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Role of Tissue Inhibitors of Metalloproteinases (TIMPs) in Colorectal Carcinoma

Increased production of matrix metalloproteinases (MMPs) has been associated with increases in invasive and metastatic potential in many types of human carcinoma. Tissue inhibitors of metalloproteinase (TIMP)-1 inhibits most interstitial collagenases and MMP-9. TIMP-2 binds specifically and noncovalently to the pro-form of MMP-2 and inhibits its enzyme activity. In this study, we examined TIMP-1 and TIMP-2 expressions in relation to clinicopathological variables in colorectal carcinoma with in situ hybridization and immunohistochemistry. TIMP-1 and TIMP-2 expressions were localized overwhelmingly to pericancer stromal cells, while malignant and normal mucosal cells were weak or negative. Strong stromal TIMP-1 immunoreactivity correlated with Dukes' stage (p=0.022), status of lymph node metastasis (p=0.044) and poor survival (p=0.005). The degree of immunohistochemical staining of TIMP-2 did not correlate with all clinicopathological variables. The correlation between enhanced TIMP-1 expression and advanced stage and poor survival suggest a growth promoting activity of TIMP-1 in colorectal carcinoma.

Key Words: *Tissue inhibitors of metalloproteinases; Colorectal neoplasms; Immunohistochemistry; In situ hybridization*

Young-Eun Joo, Kang-Seok Seo, Jin Kim†, Hyun-Soo Kim, Jong-Sun Rew, Chang-Soo Park*, Sei-Jong Kim

Departments of Internal Medicine and Pathology*, Chonnam National University Medical School, Kwangju, Korea
Department of Parasitology†, College of Medicine, Seonam University, Namwon, Korea

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Address for correspondence
Jong Sun Rew, M.D.
Department of Internal Medicine, Chonnam National University Medical School, 8 Hak-dong, Dong-gu, Kwangju 501-757, Korea
Tel: +82.62-220-6296, Fax: +82.62-225-8578

INTRODUCTION

An essential step in the process of tumor invasion and metastasis involves the degradation of tissue barriers in the extracellular matrix (ECM), particularly in the basement membrane. Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that are capable of degrading the ECM (1-2).

Increased production of MMPs has been associated with increased invasive and metastatic potential in many types of human carcinoma (3-5). Among MMPs, MMP-2 and MMP-9 (72 kDa and 92 kDa type IV collagenases) degrade high activity type IV collagen molecules, which form the major component of the basement membrane (6). These MMPs are dependent on the balance between the levels of activated enzyme and tissue inhibitors of metalloproteinases (TIMPs) (7).

Four types of TIMPs have been described: TIMP-1, TIMP-2, TIMP-3, and TIMP-4 (8-11). TIMP-1 inhibits most interstitial collagenases and MMP-9 (12). TIMP-2 binds specifically and noncovalently to the pro-form of MMP-2 and inhibits its enzyme activity (13). Thus the TIMPs serve as selective inhibitors of MMP activity. But recent reports demonstrated that enhanced expression of TIMPs correlated with advanced cancer (14-17).

In this study, we examined TIMP-1 and TIMP-2 expressions in relation to clinicopathological variables in colorectal carcinoma with in situ hybridization and immunohistochemistry.

MATERIALS AND METHODS

Study populations

Fifty-four patients who underwent surgery for colorectal carcinoma in 1992 at Chonnam National University Hospital were selected for this study. The primary criteria for selection were the availability of formalin-fixed and paraffin-embedded blocks and sufficient clinical follow-up for tumor-specific survival analysis. Patient characteristics including sex, age at time of surgery, histologic grade, location, tumor size, stage, survival and follow-up information were all obtained by chart reviews and pathologist and physician contact when necessary. Survival was measured from the time of surgery until follow-up at September 31, 1998. This study group comprised 32 males and 22 females. The mean age was 59.8±12.0 (mean±SD) with a range from 27 to 83 years.

Characteristics of patients

Histologically, there were 29 (53.7%) well-differentiated, 12 (22.2%) moderately differentiated and 13 (24.1%) poorly differentiated adenocarcinoma. The mean size of tumor was 5.6±1.9 cm (mean±SD) with a range from 2 to 11 cm. Thirty-nine (72.3%) cases were located in the rectum, 6 (11.1%) in the sigmoid colon, 2 (3.7%) in the transverse colon and 7 (13.0%) in the ascending colon. According to international TNM staging system, 8 (14.8%) of the cases were I, 20 (37.0%) were II, 23 (42.6%) were III and 3 (5.6%) were IV. With regard to metastases, no lymph node metastasis (N0) was found in 29 (53.7%) cases, lymph nodes involvement (N1-N3) was found in 25 (46.3%) cases, no distant metastases (M0) was found in 51 (94.4%) cases and distant metastases (M1) was found in 3 (5.6%). According to Dukes' classification system, 8 (14.8%) of the cases were A, 21 (38.9%) were B, 22 (40.7%) were C and 3 (5.6%) were D (Table 1).

Table 1. Clinicopathological variables of 54 patients with colorectal carcinoma

Variables	No. of cases (%)
Age	
≤60	27 (50.0)
>60	27 (50.0)
Sex	
Male	32 (59.3)
Female	22 (40.7)
Tumor size (cm)	
≤5.5	31 (57.4)
>5.5	23 (42.6)
Tumor location	
Rectum	39 (72.3)
Sigmoid	6 (11.1)
Transverse	2 (3.7)
Ascending	7 (13.0)
Gross findings	
Protruding	5 (9.3)
Ulcerating	24 (44.4)
Protruding-ulcerating	22 (40.7)
Infiltrating	3 (5.6)
Histologic grade	
Well differentiated	29 (53.7)
Moderately differentiated	12 (22.2)
Poorly differentiated	13 (24.1)
T stage	
T1	1 (1.9)
T2	9 (16.7)
T3	40 (74.1)
T4	4 (7.4)
Status of lymph node metastasis	
N0	29 (53.7)
N1-3	25 (46.3)
Status of distant metastasis	
M0	51 (94.4)
M1	3 (5.6)
Dukes' stage	
A	8 (14.8)
B	21 (38.9)
C	22 (40.7)
D	3 (5.6)

In situ hybridization

For in situ hybridization, two biotinylated oligonucleotide probes complementary to TIMP-1 (TGTGGGTGGGGTGGGACACAGGTGC) and TIMP-2 (CCACTTCCTTCTCACTGACCGC) mRNA were synthesized (18). All in situ hybridization experiments were carried out using manual capillary action technology on the Micro-Probe staining system (Fisher Scientific, Pittsburgh, PA, U.S.A.) with the modified one-hr method of Park et al. (19). Briefly, the slides were rapidly deparaffinized, cleared and rehydrated. The tissues were then digested with pepsin (Sigma, St. Louis, MO, U.S.A.) at 2 mg/mL for 4 min at 110℃. The probe was applied to the slides and the tissues were heated at 110℃ for 1 min to denature any secondary mRNA structures. Hybridization of the probe and mRNA target was performed by exposing the slides to an oven in which the temperature was gradually decreased from 110℃ to 85℃. Biotinylated hybrids were detected with streptavidin-horseradish peroxidase (Zymed, SanFrancisco, CA, U.S.A.) for 7 min at the 45℃. After preincubation in 3-amino-9-ethylcarbazole (AEC, Sigma, St. Louis, MO, U.S.A.) for 10 min at 45℃, the tissues were washed with distilled water. Following chromogen reaction, the tissues were counterstained with hematoxylin (Research Genetics, Huntsville, AL, U.S.A.), washed with distilled water, air dried and cover-slipped with Universal mount (Research Genetics). In situ hybridization for negative control was performed with probe diluent in hybridization.

Immunohistochemistry

All procedure for immunohistochemical staining was done by Micro-Probe staining system based on capillary action (20). Paraffin sections, of 4 μm in thickness with mounted probe on slides, were immunostained with anti-mouse monoclonal antibodies for TIMP-1 and TIMP-2 antigens (NeoMarkers, Union, CA, U.S.A.) by the avidin-biotin peroxidase complex method (20). Sections were deparaffinized and heated in a microwave oven for 7 min to retrieve the antigens. They were immersed in 0.6%

hydrogen peroxide for 5 min to block the endogenous peroxidase activity. The primary antibodies, used at concentrations of 1:150 and 1:120 respectively, were diluted in phosphate-buffered saline supplemented with 5% normal horse serum and 1% bovine serum albumin and then incubated with tissues for 90 min at room temperature. Anti-mouse immunoglobulin G (Sigma, St. Louis, MO, U.S.A.) labeled with biotin was used as a secondary antibody for the detection of primary antibodies and were incubated for 7 min at 45℃. After multiple rinses with universal buffer, streptavidin-alkaline phosphatase detection system (Biomeda, Foster, CA, U.S.A.) was applied for 7 min. As the final step, the slides were developed for 10 min with the enzyme substrate, 3-amino-9-ethylcarbazole (AEC). The slides were counterstained with hematoxylin solution for 3 min. After dehydration, the tissue was sealed with an universal mount. For TIMP-1 and TIMP-2 antibodies, degree of immunohistochemical staining was classified as 0 for absent immunoreactivity, 1 for weak immunoreactivity, 2 for strong immunoreactivity. Degree of immunohistochemical staining was evaluated by two independent observers without knowledge of the clinical outcomes. All sections for which the two observers disagreed were re-evaluated, and after discussion, there was complete agreement on the classification.

Statistical analysis

Significant bivariate associations were tested for all study variables by χ^2-test and Fisher's exact test. Degree of immunohistochemical staining was tested for prognostic significance for colorectal carcinoma specific survival using Kaplan-Meier survival curves and log-rank test. All statistical analysis were performed using the SPSS statistical software program. A value of $p<0.05$ was considered statistically significant.

RESULTS

In situ hybridization

TIMP-1 mRNA were detected in all colorectal carcinoma cases examined. Hybridization signal for TIMP-1 mRNA was found predominantly in the stroma surrounding the malignant cells whereas malignant and normal mucosal cells were weak or negative signal (Fig. 1).

All colorectal carcinoma tissues were also positive signal for TIMP-2 mRNA. TIMP-2 mRNA expression was localized overwhelmingly to pericancer stromal cells, while malignant and normal mucosal cells were weak or negative signal (Fig. 2).

Immunohistochemistry

In all colorectal carcinoma tissues, there was a clear immunoreactivity with TIMP-1 antibodies (Table 2). TIMP-1 immunoreactivity was observed predominantly

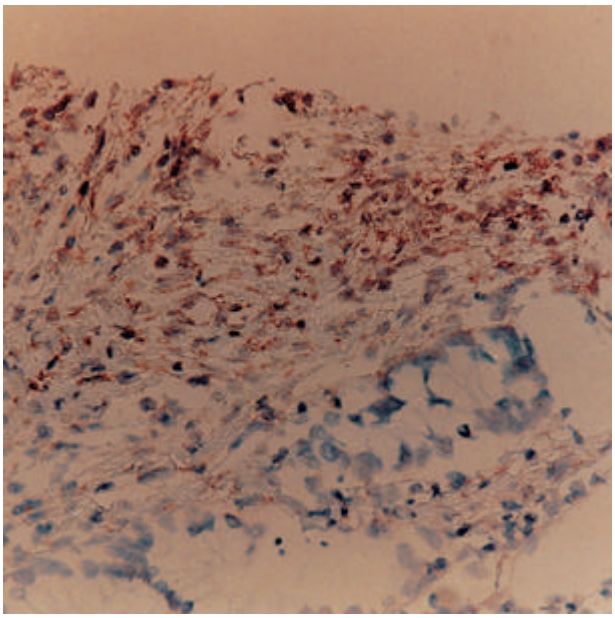

Fig. 1. TIMP-1 mRNA expression from colorectal carcinoma by in situ hybridization. Positive signal is found predominantly in the stroma surrounding the malignant cells (×200).

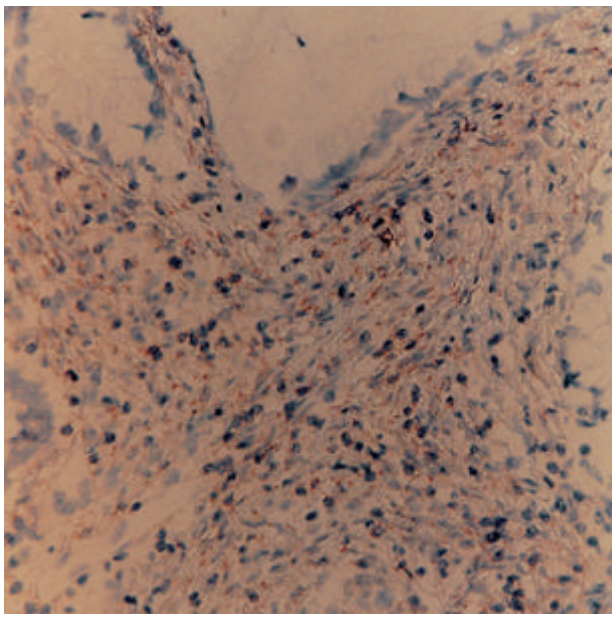

Fig. 2. TIMP-2 mRNA expression from colorectal carcinoma by in situ hybridization. Positive signal is localized overwhelmingly to pericancer stromal cells (×200).

in the stroma surrounding neoplastic glands, but it was weak or absent in malignant cells (Fig. 3). Strong TIMP-1 immunoreactivity was present in stromal cells in 31 (57.4%) of 54 cases and weak immunoreactivity was detected in 23 (42.6%) of 54 cases (Table 2). Strong stromal TIMP-1 immunoreactivity correlated with Dukes' stage (p=0.022), status of lymph node metastasis (p=0.044) and poor survival (p=0.005) (Table 3, Fig. 5). However, there was no correlation between TIMP-1 immunoreactivity and age, sex, location, tumor size, gross finding and histologic grade (Table 3).

Table 2. Results of immunohistochemical staining intensity in 54 colorectal carcinomas

Staining intensity	TIMP-1	TIMP-2
0	0 (0.0%)	0 (0.0%)
1	23 (42.6%)	33 (61.1%)
2	31 (57.4%)	21 (38.9%)
Rate of expression	54/54 (100%)	54/54 (100%)

In all colorectal carcinoma tissues, there was also clear immunoreactivity with TIMP-2 antibodies (Table 2).

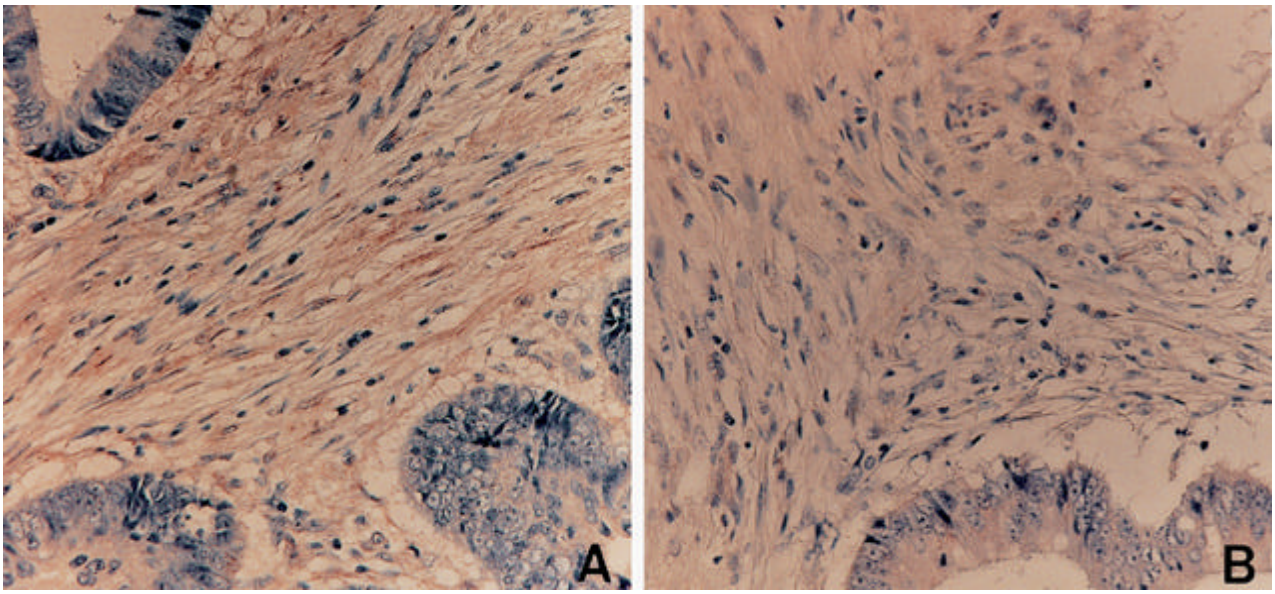

Fig. 3. Immunohistochemical staining of TIMP-1 in colorectal carcinoma. TIMP-1 immunoreactivity was observed predominantly in stroma surrounding neoplastic glands, but malignant cells were weak or absent (×200). (A) Strong, (B) Weak

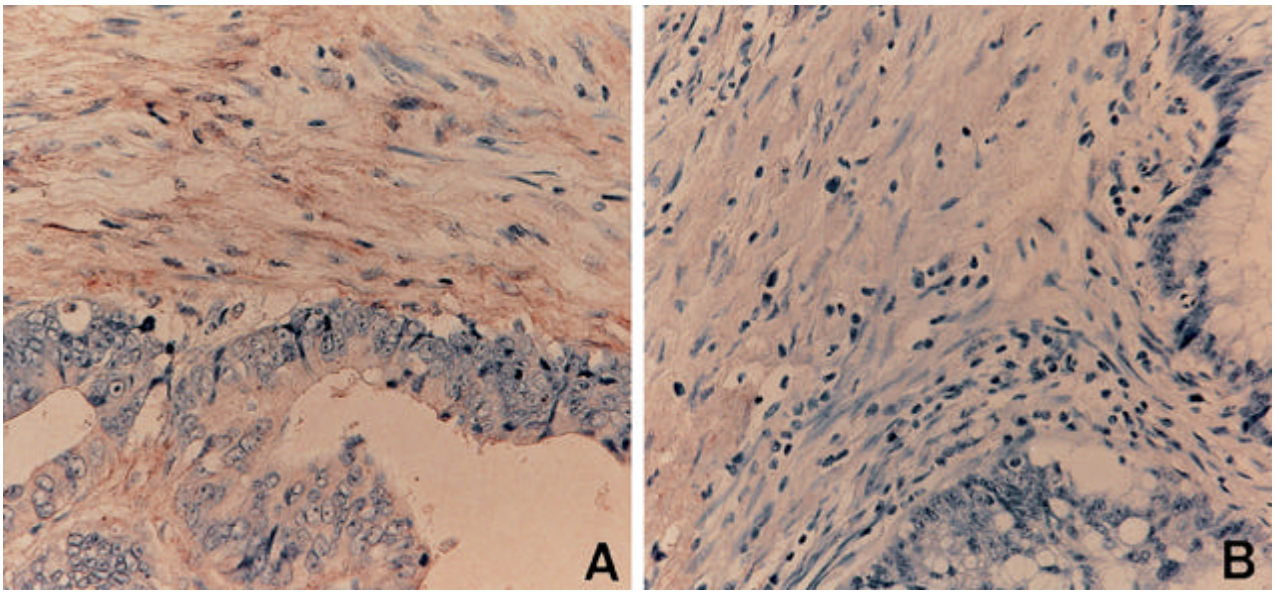

Fig. 4. Immunohistochemical staining of TIMP-2 in colorectal carcinoma. TIMP-2 immunoreactivity was observed predominantly in pericancer stromal cells, but malignant cells were weak or absent (×200). (A) Strong, (B) Weak

TIMP-2 immunoreactivity was observed predominantly in pericancer stromal cells, but malignant cells were weak or absent immunoreactivity (Fig. 4). Strong TIMP-2 immunoreactivity was present in stromal cells in 21 (38.9%) of 54 cases, and weak immunoreactivity was detected in 33 (61.6%) of 54 cases (Table 2). The degree of immunohistochemical staining of TIMP-2 did not correlate with all clinicopathological variables (Table 4, Fig. 6).

Table 3. Correlation between immunoreactivity for TIMP-1 in colorectal carcinomas and clinicopathological variables

Variables	n	TIMP-1		*p* value
		Weak (n=23)	Strong (n=31)	
Age				
≤60	27	11	16	0.783
>60	27	12	15	
Sex				
Male	32	11	21	0.141
Female	22	12	10	
Tumor size(cm)				
≤5.5	31	16	15	0.120
>5.5	23	7	16	
Tumor location				
Rectum	39	16	23	0.865
Sigmoid	6	3	3	
Transverse	2	1	1	
Ascending	7	3	4	
Gross findings				
Protruding	5	2	3	0.829
Ulcerating	24	9	15	
Protruding-ulcerating	22	10	12	
Infiltrating	3	2	1	
Histologic grade				
Well differentiated	29	13	16	0.816
Moderately differentiated	12	4	8	
Poorly differentiated	13	6	7	
T stage				
T1	1	1	0	0.583
T2	9	3	6	
T3	40	18	22	
T4	4	1	3	
Status of lymph node metastasis				
N0	29	16	13	0.044
N1-3	25	7	18	
Status of distant metastasis				
M0	51	23	28	0.125
M1	3	0	3	
Dukes' stage				
A	8	3	5	0.022
B	21	14	7	
C	22	6	16	
D	3	0	3	

Table 4. Correlation between immunoreactivity for TIMP-2 in colorectal carcinomas and clinicopathological variables

Variables	n	TIMP-2		*p* value
		weak (n=33)	strong (n=21)	
Age				
≤60	27	15	12	0.402
>60	27	18	9	
Sex				
Male	32	21	11	0.412
Female	22	12	10	
Tumor size(cm)				
≤5.5	31	17	14	0.272
>5.5	23	16	7	
Tumor location				
Rectum	39	25	14	
Sigmoid	6	3	3	0.432
Transverse	2	0	2	
Ascending	7	5	2	
Gross findings				
Protruding	5	3	2	
Ulcerating	24	16	8	0.899
Protruding-ulcerating	22	12	10	
Infiltrating	3	2	1	
Histologic grade				
Well differentiated	29	19	10	0.757
Moderately differentiated	12	7	5	
Poorly differentiated	13	7	6	
T stage				
T1	1	0	1	
T2	9	7	2	0.471
T3	40	24	16	
T4	4	2	2	
Status of lymph node metastasis				
N0	29	15	14	0.128
N1-3	25	18	7	
Status of distant metastasis				
M0	51	30	21	0.155
M1	3	3	0	
Dukes' stage				
A	8	5	3	
B	21	11	10	0.547
C	22	14	8	
D	3	3	0	

DISCUSSION

In this study, we have used in situ hybridization and immunohistochemistry to examine TIMP-1 and TIMP-2

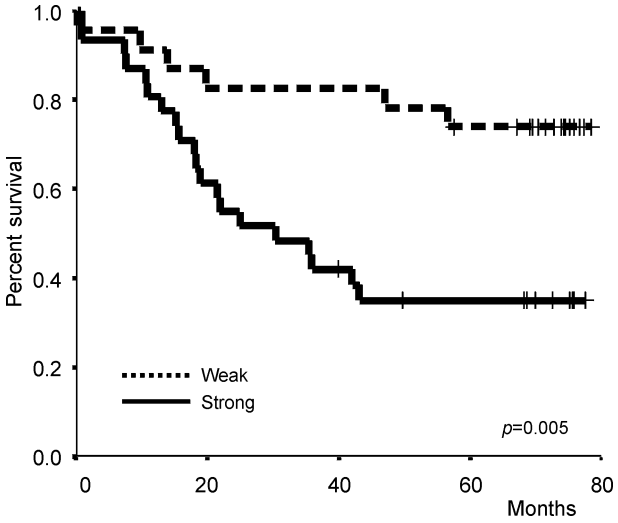

Fig. 5. Kaplan-Meier survival curves correlating survival with TIMP-1 immunohistochemical stromal staining intensity.

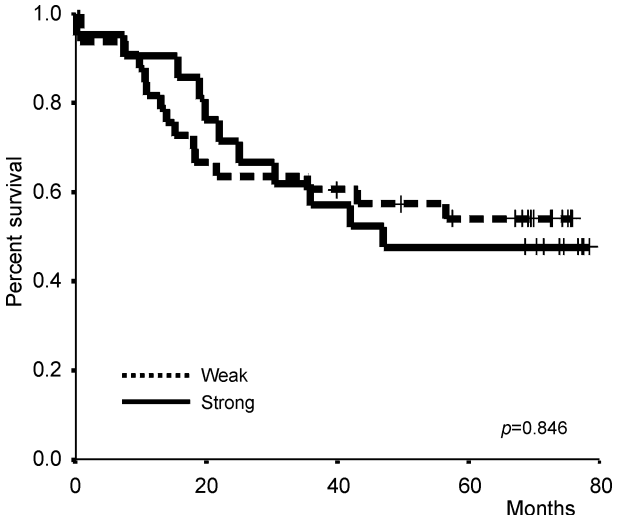

Fig. 6. Kaplan-Meier survival curves correlating survival with TIMP-2 immunohistochemical stromal staining intensity.

expressions in colorectal carcinoma. TIMP-1 and TIMP-2 were expressed overwhelmingly by peritumor stromal cells. Our results are similar to previous reports (14, 15). The cellular source of MMPs and TIMPs has important implications for our understanding of neoplastic cell behavior. The consequences of this stromal expression of TIMP-1 and TIMP-2 are not understood, but it seems to be a host response to the presence of malignant cells.

In this study, we demonstrated that enhanced expression of TIMP-1 correlated with more advanced Dukes' stage and poor survival. The correlation between TIMP-1 expression and Dukes' stage suggests the possible role of TIMP-1 in tumor invasion and metastasis. However, these findings are inconsistent with the notion that TIMP-1 acts as an important inhibitor of tumor invasion of ECM based on in vitro and animal tumor models (21, 22). Clark et al. (23) reported that TIMP-1 stimulates the secretion of collagenase from human skin fibroblasts. It suggests that TIMP-1 has growth promoting activity other than the inhibition of MMPs. Chesler et al. (24) reported that antiproteolytic and growth factor activities of the TIMP-1 molecule are physically and functionally distinct.

TIMPs are also known to be regulated by cytokines and growth factors, most notably interleukin, tumor necrosis factor-α and transforming growth factor-β (25, 26). Among those, transforming growth factor-β has been regarded as a major regulating factor. Transforming growth factor-β1 increases transcript levels of TIMP-1 but decreases those of TIMP-2 (13).

ECM remodeling is associated with physiological and pathological processes including wound healing, angiogenesis, inflammation, arthritis and more notably tumor invasion and metastasis (27). It is more than academic interest, however, to try and delineate exactly what the observations mean at a biological and functional level in neoplasia, because it seems to be a host response to the presence of malignant cells, and any pharmacological strategies aimed at modulating the balance between MMPs and TIMPs could potentially interfere with this response and promote tumor progression.

In this study, the degree of immunohistochemical staining for TIMP-2 did not correlate with all clinicopathological variables. Ring et al. (28) reported that positive expression of TIMP-2 in basement membrane and diffusely in stroma with subglandular enhancement was found significantly more often in localized tumors than in tumors with regional or distant metastases. But in breast cancer, enhanced expression of stromal TIMP-2 was a strong indicator of tumor recurrence (16). Stetler-Stevenson et al. demonstrated that TIMP-2 is regulated indepenently of TIMP-1, both in cell culture and in vivo (13). We do not explain these inconsistent results for TIMP-2 because the accurate role and regulation of TIMP-2 is presently unknown.

In summary, TIMP-2 expression did not correlate with clinicopathological variables. But there were a statistically significant stage and poor survival for cases with enhanced TIMP-1 expression, suggesting that the growth promoting activity of TIMP-1 may help determine the prognosis of colorectal carcinoma. TIMPs have growth promoting activity, in addition to their roles as MMP inhibitors. This mechanism of TIMPs' multiple functions remains elusive. Further studies are needed to define the regulation of TIMPs production at the tumor-stromal interaction may in certain situations stimulate tumor growth promoting activity rather than the inhibitory effect of MMPs.

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