenocarciomas and negligible in adjacent normal colonic mucosa, stromal fibroblasts, and infiltrated macrophages by in situ hybridization (8).

In our study, microvessel density (MVD) showed positive correlation with the A-C stage (A to C), as previously described (18, 37), and has been considered as a reliable prognostic predictor in node-negative colorectal adenocarcinomas (A-C stage A/B) (24). The absolute values of MVD in colorectal adenocarcinomas in our study appear to be slightly lower than in previous studies performed by Mlynek et al. (38). This difference can be partly explained by the vessel marker used in these studies, the different counting method, or heterogenous intratumor MVD area by area (17). Although anti-CD31 antibody has been provided as the most sensitive endothelial cell marker for microvessels, anti-CD34 antibody is an acceptable alternative and the most reproducible endothelial cell marker for microvessels (39). Also anti-CD34 antibody highlighted significantly more vessels than anti-vWF, because anti-CD34 reveals young abluminal endothelial cells associated with sprouting during angiogenesis, while anti-vWF, particularly, was expressed on grown cells (40). We selected only the central and deepest portion of the tumor and examined areas containing the highest angiogenic concentration by scanning at $\times 100$ magnification field. Although Saclarides et al. (18) evaluated angiogenesis and reported a correlation between MVD, the depth of invasion within the bowel wall, and shorter survival, however reported no statistically significant MVD differences in rectal adenocarcinomas with or without metastasis. Migration or sprouting of endothelial cells need MMPs. Some oncogenes such as *H-ras* are capable of activating the angiogenic switch accompanied by upregulation of MMP bioactivity (41). The metastatic process depends on several important factors, including angiogenesis and others (37). We found no statistically significant relationship between the expression of MMPs and angiogenesis (Table 1). The reason might be due to both angiogenesis and function of MMPs directly blocked with TIMPs (41).

Metastatic process involves a cascade of the linked sequential steps involving multiple host-tumor interactions (42). Angiogenesis is initially required for the expansion of primary tumor mass and also for that of metastatic tumor colony, so new blood vessels penetrating the tumor are frequent sites for tumor cell entry into the circulation (43). In our study, microvessel density (MVD) correlated with the stage of colorectal adenocarcinomas. At angiogenesis, endothelial cells secreted and overexpressed MMPs for sprouting themselves. In addition, the overexpression of MMPs in tumor cells is suggested to contribute to the lateral expansion of new blood vessels (14). Recently, it has been proven that oncogenic H-ras stimulated angiogenesis via upregulating mRNA for MMPs and vascular endothelial cell growth factor (VEGF) and via downregulating mRNA for tissue inhibitor of metalloproteinases (TIMPs) (41). In this study, the angiogenesis increased in patients with colorectal adenocarcinoma expressing MMP-2, and decreased in patients with colorectal adenocarinoma, which did not express MMP-9 (Table 1).

We found no siginificant relationship between the histologic grade of colorectal adenocarcinoma and the degree of angiogenesis as previously described (37) while others described a direct relationship between them (45). We found that angiogenesis had a tendency to increase in well-differentiated colorectal adenocarcinomas, opposite to previous results (45). This tendency is similar to that of MMPs, as previously described.

In summary, immunohistochemical staining revealed that aniogenesis and the expression of MMPs, especially MMP-2, and angiogenesis were semiquantitatively increased in the progression of the A-C stage of colorectal adenocarcinomas. We suggested that the degree of angiogenesis and MMP-2 expression are predictive indicators for colorectal adenocarcinomas.

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