MMP7 Expression in Colorectal Tumours of Different Stages

ANDREA POLISTENA1,2,3, ALESSANDRA CUCINA1, SIMONA DINICOLA2, CHRISTINA STENE3,
GIUSEPPE CAVALLO1, ANTONIO CIARDI5, GENNARO ORLANDO1, ROSSELLA ARENA1,
GIUSEPPE D’ERMO1, ANTONINO CAVALLO3, LOUIS BANKA JOHNSON3 and GIORGIO DE TOMA1

1Pietro Valdoni Department of Surgery, Departments of 2Clinical and Molecular Medicine and
4Experimental Medicine, Sapienza University, Rome, Italy;
3Department of Surgery, Skånes University Hospital, Malmö, Sweden

Abstract. Background/Aim: Matrix metalloproteinases (MMPs) are involved in cancer biology. Expression of MMP7 (matrilysin) in colorectal cancer is associated with metastatic disease even though it is expressed in most tumour states. In the present study, our purpose was to analyze MMP7 in bowel and lymph nodes of different tumour stages and to evaluate its expression as a cancer biomarker. Patients and Methods: 28 patients surgically-treated for benign and malignant colorectal tumours were recruited and analyzed for MMP7 in tumoural tissue, lymph nodes and serum by histology, immunohistochemistry, ELISA and western blotting. Results: Immunohistochemistry showed prevalent expression of MMP7 in advanced cancer. A significant increase (p<0.001) was evident in serum of stage III/IV cancers compared to both adenomas and non-metastatic disease. MMP7 was increased in cancer tissues with prevalence in stage I/II. Lymph nodes presented a significant increase of MMP7 (p<0.05 adenoma vs. stage I/II and p<0.001 vs. stage III/IV). Conclusion: MMP7 increases with dysplasia and cancer disease stage in tumour tissue as well as in the regional lymph nodes. It may be used as a complement in investigating suspected locally advanced cancer.

Matrix metalloproteinases (MMPs) are a family of a Zn2+-dependent proteolytic enzymes involved in physiological and pathological remodeling of extracellular matrix in proliferation, angiogenesis and wound healing. MMP7 matrilysin is the smallest metalloproteinase and is known to degrade collagen type IV and X, but also MMP2 and MMP9 (1). Previous studies demonstrated its peculiarity among MMPs, based on its expression in normal, non-inflamed, non-injured epithelia in several organs, as opposed to other MMPs which are normally or seldom not expressed at those sites (2, 3). MMP7 is produced as an inactive zymogene (28 kD). Experimental data suggest it is transformed into an active form (19 kD) by proteolysis and is capable of degrading itself in a concentration-dependent manner (1-5).

An overexpression of MMP7 in advanced stages of colorectal cancer has been linked to increased metastatic disease (6, 7). MMP7 can be found in cancer tissue, as well as in serum and in peritoneal fluid during peritoneal carcinosis (8-12). MMP7 was found to be a requirement for tumour formation but not related to depth of tumour invasion nor to surrounding stromal fibrosis (13). A rodent model for colorectal carcinoma using mice deficient for matrilysin with an MMP7-knockout mutation, presented a reduced number and size of tumours. The mutation did not block invasion since that seems to be related to activation of other MMPs, such as MMP2 and MMP9 which are produced in stromal and not in epithelial cells, where MMP7 is produced (13).

Previous studies also showed an increase in MMPs (especially MMP2, MMP7 and MMP9) after neoadjuvant radiotherapy for rectal cancer, suggesting their possible role in abnormal tissue remodeling after radiotherapeutic injury (14-16). The expression of MMP7 mRNA in humans has a high specificity in colorectal cancer, especially in malignant epithelial cells, but some studies have shown its expression in normal colorectal mucosa, as well as in different grades of dysplasia to cancer (17, 18).

The purpose of the present study was to determine whether matrilysin expression at different stages of tumour progression to cancer within the bowel can be correlated to levels in adjacent lymph nodes in the resected specimen as this would be useful for the assessment of colorectal cancer prognosis.
Patients and Methods

Patients. A prospective study was undertaken of patients referred for elective colorectal cancer treatment to the Pietro Valdoni Department of Surgery, Sapienza University of Rome. Exclusion criteria were neoadjuvant radiotherapy, chemoradiotherapy, language problems and consent withdrawal. Twenty-eight patients were recruited prospectively according to guidelines for treatment of colorectal disease after routine clinical assessment. The study was approved by the Human Ethics Committee at the Sapienza University of Rome and registered at Clinical Trials, ID NCT 01570452. The study was carried-out over a period of three years (September 2005- September 2008). Twenty-eight patients completed the study (eight with benign and 20 with malignant colorectal tumours) after acceptance of informed consent. Among benign tumours, only polyps not suitable for endoscopic resection were included. Serum controls were taken from 10 healthy volunteers after acceptance of informed consent. Staging was performed after colonoscopy and biopsy, with abdominal computed tomography (CT) and chest radiography. Clinical variables were included in a database. Patients with severe dysplasia or large symptomatic low-to-moderate dysplastic adenomas and adenocarcinoma underwent surgery. Intraoperative blood samples were collected to determine baseline parameters, specific oncomarkers (carcinoembryogenic antigen, CEA; cancer antigen 19-9, CA19-9, cancer antigen 50, CA 50) and MMP7. Intraoperative specimens were collected from cancer tissues and from normal surrounding mucosa (about 2-2.5 cm from the tumour edge). Lymph nodes from the colonic mesentery close to the tumour were collected. The specimens were fixed in 4% formaldehyde before histopathological examination.

Samples and serum preparation. Tissue samples from tumoural and mucosal tissue, as well as from lymph nodes, were kept in sterile tubes at –80°C. They were then cut obtaining aliquots weighing between 50 and 100 mg. These were treated with 300 μl of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% NP-40, 1% CHAPS, 2 mM EDTA dissolved in tetra-distilled water). A mixture of protease inhibitors (Complete-Mini Protease Inhibitor Cocktail Tablets; Roche, Mannheim, Germany) was added just before use. Samples were first homogenized by Ultra-Turrax® (T10 basic; IKA®, Staufen, Germany), then sonicated for 20 sec and centrifuged at 14,000 rpm for 20 min (19). The supernatants were then collected. Venous blood samples were drawn into sterile vacuum tubes and left at room temperature for 30 min, then centrifuged at 4,000 rpm for 15 min, to divide serum from pellet, as standard laboratory protocol. Serum was immediately aliquoted and stored at –80˚C until assayed. The protein content of supernatants and serum samples was determined by using the Bradford assay.

Histology. Samples were fixed in 4% phosphate-buffered formaldehyde and later embedded in paraffin. Sliced specimens stained with hematoxylin and eosin were analyzed under light microscopy. At least three slides were studied from each specimen by a blinded observer. Stage definition was stated according to 2002 UICC classification (20).

Immunohistochemistry. For immunohistochemistry, standard avidin-biotin procedures for human MMP7 were used. After deparaffinization and washing in phosphate-buffered solution (PBS), endogenous peroxidase activity was blocked by incubating the sliced sections in 3% hydrogen peroxide in PBS for 10 min. Analysis for MMP7 was performed using anti-MMP7 (MAB-10756; Immunological Sciences, Rome, Italy) following the manufacturer’s instructions. Biotin-conjugated secondary antibody and streptavidin-conjugated horseradish peroxidase (Dako North America, Inc., CA, USA) was applied to sections for 45 min at room temperature, and developed using 3,3′-diaminobenzidine (DAB) as substrate. Finally, counterstaining with haematoxylin was performed. Sections were mounted and the grade of staining was determined on randomly selected areas counter-checked for intensity by a blinded observer.

MMP7 determination. In supernatants and serum samples, total human MMP7 levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Quantikine®, R&D Systems, Minneapolis/USA). Diluted samples (150 μl) were added to a 96-well microtiter plate, pre-coated with a monoclonal antibody to human MMP7 and incubated at room temperature for a further 2 h on a microplate shaker. After washing, 200 μl of the secondary antibody solution were added, and the plate was incubated for 2 h at room temperature on the shaker. After washing, the substrate solution was added and incubated at room temperature in the dark. A 50 μl stop solution was added after 30 min and the optical density was measured using a microtiter plate reader (Opxsys MR®; Dinex Technologies, Inc.; Chantilly, VA, USA) at 450 nm, with correction wavelength set at 570 nm.

Western blot analysis. For western blot analysis, supernatants obtained from lymph node specimens were separated on a sodium dodecyl sulphate-polyacrylamide electrophoresis gel with a concentration of acrylamide specific for MMP7 and β-actin. Proteins were blotted onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA) and probed with the following antibodies: anti-MMP7 (MAB-10756 Immunological Sciences) and anti-β-actin (A 5060; Sigma Chemical Co., St. Louis, MO, USA). Antigens were detected with an enhanced chemoluminescence (ECL) kit from Amersham (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). All western blotting images were acquired and analyzed through an Imaging Fluor S densitometer (Bio-Rad Laboratories, Hercules). The optical density (O.D.) of each condition was correlated to the signal of the β-actin internal control.

Statistical methods. Data are expressed as the mean±standard deviation (SD). The statistical comparisons between groups were performed by using the analysis of variance (ANOVA) followed by the Bonferroni post hoc test. Differences were considered significant at p<0.05. Analysis was performed by using a statistical software (GraphPad Software, Inc., San Diego, CA, USA).

Results

Patients’ characteristics. The study group consisted of 28 patients (16 males, 12 females) with mean age±SD (standard deviation) of 74±6 years and a median of 72 years (range=55-88 years). Age was equally distributed between males and females. Tumour sites were: right colon in eight, left colon in twelve, rectum in eight. The surgical techniques used were: right hemicolectomy in eight, left hemicolectomy in six, sigmoidectomy in four, subtotal colectomy in two,
anterior rectal resection in six, abdominoperineal rectal excision in two. Among eight patients with rectal cancer, none underwent neoadjuvant radiotherapy or chemotherapy. No major complications were observed after surgery. No mortality was registered within 30 days after operation. Specimens were divided into four groups: A, serum controls of healthy volunteers; B, benign tumors (dysplastic adenomas); C, stage I and II disease (adenocarcinomas); and D, stage III and IV disease (adenocarcinomas).

CEA was increased in seven patients (>5 ng/ml) belonging to group D. CA19-9, CA50 and the clinical variables recorded pre-operatively did not show any significant differences.

Histology. Stained specimens of the tissues were examined and staged according to three groups as dysplastic adenomas (n=8); no disseminated disease (n=10), including stage I (n=7) and II (n=3); and disseminated disease (n=10), including stage III (n=8) and IV (n=2).

Immunohistochemistry. No MMP7 expression was observed in normal mucosa (Figure 1A). MMP7 was expressed in benign tumours (Figure 1B). A tendency for more evident expression in well-differentiated (Figure 1C) compared to non-differentiated carcinomas (Figure 1D) was observed.

ELISA. MMP7 expression was significantly (p<0.01) higher in stage I and II cancer tissues compared to adenomas (low and moderate dysplasia) and significantly lower compared to stage III and IV cancers (Figure 2). Levels in adenomas were also significantly lower (p<0.001) compared to those in stage III and IV disease. Normal mucosa (negative tissue control) did not show any measurable levels of MMP7 in any of the samples (data not shown). No significant difference was observed in serum levels of MMP7 comparing patients with benign adenomas to those with stage I and II cancers.

A significant increase (p<0.01) was evident in serum comparing patients with adenomas to those with stage III and IV cancer. However, no significant differences were observed for MMP7 expression within the groups comparing stage I to II and III to IV in both tumour and serum. Serum obtained from healthy controls showed very low or undetectable levels of MMP7. Lymph nodes presented lower levels of MMP7 compared to serum and tumoural tissue. Significant differences in in expression in lymph nodes were noticed.

Figure 1. Immunohistochemistry for matrix metalloproteinase-7 (MMP7). Negative staining in healthy mucosa (A). MMP7 expression in low-grade dysplastic adenoma (B), well-differentiated carcinoma T2N0M0 (C) and in poorly-differentiated carcinoma T3N2 M0 (D), original magnification, ×20.
among groups, adenoma vs. stages I and II ($p<0.05$) and stages III and IV vs. both stages I and II and adenomas ($p<0.001$) (Figure 2).

**Western blot analysis.** Further analysis of lymph nodes by western blotting, with a semi-quantitative measurement of MMP7 expression, confirmed the ELISA results. We observed a significant positive trend in the expression of MMP7 from adenoma to increasing cancer stage. MMP7 was evaluated by comparative detection of β-actin. Lymph nodes of patients with stage I and II tumours had significant higher expression than those in patients with adenomas ($p<0.05$) and those in patients with stage III and IV tumours had significantly higher levels of MMP7 ($p<0.05$) compared to those with stage I and II adenocarcinomas and those with adenomas ($p<0.001$) (Figure 3).

**Discussion**

The prognostic significance of MMP7 and its role in tumour biology has been widely investigated. Nevertheless the specific mechanism through which it promotes tumour invasion and spread is still unclear. The significance of MMP7 increase in resected specimens and in serum for the definition of oncological risk and prognosis for patients is still not clearly defined. In cancer immunology, a clear role of MMP7 and other MMPs has been shown for tumour growth, invasion and spread (6, 7, 17). Tumour specimens in our study had significantly higher levels of MMP7 in adenocarcinoma compared to varying grades of dysplasia ($p<0.001$). Even though the level of MMP7 in those with disseminated disease was less than in those with stage I and II cancer, it was nevertheless still significantly higher than that in adenomas ($p<0.001$). Immunohistochemistry showed a progressive increase with increasing dysplasia and cancer disease stage. Western blot showed a significant progressive increase in lymph node MMP7 with increasing dysplasia and infiltrative disease stage. MMP7 levels were lower in lymph nodes compared to tumour tissue, but here again, we found successive significant increases compared to adenoma through stage I and II disease to locally advanced cancer. Interesting results presented in a study by Ichikawa et al. who studied MMP7 expression by RT-PCR in lymph nodes from patients with colon cancer and showed that its expression increased accuracy in diagnosis compared to ordinary histology (17). MMP7 detected in adenocarcinoma (by RT-PCR) was associated with over 90% of histologically-positive cancer, whereas 30% of lymph nodes primarily defined as negative at histology were found to be positive for MMP7 RNA (21). Neoplastic infiltration is related to degradation of elastin, laminin, proteoglycans, osteopontin, fibronecin and type IV collagen which is mediated by MMP7. MMP7 is even overexpressed after radiation compared to preoperative levels in patients with rectal cancer (14). In a previous study, our group confirmed the correlation of MMP7 expression and immune system status, where levels increased significantly whenever the intra-luminal microflora was suppressed with antibiotics. We found that microfloral regulation does not affect MMP7 stimulation after surgical or radiological trauma (22).
Increased MMP7 expression in serum in multivariate analysis generally correlates with worse prognosis, local invasiveness, a tendency for metastatic disease and with reduced overall survival (16, 22, 23). In colorectal cancer, levels of MMP9 (which is activated by MMP7) investigated in peripheral and portal blood showed increased levels and correlated with advanced and metastatic disease stage (8, 17). Our serum ELISA results showed a clearly significant increase of MMP7 in locally advanced disease over adenoma and stage I and II cancer. No significant differences were observed in MMP7 between adenoma and non-invasive cancer, but within the two cancer groups, there was again a significant increase in MMP7 from non-metastatic to metastatic disease.

Our study, although lacking the sensitivity of RT-PCR analyses, shows that with simpler cost-benefit methods, similar results or trends can be observed when examining tumor behaviour with resected specimens. The complexity of cancer warrants combinations of different analyses in order to achieve better prognostic goals.

The results of the analyses of lymph nodes suggest a reliable application of ELISA and western blotting as an alternative to RT-PCR in staging for advanced local cancer in order to avoid down-staging in cases with histologically-negative nodes. Furthermore, immunohistochemistry seems to be a good complement.

MMPs, including MMP7, continue to be an interesting group in the quest for choosing biomarkers that can help us in the clinical setting. Evidence that T-cells generated in vitro may target MMP7-derived antigen expressed on the cellular surface of antigen-presenting cells might represent a potential option in immunotherapy against cancer (24).

Conclusion

Significantly increased concentrations of MMP7 were observed in tumor tissues, lymph nodes and in serum in and were associated with increasing grade of dysplasia and adenocarcinoma infiltration. We showed a correlation between MMP7 expression and the risk of lymph nodal involvement. This suggests a possible role for MMP7 in the determination of locally advanced cancer in resected specimens, in staging and in planning for eventual adjuvant therapy. Further investigations are, however, required to determine the exact role the complex molecule MMP7 may play in cancer treatment.

Competing Interests

None of the Authors of the study have any conflicts of interest with regards to funding or support of any kind of the study.

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