Featured Article

Matrix Metalloproteinase/Tissue Inhibitors of Matrix Metalloproteinase Phenotype Identifies Poor Prognosis Colorectal Cancers

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ABSTRACT

Purpose: **The matrix metalloproteinases (MMPs) are a family of proteolytic enzymes involved in tumor invasion; several individual members of which have been implicated in tumor prognosis. These enzymes and their physiologic inhibitors, the tissue inhibitors of matrix metalloproteinases (TIMPs), act in a coordinated manner to form an integrated system. Therefore, to understand their role in tumor invasion, it is necessary to evaluate them collectively.**

Experimental Design: **In this study all of the major members of the matrix metalloproteinase (MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-13, MT1-MMP and MT2-MMP)/tissue inhibitor of matrix metalloproteinase (TIMP-1, TIMP-2, and TIMP-3) system have been investigated by immunohistochemistry in a series ($n = 90$) of stage III (Dukes' C) colorectal cancers. An immunohistochemical score based on the intensity of immunoreactivity and proportion of immunoreactive cells was established for each MMP and TIMP.**

Results: **The MMP/TIMP profile defined by hierarchical cluster analysis of the immunohistochemical score identifies a distinct group of colorectal cancers with poor prognosis (log-rank test, 12.22, $P = 0.0005$). The median survival time of patients in this survival group was 18 months compared with a median survival of 49 months in the "good" survival group. Multivariate analysis showed that this profile was independently the most significant prognostic factor ($P = 0.001$).**

Conclusions: **This study has identified that the MMP/TIMP profile is an independent indicator of poor prognosis in colorectal cancer.**

INTRODUCTION

The matrix metalloproteinases (MMPs) are a key family of proteolytic enzymes involved in extracellular matrix degradation (1–4). These enzymes collectively can degrade all components of the extracellular matrix and are broadly classified into groups including collagenases, gelatinases, stromelysins, and membrane-type MMPs (1, 3, 4). Tissue inhibitors of metalloproteinases (TIMPs) are the main physiologic inhibitors of the MMPs (5, 6). The TIMPs are secreted proteins that complex with individual MMPs and regulate the activity of individual MMPs. Together, the MMPs and TIMPs form a complex biological system strictly controlling degradation of extracellular matrix. The MMPs and TIMPs have a significant role in facilitating tumor invasion and metastasis (1–6), not only through their direct role in degrading extracellular matrix but also by interaction with other biological systems implicated in tumor invasion, including cell adhesion molecules, cytoskeletal proteins, and growth factors (7, 8).

Colorectal cancer is one of the commonest malignant tumors in the Western world with a 5-year survival rate of ~45%. At present, the pathological stage of disease, based on the extent of both local tumor invasion and metastatic tumor spread is the most clinically useful prognostic indicator. However, tumors of the same stage can follow significantly different clinical courses, indicating the necessity for the identification of novel prognostic factors. A number of studies have investigated specific MMPs and TIMPs in colorectal cancer and several of these studies have proposed prognostic significance for individual MMPs [MMP-1 (9), MMP-7 (10), MMP-9 (11)], and TIMP-1 (12) and TIMP-2 (13) in this type of tumor. However, each of these studies has usually focused on a single MMP or TIMP, there have been no extensive studies of the expression of MMPs and TIMPs in colorectal cancer. These diverse findings of individual MMPs and TIMPs in different groups of colorectal cancer highlight the need to perform a comprehensive study of MMPs in a well-characterized series of colorectal cancers to understand the interaction between the individual MMPs/TIMPs and to further understand the biology of MMPs/TIMPs in colorectal cancer. In this study we have analyzed by immunohistochemistry the expression of all of the major MMPs and TIMPs in a well-characterized group of colorectal cancers, and we demonstrate that the MMP/TIMP molecular profile identifies a group of patients with very poor outcome.

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MATERIALS AND METHODS

Tumor Samples. Tumor samples were collected, with approval of the regional research ethics committee, from consecutive patients undergoing primary curative resection for colorectal cancer between 1994 and 1998 at Aberdeen Royal Infirmary. All of the tumors were fixed in neutral buffered formalin and representative blocks embedded in wax. Two expert gastrointestinal pathologists (S. C. and G. I. M.) reviewed the histopathology of each case by examination by light microscopy of hematoxylin and eosin-stained sections. All of the tumors ($n = 90$) in the study were Dukes' stage C adenocarcinomas, UICC stage III (*i.e.,* tumors with lymph node metastases), because these constitute a well-defined group of tumors. The clinicopathological characteristics of the patients and their tumors are summarized in Table 1. Follow-up ranged between 60 and 100 months, at which time 55 of the patients (61%) had died from colorectal cancer (median survival, 42 months; 95% confidence intervals, 27–57 months; mean survival, 53 months (95% confidence intervals, 45–61 months).

Antibodies. Immunohistochemistry for eight MMPs (MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-13, MT1-MMP, and MT2-MMP) and three TIMPs (TIMP-1, TIMP-2, and TIMP-3) was done on each of the tumors described above. The monoclonal antibodies to MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1, and TIMP-2 have been developed in our laboratory (9, 14, 15). Briefly, each antibody was produced by using a synthetic peptide conjugated to carrier protein as the immunogen. Each peptide corresponded to a unique sequence (based on amino acid sequence alignment and protein homology modeling) for the individual MMP or TIMP (9, 14, 15), and each antibody recognizes the appropriate MMP (both latent and activated form) or TIMP (9, 14, 15). The criteria used for the selection of peptide immunogens, the peptide sequences used for immunization, the production of the monoclonal antibodies, the characterization and specificity of each antibody, and also the use of these antibodies in immunohistochemistry on formalin-fixed, wax-embedded tissue section have been described previously (9, 14–17). Each of these antibodies was available as tissue culture supernatant. Antibodies to MMP-7, MMP-13, MT1-MMP, MT2-MMP, and TIMP-3 were obtained from Chemicon International Inc, Temecula, CA, and each antibody was supplied as a purified immunoglobulin fraction (information from data sheets supplied by Chemicon).

Table 1 Clinicopathologic characteristics of colorectal cancer patients

Characteristic	No. of patients (%)
Sex	
Male	48 (53.3)
Female	42 (46.7)
Age (y), mean (range)	65 (33–89)
≤65	46 (51.1)
>65	44 (48.9)
Site	
Proximal colon	29 (32.2)
Distal colon	36 (40)
Rectum	25 (27.8)
Tumor stage	
Dukes' stage C (UICC stage III)	90 (100)
Dukes' stage C1	69 (76.7)
Dukes' stage C2	21 (23.3)
TNM stage	
pN1	64 (71.1)
pN2	26 (29.9)
Tumor differentiation	
Well	0
Moderate	75 (83.3)
Poor	15 (16.7)

Immunohistochemistry. Formalin-fixed, wax-embedded tumor sections (4 μm thick) were mounted on adhesive-coated capillary gap glass slides (DakoCytomation, Ely, United Kingdom), dewaxed in xylene and rehydrated in EtOH, and an antigen retrieval step was done. This was required for all of the antibodies except MMP-7 and MMP-13 and was achieved by microwaving the sections in 0.01 mol/L citrate buffer at pH 6.0 for 20 minutes in an 800W microwave oven. The sections were then allowed to cool to room temperature and were immunostained with a Dako TechMate500 autostainer (DakoCytomation) as described previously (18, 19). Details of antibody dilutions are noted in Table 2. Primary antibody appropriately diluted in antibody diluent buffer (DakoCytomation) was applied for 50 minutes at room temperature, followed by washing with buffer (DakoCytomation) and endogenous peroxidase blocking. Biotinylated goat antimouse/rabbit secondary antibody (1:1,000, DakoCytomation) was applied for 25 minutes at room temperature, followed by further washing with buffer to remove unbound antibody. A complex of avidin with horseradish peroxidase was then applied for 25 minutes at room temperature. After further washing with buffer, diaminobenzidine was applied to the sections for three successive 5-minute periods to demonstrate sites of peroxidase activity (18, 19). The sections were then washed in buffer and water, lightly counterstained with hematoxylin, dehydrated sequentially in EtOH and xylene, and mounted.

MMP and TIMP immunoreactivity was evaluated by examination of the sections with bright- field light microscopy. A scoring system was used to describe both intensity of staining (negative, weak, moderate, and strong) and proportion of tumor cells (0%, 1–5%, 6–75%, and 76–100%) staining in each case, as described previously (18). To enable analysis of the individual immunostaining results, integer values were assigned to the intensity scores (0–3) and the proportion of cells stained (0–3). These values were multiplied together to provide a single integrated score for each MMP or TIMP and the data reduced to an ordinal scale of 0–6 as described previously (18).

Statistical Analysis. Statistical analysis, including hierarchical cluster analysis, χ^2 test, Kaplan–Meier survival analysis, and Cox Multivariate Regression analysis was done with SPSS version 11.5 (SPSS UK Ltd, Woking, UK) for Windows XP. Unsupervised two-dimensional hierarchical cluster analysis of the MMP and TIMP data were done using the between-groups linkage method with the χ^2 measure for ordinal data to identify individual groups of tumors with specific MMP/TIMP profiles. The log-rank test was used to determine survival differences between individual groups.

Table 2 Details of the antibodies to individual MMPs and TIMPs used in this study

Antibody	Type	Source (clone/ID number: ref.)	Antigen retrieval	Optimum dilution
MMP-1	Monoclonal	Own laboratory (3B6: 9, 14, 15)	Yes	1:2
MMP-2	Monoclonal	Own laboratory (4D3: 14, 15)	Yes	1:20
MMP-3	Monoclonal	Own laboratory (1B4: 14)	Yes	Undiluted tissue culture supernatant
MMP-7	Monoclonal	Chemicon (MAB3315)	No	1:3200
MMP-9	Monoclonal	Own laboratory (2C3: 14, 15)	Yes	1:2
MMP-13	Monoclonal	Chemicon (MAB181–15A12)	No	1:200
MT1-MMP	Polyclonal	Chemicon (AB815)	Yes	1:200
MT2-MMP	Monoclonal	Chemicon (MAB3320)	Yes	1:400
TIMP-1	Monoclonal	Own laboratory (2A5: 14)	Yes	1:2
TIMP-2	Monoclonal	Own laboratory (3A4: 14)	Yes	1:64
TIMP-3	Monoclonal	Chemicon (MAB3318)	Yes	1:2,000

Abbreviation: ID, identification.

RESULTS

All of the MMPs and TIMPs showed immunoreactivity in a proportion of colon cancers (Fig. 1). The proportion of cells and the intensity of immunoreactivity for each MMP and TIMP was variable in each tumor and was assessed as described in the Materials and Methods to establish an immunohistochemical score for each MMP and TIMP. To identify specific groups of tumors with distinct MMP/TIMP immunohistochemical expression profiles the data were analyzed by unsupervised hierarchical cluster analysis.

The results of the unsupervised two-dimensional hierarchical cluster analysis of the MMP and TIMP data are shown in

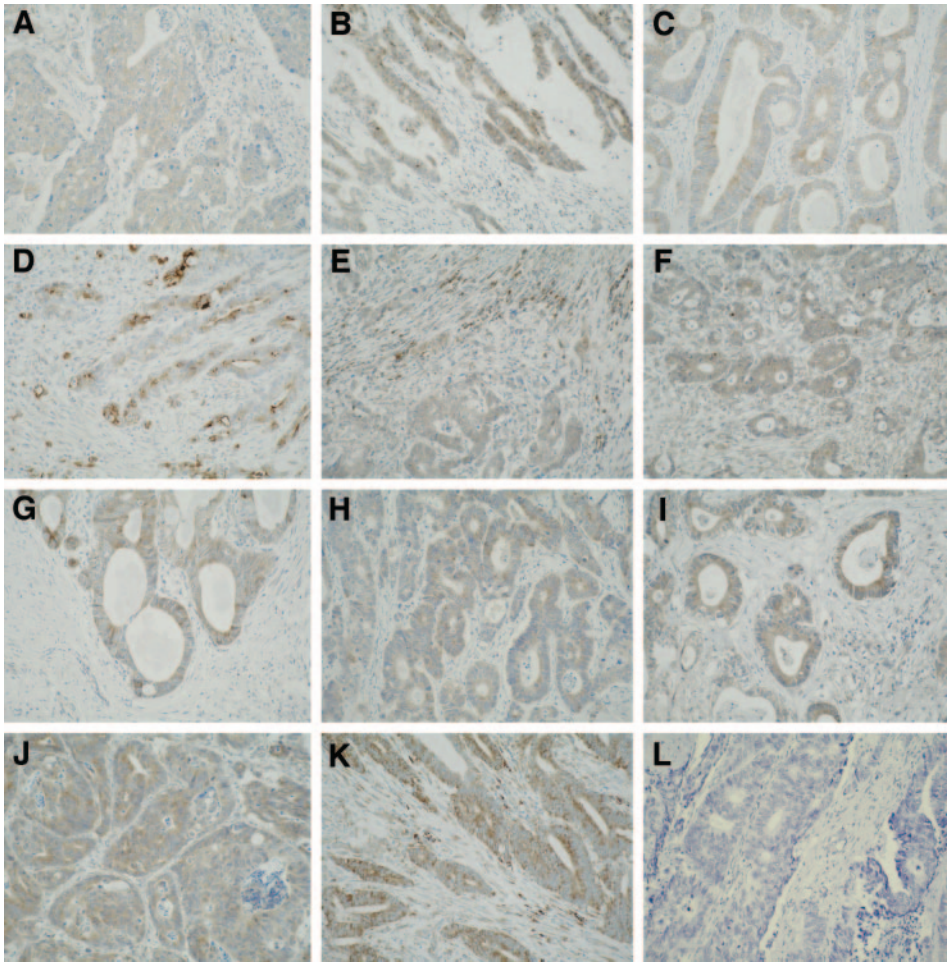

Fig. 1 Immunohistochemical localization of individual MMPs and TIMPs in colorectal cancer showing variation in the intensity and proportion of tumor cells displaying immunoreactivity. *A*, MMP-1; *B*, MMP-2; *C*, MMP-3; *D*, MMP-7; *E*, MMP-9; *F*, MMP-13; *G*, MT1-MMP; *H*, MT2-MMP; *I*, TIMP-1; *J*, TIMP-2; *K*, TIMP-3; *L*, negative control (Tris-buffered saline in place of primary antibody).

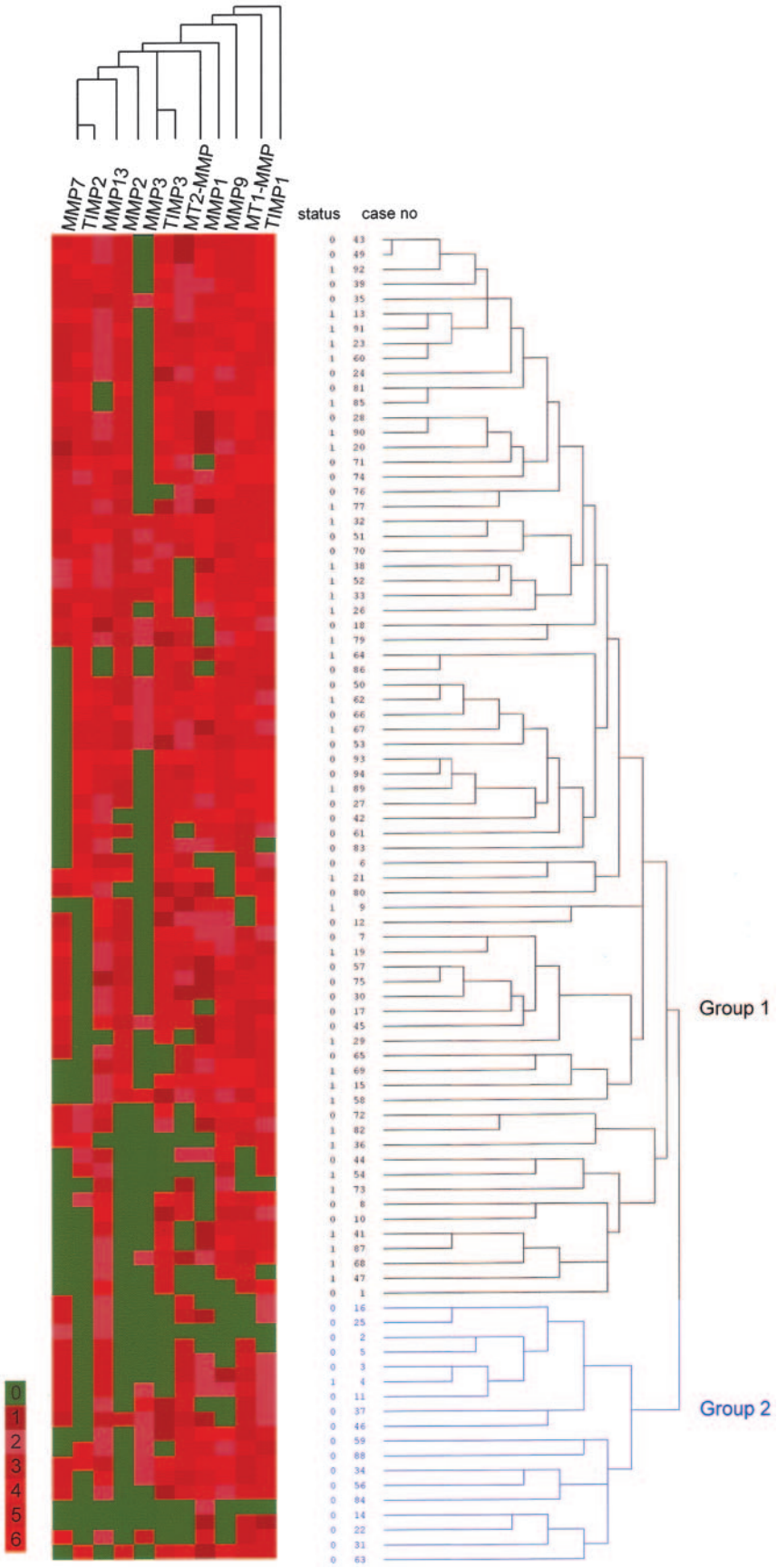

Fig. 2 Two-dimensional unsupervised hierarchical cluster analysis of MMP/TIMP profile in colorectal cancer. *On the left*, a graphical representation of the MMP/TIMP immunohistochemistry score; *on the right*, the dendrogram produced by the hierarchical cluster analysis. *Columns*, specific MMPs and TIMPs; *rows*, individual cases. In the graphical representation, *green*, zero values; *red*, positive values. *Brighter shades of red*; a higher MMP/TIMP score. There is a primary division of the dendrogram into two first-order clusters (group 1, *black* ; group 2, *blue*), with distinct MMP/TIMP expression profiles. *The status column*: *1* = alive; *0* = dead, at the census point. All but one of the patients in group 2 were dead at the time of censoring the patient survival data.

Fig. 2, which illustrates by a dendrogram the grouping of the tumors according to their MMP/TIMP profile. The dendrogram provides a visual representation of the hierarchical clustering process and identifies those cases that are most similar and that form groups that are distinct from other groups or clusters. The dendrogram shows a first-order division of the tumors into two distinct MMP/TIMP molecular profiles, designated group 1 ($n = 72$) and group 2 ($n = 18$).

In group 1, there are 38 patients who are dead, and in group 2 there is only one patient alive with all 17 other patients dead. The median survival time of patients in group 2 was 18 months (95% confidence intervals, 10–26 months; mean, 30 months; 95% confidence intervals, 17–43 months), compared with a median survival of 49 months (95% confidence intervals, 49–67 months; mean, 58 months; 95% confidence intervals, 49–67 months) for those in group 1. Kaplan-Meier analysis shows that the difference in survival between these two groups is highly statistically significant (log-rank test, 12.22; $P = 0.0005$; Table 3; Fig. 3). Importantly, there is no significant difference between the two groups in terms of the distribution of tumor site, degree of tumor differentiation, nodal status (*i.e.,* Dukes' stage C1 *versus* C2 or stage pN1 *versus* pN2), patient age, or gender.

The specific MMP/TIMP profile emerges as the single most significant independent factor ($P = 0.001$) in Cox multivariate analysis, which was done with the following variables for each case: tumor site, tumor differentiation, nodal status, patient gender, age, and "cluster group," *i.e.,* group 1 or group 2 (Table 4).

Table 3 Results of survival analysis for individual factors

Factor	Log rank	*P*
Cluster grouping (group 1 *vs.* group 2)	12.22	0.0005
Dukes' C1 *vs.* Dukes' C2	4.56	0.03
Gender	4.12	0.04
Tumor site (proximal *vs.* distal *vs.* rectum)	3.08	0.21
Age (≤65 and >65)	2.05	0.15
pN1 *vs.* pN2	0.33	0.57
Tumor differentiation	0.07	0.79

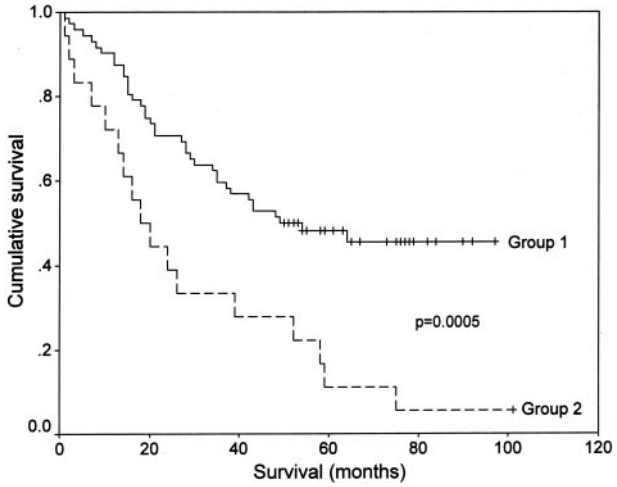

Fig. 3 Kaplan–Meier survival plot of the two primary clusters identified by unsupervised hierarchical cluster analysis (group 1 and group 2). There is a highly significant difference in survival between these two groups (log-rank test = 12.22, $P = 0.0005$).

Table 4 Result of Cox's multivariate regression analysis

Factor	Wald value *	*P* value
Cluster grouping (group 1 *vs.* group 2)	11.175	0.001
Gender (male *vs.* female)	6.093	0.01
Tumor site (proximal *vs.* distal *vs.* rectum)	3.624	0.06
Age (≤65 and >65)	1.114	0.29
Dukes' C1 *vs.* Dukes' C2	0.72	0.4
pN1 *vs.* pN2	0.520	0.471
Tumor differentiation	0.02	0.882

* Wald value, A test of the statistical significance of each variable in multivariate analysis.

MMP-2 ($\chi^2 = 32.34$, $P < 0.001$), MMP-3 ($\chi^2 = 4.89$, $P = 0.03$), MMP-9 ($\chi^2 = 27.4$, $P < 0.001$), TIMP-2 ($\chi^2 = 21.21$, $P < 0.001$), and TIMP-3 ($\chi^2 = 40.5$, $P < 0.001$) were identified as showing significantly altered expression in group 2 compared with group 1. In all cases, expression of these MMPs and TIMPs was reduced in tumors of group 2 compared with group 1 tumors. Survival analyses showed that TIMP-2 (log-rank = 9.7, $P = 0.01$), MMP-2 (log-rank = 5.2, $P = 0.02$), and MMP-3 (log-rank 3.87, $P = 0.05$) demonstrated significant differences in outcome between group 1 and group 2.

DISCUSSION

We have investigated the presence of all of the major MMPs and TIMPs simultaneously in a large series of well-characterized colorectal cancers of a single stage, all with long-term follow-up. Although the presence of several individual MMPs [MMP-1 (9), MMP-7 (10), MMP-9 (11), MMP-13 (18)], and TIMPs [TIMP-1 (12) and TIMP-2 (13)] in colorectal cancer have been suggested to be of prognostic significance; these studies have included cases of different stages and it is important to investigate tumor markers in well-defined groups of colorectal cancer of a single pathologic stage to establish their clinical significance (20). Furthermore, because it is the concerted action of the MMPs and TIMPs that determine their biological actions (1–6), it is essential to study them in an integrated fashion.

We have used an innovative approach to analyze the large amount of immunohistochemical data generated in this study. Unsupervised hierarchical cluster analysis groups data together agglomeratively on the basis of shared characteristics (in this study MMP and TIMP immunohistochemical score) without an *a priori* hypothesis. When this approach to data analysis is used, two specific MMP/TIMP molecular phenotypes emerge, and testing the hypothesis that these two groups had distinct survival outcomes show that the individual groups have distinctly different prognosis ($P = 0.0005$). Importantly, these groups are similar with respect to patient age and gender, tumor site, histologic differentiation of the tumor. We have tested our results against the main clinicopathological prognostic factors (Dukes' stage C1 *versus* C2, gender, age, tumor site, age, and tumor differentiation) confirming the highly significant inde-

pendent prognostic significance of the groupings. This demonstrates that the MMP/TIMP profile is not simply a surrogate for any these known and established prognostic indicators and shows that the MMP/TIMP profile is a highly significant prognostic factor in colorectal cancer. Our finding of two distinct groups within stage III colorectal cancers is also consistent with the clinical observation that within this disease stage, some patients have a relatively indolent course, whereas others progress much more rapidly.

We also identified the main MMPs and TIMPs that contributed to the aggressive phenotype and identified that, although the loss of TIMP-2 was the most significant individual MMP or TIMP that contributed to poor survival of patients in group 2, its level of statistical significance in relation to survival was much less than the significance of the overall MMP/TIMP profile. This highlights the importance of identifying the MMP/TIMP profile. The loss of TIMP-2 expression would potentially result in greater MMP activity and biologically would contribute to enhanced tumor invasion and metastasis (1, 5).

This study, by analyzing the MMPs and TIMPs collectively, is the first to demonstrate that this system produces an aggressive phenotype in colorectal cancer, and these findings represent a conceptual breakthrough in the understanding of the molecular pathology of this type of tumor. Because colorectal cancer represents a well-characterized model of tumor development and progression, the results of this study provide the basis for the reevaluation of the clinical use of MMP inhibitors in colorectal cancer and other malignant diseases (21, 22).

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