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Active MMP-2 Effectively Identifies the Presence of Colorectal Cancer

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Abstract

Fully active MMP-2 is expressed at such low levels in human tissues that studies often fail to confirm its value as a cancer marker despite strong associations with malignancy. This study utilized careful extraction, accurate activity measurements, standardization to purified controls and a new statistical metric to determine whether active MMP-2 is an effective indicator of colorectal cancer compared to pro-MMP-2 or pro-MMP-9. MMP-2 and MMP-9 activities were analyzed in matched normal and cancer samples from 269 patients by gelatin zymography, computer-assisted image analysis, serial dilutions of strong samples and standardization to controls. An index of effect size was designed for comparative evaluation of active MMP-2, pro-MMP-2 and pro-MMP-9 activities. For each gelatinase, mean activity and protein levels/mg soluble protein in normal mucosa and colorectal cancer were calculated for the first time with respect to commercial standards. Active MMP-2 activity, detected in 99% of colorectal cancers, was higher in 95% of cancers (on average 10-fold) than in normal mucosa. Levels of pro-MMP-2 and pro-MMP-9, but not active MMP-9, activities were also significantly higher in cancers versus normal. However, active MMP-2 activity provided the most effective test for the presence of cancer ($P < 0.0001$) with

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Statement of Novelty: We have measured MMP-2 and MMP-9 activities and protein content for the first time in units referenced to external controls to permit lab-to-lab reproducibility and a baseline for development of clinical assays. We have used the effect size statistic to compare matrix metalloproteinase activities in patient-matched normal and cancer tissues, optimizing analysis of matched-pair data. Finally, receiver operating characteristic (ROC) curves demonstrated that a cut-off for active MMP-2 activity of 44 SDU/mg total soluble protein, which is three times mean normal levels, will permit detection of colorectal cancer with an estimated sensitivity of 84% and estimated specificity of 93%. Comparisons to ROC curves for pro-MMP-2 and pro-MMP-9 show that active MMP-2 activity has a much higher estimated probability of correctly distinguishing colorectal cancer from normal mucosa than pro-MMP-2 and pro-MMP-9.

Statement of Impact: Definition of active MMP-2 activity and protein levels in normal and cancer tissues with reference to a commercial standard is a significant step in development of this marker for clinical or research applications. Statistical analysis of normal and cancer paired samples from 269 colorectal cancer patients (the largest such database for these markers) demonstrates that the active form of MMP-2 is a much more effective indicator of the presence of colorectal cancer than either pro-MMP-2 or pro-MMP-9 activities. Our results suggest that targeting the activation of MMP-2 may prove more effective as a cancer therapy than broad spectrum inhibition of MMP-2 and MMP-9 activities. In addition, it may be possible to extend this assay to the detection of cancer in other organs, including lung, pancreas, thyroid and breast.

an effect size statistically significantly larger than for either pro-MMP-2 or pro-MMP-9. Receiver operating characteristic (ROC) curves demonstrated that a cut-off for active MMP-2 of >44 SDU activity/mg soluble protein (>180 pg/mg), which is three times mean normal levels, would permit detection of colorectal cancer with an estimated sensitivity of 84% and estimated specificity of 93%.

Keywords

cancer diagnosis; colorectal carcinomas; active MMP-2

INTRODUCTION

The ability of matrix metalloproteinases (MMPs) to degrade components of the extracellular matrix has led to extensive characterization of these enzymes for their roles in cellular function under both physiological and pathological conditions (1–3). More than 20 MMPs have been described thus far, with the gelatinases, MMP-2 and MMP-9, of particular interest for their potential contributions to cancer invasion and metastasis (4,5). Significant increases in MMP-2 and MMP-9 expression levels have been reported for many types of human cancer (6–13), as well as in cell culture and animal models of cancer, providing evidence for a strong association between gelatinase levels and cancer invasion or metastasis (14,15). The development of MMP inhibitors (MMPIs) for cancer therapy proved effective in reducing cancer growth and spread in animal models (reviewed, 4). However, subsequent clinical trials to treat human cancers by inhibiting MMPs with either broad spectrum MMPIs, such as marimastat and batimastat (British Biotech) or “deep pocket” MMPIs such as prinomastat (Agouron) and tinomastat (Bayer), have been unsuccessful (16). As a result, researchers are attempting to characterize in more specific detail the mechanisms by which abnormal expression of MMPs contribute to cancer. These proteinases do not function merely by digestion of the extracellular matrix and the removal of physical barriers to cancer spread. They are also capable of activation cleavages of angiogenic factors, latent growth factors, growth factor binding proteins, adhesion molecules, cell surface receptors, cytokines and other proteins (5). They are therefore implicated in pathways that regulate angiogenesis, growth control and inflammation as well as invasion and metastasis.

With the elucidation of multiple substrates and functions for MMPs, it has become important to sort out the distinctive roles and relative contributions of individual MMP activities at different cancer stages and in various types of cancer. Our goals for this study were to generate a more complete and accurate database on the comparative levels of the individual forms of active and pro-MMP-2 and MMP-9 activities in a large set of primary human colorectal carcinomas and patient-matched control mucosa (n = 269 paired samples) and to improve evaluation methods for studying matched pair data on several gelatinases simultaneously. We have approached these goals by measuring gelatinase activity with respect to purified commercial matrix metalloproteinases that served both as internal controls for gel-to-gel variation and as external standards for defining specific activities and protein concentrations in normal and cancer tissues. Serial dilution and computer imaging of both standards and samples were used to assess and quantify the full range of expression for the active and pro-forms of MMP-2 and MMP-9 in multiple tissues. We have estimated an effect size statistic to evaluate individual gelatinase activities as markers of the presence of colorectal cancer. We have also plotted receiver operating characteristic (ROC) curves to estimate the sensitivity and specificity profiles of these markers for identification of colorectal cancer versus normal mucosa.

MATERIALS AND METHODS

Patient and Tissue Samples

Paired sets of patient-matched normal colon mucosa and carcinoma tissues were obtained from 269 patients following surgery for colorectal cancer (154 men, 115 women; age range: 33 to 99 years; median age: 70). Normal mucosa samples were obtained at least 5–10 cm from the tumors and were separated from submucosa, muscle layer, serosa and surrounding fat prior to being snap frozen and stored at -80°C . Thirty percent of our samples were collected from Boston University Medical Center (BUMC, 76 matched pairs) while 70% were collected through the Cooperative Human Tissue Network (CHTN, 193 matched pairs) in accordance with human research protocols approved by the Boston University Medical Center Institutional Review Board. Matched pairs were numbered sequentially as they arrived, irrespective of site of origin. CHTN samples were snap frozen, sent on dry ice from the Midwestern (Ohio), Eastern (Pennsylvania) and Southern (Alabama) divisions and stored at -80°C immediately upon arrival. Information on cancer stage and histopathology for individual carcinomas was obtained from pathology reports. Cases were excluded from this study if only normal or only cancer tissue was obtained (no matched pair); polyp, lymph node or metastatic tissue was obtained rather than primary cancer; wrong type of cancer was sent; no pathology report was available; sample was mishandled (placed in saline or not kept frozen) or was too small for analysis. We analyzed normal mucosa and matched colorectal carcinoma samples from cancer cases at all stages (Stage I, $n=18$; Stage II, $n=117$; Stage III, $n=90$; Stage IV, $n=44$). Staging of carcinomas was done in accordance with the *American Joint Committee on Cancer (AJCC) Tumor Node Metastasis (TNM)* system (17), with stage I cancers defined as invading into the submucosa or muscularis propria (T1, T2), stage II cancers defined as having spread through the muscularis propria into the subserosa, pericolic or perirectal tissues (T3) or directly into other organs or structures or perforating visceral peritoneum (T4) without involving lymph nodes, stage III cancers (any T) as having regional lymph node metastases, and stage IV cancers (any T) as having distant metastases, most commonly to the liver or lungs. Normal mucosa was selected as the appropriate patient-matched control tissue as it contains the epithelium from which carcinomas arise. These samples provided “baseline” enzyme measurements that were incorporated into the effect-size statistic that became the focus of some of our data analyses, as described below.

Tissue Extraction

To minimize variation, each pair of normal mucosa and matched carcinoma was extracted and assayed at the same time. To achieve more consistent detection of endogenous active MMP-2 in particular, an extraction method was used to optimize cell lysis with minimum auto-degradation in stored samples (18). Tissue samples (60–80 mg) were homogenized in 500 μl of distilled, deionized water, frozen and thawed three times, and centrifuged for 50 min at 4°C at 17,210 g in a Sorvall 5B centrifuge. Supernatants were removed and protein content of extracts determined by the method of Lowry (19) using bovine serum albumin as the standard.

Gelatin Zymography

Tissue extracts were analyzed on gelatin zymograms for identification of gelatinolytic type IV collagenases, including all pro-and active MMP-2 and MMP-9 bands. Zymograms were performed as described by Rao et al. (20) with minor modifications as published (21). Briefly, 25 μg soluble protein were electrophoresed on a commercially prepared 10% SDS-PAGE gel ($8\times 8\times 0.1\text{cm}$) containing gelatin (1 mg/ml) (Invitrogen/GIBCO, Grand Island, NY, USA). Gels were subjected to two 30-min washes in 2.5% Triton X-100 prior to development of enzyme activity bands in substrate buffer (50mM Tris-HCl, 10 mM CaCl₂, 0.15M NaCl, 0.05% NaAzide, pH 7.5) for 18 hours. Resulting gelatinolytic enzymes were

detected as transparent bands of digested gelatin against a Coomassie blue-stained gel background.

Molecular weight standards (30,000–200,000; Sigma, St. Louis, MO USA) were electrophoresed with samples to identify band sizes for different MMP forms. To verify the sizes of activated MMP forms on zymograms, several samples were also treated with 1mM p-aminophenylmercuric acetate (APMA, Sigma, St. Louis, MO USA) for 1.5 hr at room temperature before gelatin zymography was performed (22). To ensure that we were measuring MMP activities, enzyme specificity was tested in both normal and cancer samples by incubating sample gel slices in different proteolytic inhibitors as previously described (21), including 10 mM EDTA (Fisher, Fairlawn, NJ USA) 100mM E-64 (Sigma), 200uM phenylmethylsulfonyl fluoride (PMSF) (Sigma), 1ug/ml pepstatin (Sigma) and 10mM 1,10 phenanthroline (Sigma, St. Louis MO USA). In addition, to confirm identity of specific gel bands and to standardize activity measurements, purified active gelatinase controls for active MMP-2 (human recombinant active MMP-2), pro-MMP-2 (human synovial fibroblast proenzyme MMP-2) which is separated from a non-covalent 1:1 complex with TIMP-2 and activated in the presence of SDS during gel electrophoresis (23) and human neutrophil MMP-9 (all from Calbiochem, La Jolla, CA USA) were electrophoresed next to the tissue extracts, as detailed below. Purified standards were diluted in recommended buffers with the addition of BSA (5 µg/µl) (Sigma, St. Louis MO USA) to increase enzyme stability during storage.

Normal mucosa and colorectal tumor samples from the same individual were run side-by-side on zymograms to control for intrinsic genetic variability in normal banding patterns in individuals and for sample handling of a given case. A constant amount of soluble protein was loaded into each gel slot. As an additional control for loading, the bottom section (2–4 cm) of each gel was cut off before the gelatinase assay and stained with Coomassie Blue to permit detection of a low molecular weight protein that is relatively constant across samples and does not demonstrate a cancer-specific change from normal to cancer tissue (24). Precise gel buffer pH was important in generating sharp gel bands. Careful protein loading per lane and extensive gel washing before the substrate incubation and after Coomassie Blue staining contributed to assay reproducibility.

Band Quantification

Computer-assisted imaging employed Total Lab software (also called 'Phoretix ID Quantifier'; Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK; see www.nonlinear.com for references) to score each MMP band in activity units, based on band area×pixel intensity with resulting activity measured in "Total Lab Units" (TLU). Purified commercial controls (Calbiochem, EMD Biosciences, La Jolla, CA USA) were also used to standardize MMP-2 or MMP-9 enzyme activity in µUnits /mg soluble tissue protein with resulting activity based on commercially defined measurements of purified MMP-2 or MMP-9 controls. For this report, commercial activity units for active MMP-2 are designated "TPH units" (thiopeptolide hydrolysis units) based on the control for active MMP-2, defined as having 9×10^3 µUnits activity/ng protein (Calbiochem, EMD Biosciences, La Jolla, CA USA) in a standard thiopeptolide hydrolysis assay (25). Commercial activity units for pro-MMP-2 and pro-MMP-9 (Calbiochem, EMD Biosciences, La Jolla, CA USA) were designated "SPS units" (synthetic peptide substrate units) based on pro-MMP-2 control activity (190×10^{-3} µUnits/ng protein) against the dinitrophenyl-linked synthetic peptide substrate 2,4-DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH or on the purified MMP-9 control activity (2.07 µUnits/ng protein) against the same synthetic peptide substrate (26).

Although Total Lab imaging software permitted digital assessment of gel bands and a standardization to commercial standards, this methodology was limited by the dynamic

range of a computer imaging in measuring samples across a broad spectrum of activity on a single gel for comparisons of MMP activities in normal versus cancer samples. Accurate comparisons of such samples were determined by serial dilution of the sample to generate the weakest complete band for each gelatinase activity, defined arbitrarily as 1 serial dilution unit (SDU), and then measured by computer imaging and with reference to purified controls to generate activity units for MMP-2 and aMMP-9 that can be interconverted and compared to similar activity measurements made in other laboratories. For serial dilution units (SDU) the activity levels in the original undiluted sample are defined by the extent of dilution required for a given sample to generate the weakest complete band of activity for a particular gelatinase form (with controls for this definition coming from computer imaging of purified standards and samples).

Thus, we have defined MMP bands for this report in 3 types of unit as described above, including commercial activity units (either TPH or SPS units), Total Lab software imaging units (TLU) and serial dilution units (SDU), which are interconvertible as each can be referenced to the other and to external standards. Data for figures and tables have typically been given in serial dilution units for this report as these are units that can be readily and universally calculated for each marker but also can be standardized here to commercial activity units or converted to Total Lab imaging units using the data given in Table 1.

Statistical Methods

To compare gelatinase activity levels with respect to tissue procurement sites, we used a two-tailed t-test to assess whether there was a difference between mean active MMP-2 activities in the tissue samples that were obtained locally (from Boston Medical Center) and those obtained distantly (through the Cooperative Human Tissue Network).

We also determined whether activity levels varied with cancer stage. Preliminary data-analyses indicated that sample distributions of the levels of each marker, in both normal and cancerous tissues, were positively skewed. The presence of the skewness impacts estimates of location and variability, and renders data inhospitable to parametric analysis based on normal-theory assumptions. Methodologists (27) recommend that such data be square-root transformed before analysis. We adopted this recommendation. After transformation, the Wilcoxon matched-pairs signed rank test was used to determine whether activity levels of each gelatinase marker differed in normal and cancer tissues at each cancer stage.

It was not possible to compare enzyme levels for active MMP-2, pro-MMP-2 and pro-MMP-9 directly, as each marker required different scales for measurement of cancer-specific changes. We could, however, compare enzyme levels between normal and cancerous tissues for each of the individual markers. To compare the relative effectiveness of the three markers for cancer detection, we created an index of marker effectiveness that borrowed the notion of effect size (i.e. a standardized mean difference) from statistical power analysis (28) and meta-analysis (29). For each marker, we defined effect size as the difference between the square-root transformed enzyme levels in cancerous and normal tissues, expressed in units of the standard deviation of the square-root transformed enzyme levels in the normal tissue, as follows:

$$ES_i = \frac{(CT_i - NT_i)}{s_{NT}}$$

where CT and NT represent the square-root transformed enzyme levels in cancerous and normal tissue, respectively, for the i^{th} patient and s_{NT} is the standard deviation across patients of NT . For each patient we then estimated an effect size for each of three markers:

active MMP-2, pro-MMP-2 and pro-MMP-9. Repeated-measures analysis of variance (30) was used to test whether average effect size differed across markers.

Finally, in order to determine the potential effectiveness of gelatinase activity assays for detecting the presence of cancer in individual colorectal tissue samples, receiver operating characteristic curves (ROC) were plotted for active MMP-2, pro-MMP-2 and pro-MMP-9 activity in 269 normal colorectal mucosa versus 269 colorectal cancer samples (30).

RESULTS

MMP-2 and MMP-9 Activities and Assay Controls

MMP-2 and MMP-9 activities in matched normal (N) and cancer (C) tissues are shown with respect to size markers (M) (Fig 1A). Reproducibility of gelatinolytic banding was demonstrated by a strong correlation coefficient (0.86; $p < 0.01$) for duplicate assays run on different days for 24 normal/cancer pairs (Fig 1B). A slightly lower, but still strong, correlation (0.79; $p < 0.01$) was measured for duplicate assays on 29 matched tissue pairs that had been re-extracted as well as re-assayed, with minimal tissue heterogeneity revealed by reextraction (Fig 1C). We also found no statistically significant differences in mean active MMP-2 activities for either normal or cancer samples obtained locally (BUMC) versus distantly (CHTN) ($p = 0.46$ for normal tissues; $p = 0.55$ for cancer tissues, $n = 269$).

Treatment of samples with 1mM APMA resulted in activation to lower molecular weight bands as expected (22), with a corresponding decrease in pro- forms of MMP-9 and MMP-2 and an increase in active forms (Fig 1D, left side). Active MMP-9 demonstrated a slightly lower molecular mass in normal tissue versus cancer, whether APMA treated or not, possibly reflecting normal/cancer differences in glycosylation. APMA treatment generated the intermediate form of active MMP-2 at 62 kD that can be seen in both APMA-treated normal mucosa and cancer samples, as expected (22), and that is distinguishable from the faster migrating, fully active 60 kD MMP-2 present in cancer samples with or without APMA treatment. Metalloproteinase inhibitors, EDTA and phenanthroline completely inhibited all gelatinolytic bands detected in this same colorectal cancer sample (Fig. 1D, right side), confirming that these bands are due to metalloproteinase activities. No inhibitors for other classes of proteolytic enzyme, including cysteine (E-64), serine (PMSF) or aspartyl (pepstatin) resulted in inhibition of these bands (data not shown).

Quantitative Measurement of MMP-2 and MMP-9 Activity Levels with Respect to Commercial Controls

Dilutions of purified active MMP-2, pro-MMP-2 and MMP-9 standards (Fig 2) run on zymograms were quantified using Total Lab software and graphed in Total Lab Units (TLU) vs. commercially defined μ U of activity (Fig 2). Measurements in TLU of active MMP-2, pro-MMP-2 and pro-MMP-9 resulted in linear standard curves over a 4- to 5-fold range but were not linear over a 10-fold range of activity. To improve measurement of gelatinase activities over the broad range observed in normal and cancer samples (>200-fold for some comparisons as seen in Fig 3A), serial dilution of samples was done to further define activity units (SDU), as illustrated for Case 174 (Fig 3B) which had very high gelatinase activities in the cancer (C174) compared to matched normal tissue (N174). The active MMP-2 band in C174 contained 10 SDU of activity while the pro-MMP-9 band contained 50 SDU. Using data from activity bands in multiple cases ($n = 363$ bands), the estimated correlation for measurements calculated in TLU by imaging software versus measurements of the same bands evaluated independently in SDU was 0.8 ($p < 0.01$).

Known quantities of commercial standards for active MMP-2, pro-MMP-2 and pro-MMP-9 were electrophoresed on gelatin zymograms next to colorectal tissue extracts to confirm the

location of specific activities and provide external controls with defined activity for comparison to unknown samples (Fig 3C). These purified controls together with computer imaging and serial dilution measurements made it possible to calculate enzyme activity for each gelatinase in 3 types of interconvertible units/mg protein for the set of 269 tissue pairs (Table 1).

In addition to activity measurements, estimates of protein content/mg total soluble protein in normal and cancer tissues for each gelatinase could also be approximated by comparison of average activity units per mg protein for each gelatinase (Table 1) to purified commercial standards. Thus, mean protein concentrations/mg total soluble protein in normal mucosa and colorectal carcinoma extracts were estimated at 59 pg/mg and 610 pg/mg respectively for active MMP-2, 2.3 ng/mg and 4.2 ng/mg respectively for pro-MMP-2 and 13.5 ng/mg and 42.9 ng/mg respectively for pro-MMP-9. These calculations assume equivalent specific activities for purified standards and corresponding proteins within a tissue extract and therefore may overestimate the MMP protein content within tissue samples as purified control proteins may have lost activity during purification while the same proteins within an extract are often stabilized by the presence of other proteins.

Normal vs. Cancer Activity Levels for Pro- and Active Forms of MMP-2 and MMP-9

Active MMP-2—Levels of 60 kD active MMP-2 activity were very low in 70% and not detectable in 30% of normal (N) tissue extracts, despite clear detection of other gelatinases in the same samples (Fig 1 and Fig 3). However, the 60 kD active MMP-2 activity was detectable in 265/269 cancer samples (98.5%) and was higher in cancer vs. matched normal for 256/269 cases (95.2%) with mean active MMP-2 activity ten times higher in cancer vs. normal mucosa ($p < 0.0001$) (summary data, Table 1 and Fig 5).

Pro-MMP-2—Compared to other gelatinases, the 65 kD pro-MMP-2 demonstrated a smaller range of variation in normal and cancer samples. Pro-MMP-2 had higher baseline levels in normal tissues than active MMP-2 (see Fig 1 and Fig 3) but a much smaller average difference between cancerous and matched normal samples. Nevertheless, in 80.7% of the cases analyzed, pro-MMP-2 activity was higher in the cancer than in patient-matched normal mucosa ($p < 0.0001$).

Active MMP-9—The 80 kD active MMP-9 activity band was only present in half of the samples analyzed, normal or cancer, and seldom expressed in both normal and cancer from the same patient. In contrast to the other three markers, this gelatinase activity was lower in cancers versus normal as frequently as it was higher (Fig 1 and 3). Active MMP-9 activity levels in 97 cases (194 tissues) demonstrated no statistically significant average difference between cancer and normal mucosa. Once initial data on these 97 cases had been collected, active MMP-9 was not evaluated further as a marker for cancer detection.

Pro-MMP-9—Of the 4 gelatinases analyzed, the 97 kD pro-MMP-9 activity demonstrated the broadest range of activity and greatest variation from case to case for both normal and cancer tissues. Pro-MMP-9 activity levels were dramatically higher in some cancer samples than in matched normal (e.g., Fig 1A, Case 29; Fig. 1B, Cases 278 and 280; Fig 1C, Case 134; Fig 3A, Cases 128, 331 and 376; Fig 3B, Case 174) but differed minimally in other cases (e.g., Fig 1A, C31; Fig 1B, Case 282; Fig 3A, Cases 129 and 130), and in some cases were even lower in cancer than normal (e.g. Fig 3A, Cases 160, 359 and 311). However, mean pro-MMP-9 levels in cancer were three and a half times those of patient-matched normal mucosa ($P < 0.0001$). In 81.8% of 269 cases, pro-MMP-9 activity was greater in cancer than matched normal mucosa ($p < 0.0001$).

MMP-2 and MMP-9 Activities with Cancer Stage

Notable variation in expression was observed for different MMP-2 and MMP-9 bands in the same tissues but no stage-specific patterns were observed (see Fig 3A), illustrated further in box plots of median active MMP-2, pro-MMP-2 and pro-MMP-9 activities for 269 normal and cancer extracts graphed with respect to cancer stage (Fig 4). Normal mucosa showed no significant changes in marker levels from stage to stage. However, the median difference between normal and cancer levels for every marker, comparing individual matched pairs at each stage, was elevated in cancers (C) versus normal mucosa (N). Using the Wilcoxon signed-rank test, we rejected the null hypothesis that distribution of values in normal and cancer tissues were the same ($p < 0.01$ for normal vs. cancer median values for pro-MMP-2 in Stage I cancers; $p < 0.001$ for all other comparisons at all stages). Highest median activity for each marker was measured in Stage II cancers. None of the 3 gelatinase activities demonstrated progressive increases at later stages.

To test whether MMP activity levels in cancers were related to corresponding levels in matched normal tissues, correlation coefficients were estimated. The resulting correlation coefficients were very low (0.158, 0.392 and 0.130, for active MMP-2, pro-MMP-2 and pro-MMP-9 respectively), a pattern also evident in the 18 cases seen in Figure 1 and Figure 3.

Effect Sizes for MMP-2 and MMP-9 Activities

Univariate descriptive statistics on enzyme levels in cancerous and normal tissues for active MMP-2, pro-MMP-2 and pro-MMP-9, both before and after applying a square root transformation, demonstrate that mean enzyme levels are consistently higher in cancerous than normal tissues, regardless of marker. Thus, all three gelatinase activities successfully detected the presence of colorectal cancer. However, active MMP-2 was a much more effective indicator of colorectal cancer than pro-MMP-2 or pro-MMP-9, with an average effect size for active MMP-2 of 6.21, more than four times as large as average effect sizes for the other activities (which were 1.34 and 1.53, respectively).

This same pattern was replicated for transformed enzyme levels in cancerous and normal tissue and the corresponding effect size indices (Fig 5A, right side). Even in the more conservative transformed metric the average effect size for active MMP-2 (3.17) was more than twice as large as those for pro-MMP-2 and pro-MMP-9. The parallel schematic plot for transformed data (Fig 5C) illustrates the much larger median effect size for the active MMP-2 marker (represented by the solid vertical line intersecting the central box) than for the pro-MMP-2 and pro-MMP-9 markers.

Using repeated-measures analysis of variance testing (Fig 5B), we were able to reject the null hypothesis that effect size was identical across the three markers ($F[2,536]=185.61$, $p < 0.001$) and to conclude that effect sizes differed dramatically across markers, on average, in the population. The corresponding null hypothesis was similarly rejected for analyses on effect sizes computed on the untransformed data despite failure of the normal-theory assumptions due to skewness of the untransformed data. Subsequent multiple comparisons, conducted post-hoc, indicate that effect sizes for pro-MMP-2 and pro-MMP-9 do not differ from each other at the 0.05 level but that the effect size for active MMP-2 is larger than both of the other two at the 0.0001 level ($F[1,536]=308.20$, $p < 0.0001$ and $F[1,536]=245.01$, $p < 0.0001$, respectively) and thus, that active MMP-2 activity provides the most effective test of the presence of cancer.

Receiver Operating Characteristic (ROC) Curves

In Figure 6A–C, ROC curves are presented for active MMP-2, pro-MMP-2 and pro-MMP-9 respectively. The area under the curve for active MMP-2 (0.935) provides a measure of the

probability that, for a randomly selected pair of normal and cancerous colorectal tissues, levels of this marker will correctly distinguish colorectal cancer from normal mucosa. The corresponding areas under the ROC curves for pro-MMP-2 (0.751) and pro-MMP-9 (0.779) are considerably lower, indicating lower probabilities that pro-MMP-2 or pro-MMP-9 will correctly distinguish colorectal cancer from normal mucosa. Using a cut-off of active MMP-2 activity > 44 SDU/mg soluble tissue protein (three times the average activity of normal mucosa, or >180 pg active MMP-2 protein/mg soluble tissue protein), this assay has an estimated sensitivity of 84% and a specificity of 93% for detection of colorectal cancer. This cutoff level was chosen because it was the level that maximized the percentage of the tissues that were correctly identified (88.3%). Using the same criterion, a cut-off for pro-MMP-2 activity (>180 SDU/mg soluble tissue protein) was chosen that correctly identified 69.5% of tissues. The analogous cutoff for pro-MMP-9 activity (>280 SDU/mg soluble tissue protein) correctly identified 70.8% of tissues. At these cutoffs, the estimated sensitivities and specificities (54% sensitivity and 86% specificity for pro-MMP-2; 67% sensitivity and 75% specificity for pro-MMP-9) were considerably lower than those for active MMP-2.

DISCUSSION

This study was designed to generate an accurate database on levels of individual active and pro-forms of MMP-2 and MMP-9 activities in a large set of primary human colorectal carcinomas and patient-matched control mucosa (n = 269). Improved quantitative measurements were applied to zymograms to evaluate each marker for the ability to detect colorectal cancer.

Statistical Evaluation of MMP-2 and MMP-9 Activities as Cancer Markers

Lack of detection of active MMP-2 in many normal tissues has meant that individual cancer/normal ratios often could not be obtained, minimizing the advantage of paired samples for simultaneous analysis of several markers. In some laboratories, an alternative to C/N ratios, the “activation ratio,” has been employed (10,11,31) with mean activation ratios permitting some useful conclusions to be drawn. However, both mean C/N ratios and mean activation ratios have undesirable mathematical properties and thus do not adequately support statistical analysis. To redress this problem, we compared activity levels in normal and cancer tissues using an effect size whose metric was standardized across the different markers of interest (active MMP-2, pro-MMP-2 and pro-MMP-9). We were able to demonstrate that active MMP-2 activity provides the most effective test of the presence of cancer of the three gelatinases analyzed ($p < .0001$), with an effect size for active MMP-2 statistically significantly larger than the effect size for either pro-MMP-2 or pro-MMP-9.

In an extension of these observations, we investigated the sensitivity and specificity of our active MMP-2 activity assay for detection of colorectal cancer compared to pro-MMP-2 and pro-MMP-9 activity assays, by plotting ROC curves for each marker. The resulting curves confirmed the findings of our effect size analyses and demonstrated high specificity and sensitivity of active MMP-2 levels that were greater than 44 SDU/mg protein for distinguishing colorectal cancer from normal mucosa in individual tissues. Gimeno-Garcia and colleagues, in their analyses of biopsy samples, reported that significant upregulation of pro- and active MMP-2 only occurred in carcinomas (7). However, they also reported that some adenomas did demonstrate increased levels of active MMP-2 and suggested that additional studies might be useful. Based on our data and reports from the literature, we postulate that levels of active MMP-2 activity measured with our assay in adenomas removed during colonoscopy for colorectal cancer screening could provide useful information on the malignant potential of these adenomas, supplementing routine

pathological assessments of these lesions. Such information, in turn, could be used to optimize the timing of future surveillance colonoscopy for individuals with high risk lesions.

MMP Expression Patterns with Cancer Stage

At every cancer stage, all three gelatinase activities were statistically significantly higher in cancers than patient matched (or unmatched) normal mucosa. Elevation of active and pro-MMP-2 activities in cancers over normal mucosa was greater in Stage II than Stage I cancers but not progressively higher in more advanced cancers (Stages III and IV), possibly indicating an important role, particularly for pro- and active MMP-2, in events associated with invasion of the basement membrane and local spread rather than distant metastasis. Waas and colleagues have also reported highest levels of active MMP-2 measured in Stage II colorectal cancers (32), with lower levels of active MMP-2 in colorectal cancers that are metastatic (33).

In contrast to active MMP-2, we found that active MMP-9 activity did not demonstrate any cancer-specific expression pattern, confirming previous reports (7,34–36). Lack of elevated active MMP-9 in cancers together with dramatically elevated pro-MMP-9 activity does not, however, eliminate an important role for MMP-9 in cancers, as pro-MMP-9 demonstrates significant catalytic activity even with the pro-peptide intact (5). The dramatic range of expression for pro-MMP-9 activity, particularly in cancer samples, may reflect its high inducibility and response to many growth factors and signaling molecules (4,5) that may differ from cancer to cancer due to variation in molecular profiles. The majority of sites for potential transcriptional regulation of MMP-2 do not overlap with those for MMP-9 (4), which could explain differences in MMP-2 versus MMP-9 cancer/normal expression for the same matched pair.

Methods of Studying MMP-2 and MMP-9

Different assay methodologies, (immunohistochemistry, ELISA, *in situ* zymography, western blots, *in situ* hybridization) have proven useful in characterizing cellular localization and cancer-related changes for total MMP-2 and MMP-9 protein, activity or mRNA levels (4;37–41). Immunohistochemical (IHC) studies of MMP-2 or MMP-9 have shown no detection (42) or weak to moderate detection of these proteins in scattered stromal and epithelial cells of normal mucosa (41,43) with stronger staining in both epithelial and stromal cells of colorectal carcinomas (41,42,44). Fewer MMP-9 positive cells were seen in metastatic tumors and lymph nodes compared to earlier stage cancers (43). MMP-9 protein staining was stronger and more diffuse in epithelial cell cytoplasm of cancers than MMP-2. Our studies of MMP-2 and MMP-9 activity levels are consistent with and complement these prior observations on protein and provide additional information on specific forms (pro-versus active) of MMP-2 or MMP-9 that cannot yet be determined by IHC or ELISA since antibodies to detect the active proteins also detect the larger pro-forms without distinguishing between them.

In situ zymography has been used to demonstrate gelatinolytic activity in colon cancer metastases of rat liver (44) and in cancer cell nests of thyroid carcinomas (9) but this assay does not readily distinguish MMP-2 from MMP-9 nor even MMP from non-MMP gelatinases. Use of different classes of inhibitor to further specify activities detected by *in situ* zymography, however, suggests the presence of more than one gelatinase activity at a given site (44).

Gelatin zymography continues to be the only methodology that permits simultaneous comparison of activity levels for specific pro- and active forms of MMP-2 and MMP-9. However, while quantitation of gelatinase activities by computer imaging and densitometry

readings of zymograms has been described for some time (45), issues with methodology have prevented the standardization of results. Activity units derived from various imaging programs often differ and the dynamic range for imaging is seldom described as linear over the broad range of gelatinase activities from normal to cancer. Data from most zymography studies have not been based on corresponding measurement of purified standards, even if such controls were used to identify the position of gelatinase bands. For this report, we have analyzed expression of each major gelatinase and have defined these activities in three different interconvertible units of activity that can be referenced back to purified standards, thus integrating several methods for measuring active and pro-MMP-2 and MMP-9 activities for potential comparison across studies and laboratories.

Estimates of amounts of detectable active MMP-2, pro-MMP-2 and pro-MMP-9 protein and specific activity in human colorectal normal and cancer tissues have not, to our knowledge, been previously reported. Our ability to accurately measure very low levels of each marker in human tissues in assays that can be cross-referenced to commercially available protein controls establishes inter-laboratory standards that can be utilized at a multinational level and should permit more carefully defined characterization of these metalloproteinase activities in human disease.

Active MMP-2: Cancer Marker and Target for Therapy?

Active MMP-2 has been described as a “master switch” that triggers tumor spread and metastatic progression (46). In 1991, Yamagata and colleagues reported the activated form of 72 kD gelatinase (i.e. MMP-2) as a new gelatinase species detected in culture media of 15 gastric and 15 colorectal carcinoma explants but in only 1/30 matched normal explants (34). Subsequently, activation of pro-MMP-2 was described as a “feature of the malignant phenotype” in colorectal and gastric cancers (35). Active MMP-2 activity was reported to occur selectively in pancreatic cancer versus normal pancreas (8), lung cancer compared to normal lung parenchyma (47), papillary thyroid carcinomas compared to follicular adenomas or normal thyroid (9) and in breast cancers (31) and squamous cell carcinomas of the head and neck more frequently than comparable normal tissues (11). However, most studies of active MMP-2 have focused on a small number of cancers (8,9,35,47) or failed to detect activity in many extracts (35), even when pro-MMP-2 and pro-MMP-9 activities were highly detectable.

Extremely low active MMP-2 activity in normal tissues suggests that active MMP-2 does not contribute much to routine cell functions. Indeed MMP-2 knock-out animals have a mild phenotype, with minor deficits in developmental angiogenesis, formation of skeleton and joints (5). We found that, on average, normal tissue demonstrated activity equivalent to less than 1.5 pg of active MMP-2 protein/25 µg total soluble protein. This calculation for a normal human tissue is close to the lower limits of sensitivity (2 pg) described for detection of a purified MMP-2 control (45). Such low levels of active MMP-2 in normal colon tissue together with the mild phenotype in knock-out mice and a high sensitivity and specificity for this marker in detecting colorectal cancer suggest that specifically inhibiting MMP-2 activation may prove more effective as a colorectal cancer therapy than the broad spectrum inhibition of MMP-2 and MMP-9 activities tested previously in clinical trials that were often both unsuccessful and toxic to patients (16).

A further application of our methodologies may be the accurate measurement of active MMP-2 activity levels in non-colorectal tissues. Published reports have suggested that active MMP-2 may be a valid marker for identifying malignancy in pancreas, thyroid, breast and lung, for example (8,9,31,47). However, careful assay work must be done first to determine the active MMP-2 cut-off levels for cancer detection in surgical and biopsy specimens from these organs.

Abbreviations

MMP-2	matrix metalloproteinase-2
MMP-9	matrix metalloproteinase 9
TLU	Total Lab units
TPH	thiopeptolide hydrolysis
SPS	synthetic peptide substrate

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Figure 1. A. Gelatin Zymogram of MMP-2 and MMP-9 Activities

Two pairs of patient-matched normal mucosa (N) and colorectal cancer (C) extracts from Cases 31 and 29 were electrophoresed on a gelatin zymogram together with Coomassie blue stained protein markers (M) of defined molecular mass. Active MMP-2 was detected as a 60 kD band with strong activity in cancer samples and weak activity in matched normal mucosa. Pro-MMP-2 was detected as a 65 kD band in all 4 samples, with higher activity in cancers compared to matched normal mucosa. Active MMP-9 activity was detected as an 80 kD band in one normal mucosa sample (N29). Pro-MMP-9 activity was detected as a strong 97 kD band in all 4 samples; cancer sample C29 (but not cancer sample C31) showed higher pro-MMP-9 activity than the matched normal.

1B. Zymogram Patterns on Duplicate Gels. Reproducibility of gelatin zymogram banding patterns is illustrated for the same 8 samples (4 matched pairs of normal and cancer tissue extracts numbered 278, 279, 280 and 282) electrophoresed on different gels on different days. Samples were stored at -80°C between gel runs. A Coomassie blue stained loading control, as described in the methods section, is shown below each gel.

1C. Zymogram Patterns for Reextracted and Reassayed Samples. Reproducibility of gelatin zymogram banding patterns is illustrated for 6 samples from the same 3 matched pairs of normal and cancer tissue extracts (cases 91, 134 and 130) that were reextracted and assayed twice.

1D. Chemical Activation and inhibition of MMP-2 and MMP-9 Activities. On the left are shown gelatin zymogram banding patterns for pro- and active MMP-2 and MMP-9 activities in matched normal mucosa and carcinoma samples for Case 91 in the absence (–) or presence (+) of 1.5 hr pre-treatment with 1mM APMA prior to gel electrophoresis. On the right are shown detectable gelatinolytic activities for the Case 91 cancer sample either without the addition of a proteinase inhibitor (–) or with the addition of a metalloproteinase inhibitor (either EDTA or phenanthroline) to the activity assay.

Figure 2. Standard Curves of Purified Controls

Graphs show gelatin zymography data for serial dilutions on purified commercial standards of active MMP-2, pro-MMP-2 and pro-MMP-9 respectively. Gelatin zymogram results are shown below each graph. Defined activities are given for each purified standard (Calbiochem) based on the amount of protein loaded in that lane. Band activity levels were then quantified by computer-assisted image analysis using Total Lab software (Nonlinear Dynamics) and resulting data in Total Lab Units (TLU) graphed to generate a curve for each standard. Total Lab Units (TLU) represent each activity band in units of intensity in pixels $\times$ band area, as plotted on the Y-axis versus commercial activity units for each standard on the X-axis.

Figure 3. A. Variation in MMP-2 and MMP-9 Expression Patterns

Gelatin zymogram data show higher levels of active MMP-2 (60 kD) in 8/8 cancer samples compared to matched normal mucosa. Active MMP-2 was very low or not detectable in normal tissues. In every case higher levels of active MMP-2 identify the cancer sample in a matched pair. The dramatic range of expression for pro-MMP-9 activity (97 kD) and relatively constant expression for pro-MMP-2 activity (65 kD) is also illustrated with these samples. Case number and cancer stage (I to IV) are shown below each pair. No correlation between cancer stage and expression level was observed for any of these gelatinase activities (see also Figure 4).

3B. Representative Serial Dilution: Defining Serial Dilution Units (SDU) on a Zymogram Gel. Tissue extracts (25 µg/lane) of normal mucosa (N174) and carcinoma (C174) for Case 174 were run in undiluted form (N 174, C 174) next to serial dilutions of the cancer sample for Case 174 as labeled above each lane (1:2 through 1:100). For each MMP activity band, the number of serial dilution units (SDU) in a given band for a given lane is shown below that lane. The weakest visible complete band of pro-MMP-9 activity (seen in the 1:50 dilution) was designated 1 SDU of MMP-9 activity with the SDUs for pro-MMP-9 bands in other lanes defined by the known dilution of sample in that lane. Similarly, the weakest complete band of MMP-2 (in the 1:10 dilution lane for active MMP-2) was designated 1 SDU of MMP-2 activity with SDUs for the remaining active MMP-2 (or proMMP-2) bands in other lanes defined similarly by the known dilution of sample in that lane.

3C. Purified MMP-2 and MMP-9 Standards Run with Tissue Samples. Commercially purified standards of each gelatinase are seen in lane 1 of three different zymograms, in each case run next to tissue extracts, confirming the identity of specific bands of gelatinase activity in the extracts. The amount of protein (pg) loaded for each standard is above the lane. Corresponding amounts of activity for the commercial standards are seen below each standard lane, in TPH µUnits for active MMP-2 activity and in SPS µUnits for pro-MMP-2 and pro-MMP-9 activities (see Methods for details).

Figure 4. Activity Levels with Respect to Cancer Stage

Box plots of active MMP-2, pro-MMP-2 and pro-MMP-9 activities, respectively, at each cancer stage (Stage I, n = 18, Stage II, n = 117; Stage III, n = 90; Stage IV, n = 44) for normal mucosa and colorectal carcinoma extracts, as determined from gelatin zymogram data on 269 patient-matched tissue pairs (activities are graphed in serial dilution units/mg protein). Median activity levels are represented by a solid horizontal line intersecting the central box of each plot.

Figure 5. A. Univariate descriptive statistics on enzyme levels for active MMP-2, Pro-MMP-2 and Pro-MMP-9

Statistics on untransformed enzyme levels are reported in the 2nd through 4th columns. Mean enzyme levels are consistently higher in the cancerous tissues (column 3) than in normal tissue (column 2), regardless of marker, indicating that all three gelatinase activities are successfully detecting the presence of the cancer. Estimated effect sizes, presented in the 4th column, show that the average effect size for active MMP-2 activity, at 6.21, is more than four times as large as the average effect sizes for the pro-MMP-2 or pro-MMP-9 activities (which are 1.34 and 1.53, respectively). Corresponding effect size indices for the transformed enzyme levels (column 7) also demonstrate that enzyme levels are consistently higher in the cancerous tissues (column 6) than in normal tissue (column 5).

5B. Repeated-Measures Analysis of Variance of Effect Size of the Square-Root Transformed Enzyme Levels, By Marker. These data support the conclusion that average effect sizes differ dramatically across the three markers: active MMP-2, pro-MMP-2 and pro-MMP-9 (SS = sum of squares; df = degrees of freedom; MS = mean squares).

5C. Distributions of Effect Size for each Marker. Parallel schematic plot displaying the sample univariate distributions of effect size for active MMP-2, pro-MMP-2 and pro-MMP-9 activities (after square root transformation of the raw data).

Figure 6.

ROC curves for active MMP-2, pro-MMP-2 and pro-MMP-9. Receiver operating characteristic curves (ROC) were estimated for active MMP-2, pro-MMP-2 and pro-MMP-9 activity by plotting sensitivity against (1-specificity) for each marker, in a comparison of activity levels in 269 normal colorectal mucosa samples versus 269 colorectal cancer samples.

Table 1

Mean normal and cancer activity/mg protein in 3 types of interconvertible units

Tissue	Active MMP-2 Activity			Pro-MMP-2 Activity			Pro-MMP9 Activity		
	SDU	TPH μ U	TLU	SDU	SPS μ U	TLU	SDU	SPS μ U	TLU
Normal	14.2	532.5	19.2×10^3	115.1	0.432	548.6×10^3	225.0	28.5	1934.6×10^3
Cancer	146.1	5,478.8	197.8×10^3	214.0	0.803	1019.8×10^3	715.4	90.6	6149.9×10^3