Overexpression of matrix metalloproteinase 9 in tumor epithelial cells correlates with colorectal cancer metastasis.

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Overexpression of Matrix Metalloproteinase 9 in Tumor Epithelial Cells Correlates with Colorectal Cancer Metastasis

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Short Title: Colon Cancer Cell MMP9 Predicts Metastasis

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ABSTRACT

Colorectal cancer mortality largely reflects metastasis, the spread of the disease at distant organs. Matrix metalloproteinase 9 is a key regulator of metastasis and a target for anticancer strategies in colon cancer. Here, overexpression of matrix metalloproteinase 9 in pure tumor epithelial, but nor stromal, cell populations was associated with metastatic progression of colorectal cancer as defined by RT-PCR and confirmed by immunostaining. Thus, cancer cell matrix metalloproteinase 9 represents a novel, selective prognostic and predictive factor that may be exploited for more effective disease stage stratification and therapeutic regimen selection in patients with colorectal cancer.
INTRODUCTION

Prevention or interruption of metastatic disease progression represents an unfilled therapeutic gap in the management of patients with colorectal cancer, the second leading cause of tumor-related mortality in developed nations.\textsuperscript{1,2} Metastasis results from dissemination of select cell clones, exhibiting aberrant migratory and survival abilities, from the primary tumor to distant organs where they establish extra-intestinal colonies and remodel the local parenchyma.\textsuperscript{3,4} Uniformly, this process drastically aggravates the patient outlook and less than 10\% of patients with colon cancer metastasis at distant organs survive beyond five year from diagnosis.\textsuperscript{1} Effective strategies to reduce mortality and improve patient outcomes include early detection of pre-metastatic disease and timely recognition of metastatic spread.\textsuperscript{2} However, reliable pathological markers of metastatic colorectal cancer are scarce, reflected by the genotypic and phenotypic heterogeneity of colon cancer cells and the variability in analytical methods employed.\textsuperscript{3-5} Moreover, despite the progress of current chemotherapeutic regimens, advanced colorectal cancer with distant metastatic deposits remains incurable.\textsuperscript{6-8} Thus, identification of novel, selective prognostic indicators and therapeutic targets of metastatic disease progression are of paramount importance to reduce morbidity and mortality from colorectal cancer.

Matrix metalloproteinases (MMPs) are zinc-dependent metalloendopeptidases which mediate metastasis.\textsuperscript{9,10} Cancer cells employ MMPs to cleave the extracellular matrix and invade surrounding tissues.\textsuperscript{10,11} Specifically, in colon cancer MMP-9 has emerged as a critical pro-metastatic protease regulating tumor cell growth, mobility and survival.\textsuperscript{8,11,12} A complex interplay between MMP-9 activity, dynamic membrane regions and signaling
by adhesion molecules regulates tumor cell migration, invasion and metastasis.\textsuperscript{13-15} Further, MMP-9 promotes tumor neovascularization by specifically activating angiogenic factors at the cancer cell/matrix interface.\textsuperscript{16} Consistent with the hypothesis that MMP-9 is a key regulator of the malignant phenotype, colorectal tumors from patients exhibit overexpression of MMP-9 compared to matched normal adjacent tissues.\textsuperscript{8,12} In that context, stromal cells within the primary colorectal tumor are principal MMP-9 producers and may promote invasion and metastasis by cancer cells.\textsuperscript{17,18} However, human colorectal cancer cells also exhibit cell autonomous abilities to synthesize and secrete MMP-9, an effect associated with induction of MMP-9-dependent proteolytic functions at the pericellular space which mediate metastasis.\textsuperscript{8} Indeed, tumor epithelial cell MMP-9 promotes extracellular matrix degradation, actin polymerization-driven cell spreading, and hematogenous tumor cell seeding of mouse lungs.\textsuperscript{8} In principle, tumor epithelial cell MMP-9 may represent a specific diagnostic and therapeutic target for colorectal cancer metastasis.\textsuperscript{8} Obstacles to these clinical applications include the unknown prognostic role of cancer cell MMP-9, the contribution of stroma-derived MMP-9 to cancer cell pathobiology, and the absence of approaches that selectively target MMP-9 functions mediating metastasis. Here, a previously unrecognized potential for colon tumor epithelial cell MMP-9 as a specific and selective biomarker of metastatic disease progression is revealed. Specifically, relative increase of MMP-9 expression in tumor intestinal epithelial, but not stromal, cells compared to matched normal mucosa cells was demonstrated to predict locoregional metastatic propagation and correlate with advanced disease stages. Together, these observations
suggest MMP-9 in tumor epithelial cells is a novel prognostic and predictive marker and a potential therapeutic target for patients with colorectal cancer.
METHODS

Clinical specimens

Fresh surgical specimens from 11 patients (Table I) with histologically-confirmed adenocarcinomas of the colo-rectum were obtained from the Department of Pathology, Anatomy and Cell Biology of Thomas Jefferson University (Philadelphia, PA) under a protocol approved by the Institutional Review Board.

Isolation of pure intestinal cell populations

Colorectal tumors and normal adjacent tissues were subjected to proteolytic digestion by incubation (37°C for 1 h) in L-15 media containing 10% FBS, 0.1 μg/ml insulin, 50 ng/ml EGF, 2 mM L-glutamine, 100 μ/ml penicillin and streptomycin, 125 μg/ml fungizone and 1,250 units/ml collagenase XI. Following digestion, dissociated intestinal cells were passed through a cell strainer, magnetically labeled with magnetic microbeads conjugated to a mouse antibody directed against the human epithelial antigen (HEA), and transferred into a magnetic activated cell sorting (MACS) column (Miltenyi Biotec, Germany). After washing to recover the unlabeled fraction, which includes stromal cells, magnetically-retained HEA⁺ cells were removed from the magnetic field and collected with MACS elution buffer. To further purify intestinal epithelial cells from residual cell contamination, MACS-sorted HEA⁺ cells were fluorescently labeled by incubation with PE-conjugated mouse anti-human CD104 (BD Biosciences, San Jose, CA), another specific intestinal epithelial cell marker, and subjected to fluorescent activated cell sorting (FACS) employing a FACS Caliber flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The MACS flow-through (unlabeled fraction) was also subjected to FACS
employing FITC-conjugated anti-CD45 (Miltenyi Biotec, Germany), a stromal cell marker, to obtain purified intestinal stromal cells.

**Real-time reverse transcription-PCR**

Purified cell populations from patients were pelleted and resuspended in RNAlater (Ambion, Inc., Austin, TX). Total RNA was isolated with the Qiagen RNA Easy kit (Qiagen, Valencia, CA) and subjected to one-step reverse transcription (RT)-PCR on a 7000 Sequence Detection System for 45 cycles (95°C, 5 minutes; 94°C, 20 minutes; 62°C, 1 minute) using Taqman® EZ RT-PCR Core Reagents (Applied Biosystems, Inc., Foster City, California). MMP-9 and β-actin mRNAs were specifically detected and quantified with respective fluorescently labeled primers/probe sets (Assay on Demand, Applied Biosystems). Data were analyzed using Sequence Detection Software (Applied Biosystems) with thresholds set at 0.2. Template-negative controls were run on each PCR plate.

**Immunostaining**

Paraffin-embedded intestinal sections (5 μm) from clinical specimens were deparaffinized and rehydrated with sequential washes of xylene, ethanol and water. Antigens were unmasked by heating twice at 100°C for 5 min in 10 mM citric buffer, pH 6.0. For immunohistochemistry (IHC), tissue sections were incubated overnight at 4°C with a goat polyclonal antibody against human MMP-9 (R&D Systems, Minneapolis, MN; 20 μg/ml), followed by incubation with the secondary antibody and DAB substrate (Avidin-biotin kit; Vector Laboratory, Burlingame, CA). For immunofluorescence, tissue slides were first incubated overnight at room temperature with the goat polyclonal antibody against human MMP-9 (R&D Systems; 20 μg/ml) or the rabbit polyclonal
antibody against human β-catenin (Santa Cruz, Santa Cruz, CA; 1:50). Then, the specific primary antibody binding was visualized employing AlexaFluor 555 conjugated donkey anti-goat or AlexaFluor 488 conjugated donkey anti-rabbit secondary antibodies (Molecular Probes, Eugene, OR). Sections were counterstained with Slowfade Gold antifade reagent with DAPI (Molecular Probes) to identify individual cells and nuclei and examined with a Zeiss LSM 510 Meta Confocal microscope. Secondary antibody alone was used as negative control.

**Statistical analysis**

Unless otherwise indicated, data were expressed as the mean ± SEM. The association between patient sub-groups with relative stromal or epithelial MMP-9 overexpression and the degree of lymph node involvement was evaluated employing the exact version of Cochran-Armitage Trend test. Differences in stage grouping scores and percent of positive nodes between patients with increased and decreased MMP-9 mRNA levels in tumor cell populations were determined by Wilcoxon two-sample test. Data were analyzed employing StatExact 7 (Sytel Software Corporation, Cambridge, MA).
RESULTS

Increased MMP-9 expression in colorectal tumor cells is associated with locoregional metastatic dissemination

Normal adjacent intestinal mucosa and colorectal tumors from 11 patients were subjected to collagenase digestion. Crude populations enriched in epithelial or stromal cells were first obtained by isolating cells positive and negative for HEA from the bulk of dissociated human intestinal cells employing MACS sorting. Purified intestinal epithelial (Figure 1, top panels) and stromal (Figure 1, bottom panels) cells were collected employing FACS sorting with PE-conjugated anti-CD104 (on MACS-isolated HEA+ cells) and FITC-conjugated anti-CD45 (on unlabeled cells of the MACS flow-through), respectively. Then, isolated epithelial and stromal cells were subjected to real-time RT-PCR for quantification of MMP-9 mRNA. MMP-9 was expressed in all epithelial and stromal cells from patients examined (Figure 2A). Further, within epithelial or stromal cell populations, MMP-9 expression was increased or decreased in cells purified from tumors compared to matched normal adjacent tissues (Figure 2A). Importantly, local metastasis to regional lymph nodes occurred only in patients exhibiting increased MMP-9 expression in tumor cells (Figure 2B). In contrast, all cancer patients with decreased MMP-9 levels in both epithelial and stromal cells from tumors were node-negative (Figure 2B).

MMP-9 in tumor epithelial cells is a prognostic indicator of metastatic colorectal cancer

Of significance, 100% of patients (n=5) with relative MMP-9 overexpression in tumor epithelial cells, compared to normal epithelial cells, exhibited lymph node involvement.
Among the different pathologic sub-groups defined by the relative MMP-9 expression in tumors, colorectal cancer patients with tumor epithelial cell MMP-9 overexpression exhibited the highest metastatic tumor burden in regional lymph nodes (Figure 2B). Conversely, only 83% of patients (n=6) with relative MMP-9 overexpression in tumor stromal cells, compared to stromal cells from the adjacent normal mucosa, had node-positive disease, reflected by low-to-intermediate metastatic involvement (Figure 2B). Accordingly, the proportion of patients with relative overexpression of MMP-9 in tumor epithelial, but not stromal, cells increased significantly in higher node-positive stages (N0, 0%; N1, 50%; N2, 100%), as defined employing the tumor node metastasis (TNM) system by the American Joint Committee on Cancer (AJCC) (Figure 3, left panels). Attribution of an arbitrary score to the TNM-based stage grouping of patients examined (Table II) revealed that patients with increased MMP-9 levels in tumor epithelial cells exhibited the most advanced stage of disease, which was significantly higher than that of patients (n=6) with decreased MMP-9 expression in cancer cell populations (stage score: 6.60 ± 0.27 in patients with increased cancer cell MMP-9 vs 3.83 ± 0.77 in patients with decreased cancer cell MMP-9, p=0.022; Figure 3, bottom-middle panel). No significant difference in stage grouping scores was detected between patients with relative MMP-9 overexpression in tumor stromal cells and those with relative MMP-9 downregulation in the same cell population (Figure 3, upper-middle panel). Further, patients with a relative increase in expression of MMP-9 mRNA in epithelial cells in tumors, compared to matched normal tissues, exhibited a significantly higher proportion of regional lymph nodes harboring metastases than patients with a relative decrease in MMP-9 expression (percent of lymph node-positive: 27.20 ± 9.60 in patients with increased cancer cell
MMP-9 vs 4.85 ± 4.00 in patients with decreased cancer cell MMP-9, p=0.017; *Figure 3, bottom-right panel*. In contrast, disease burden in patients did not correlate with relative changes in MMP-9 expression in tumor stromal cells compared to normal adjacent tissues (*Figure 3, upper-right panel*).

**Overexpression of MMP-9 in colon tumor cell compartments is detected by conventional histopathology**

Paraffin-embedded tissue slides from patients with colorectal cancer were immunostained for MMP-9 (*Figure 4*). IHC analysis revealed that MMP-9 protein is detected in both normal adjacent tissues and primary tumors from colon cancer patients (*Figure 4, left panels*). Moreover, confocal microscopy of tissue specimens from a patient with node-positive disease demonstrated overexpression of MMP-9 protein in primary tumor cell compartments, compared to cells from the matched normal adjacent mucosa (*Figure 4, right panels*). Importantly, MMP-9 in tumor epithelial cells was specifically identified by co-localization of immunofluorescent signals from MMP-9 and β-catenin (*Figure 4, right panels*). These analyses revealed that increased MMP-9 protein in colon epithelial cell populations of the primary tumor can be detected by routine histopathological techniques (*Figure 4, right panels*).
DISCUSSION

The mortality rate for large bowel cancer, ~50%, largely reflects metastatic disease progression, and tumor deposits at distant locations signify unresectable and untreated disease.\textsuperscript{1,19} Although the final stages are irreversible, formation of metastatic foci of colon cancer cells at extra-intestinal sites is a vulnerable, multi-step process. Thus, for hematogenous metastatic dissemination cancer cells have to detach from surrounding stroma, migrate through tissue boundaries and invade the primary organ, intravasate into blood vessels, embolize and distribute to distant tissues.\textsuperscript{4} Following tumor seeding, only select cancer cells with unique abilities to engage productive cell-matrix interactions with the host parenchyma proliferate and form the metastatic niche.\textsuperscript{3,20} Further, to establish enduring metastasis colon cancer cells have to evade local immune anti-tumor defenses, maliciously remodel the host micro-environment, and develop an autonomous vascular network.\textsuperscript{4,21} As a result, metastasis is a highly selective and inefficient process and >99.99\% of intravasated tumor cells are eliminated before reaching their final destination.\textsuperscript{22} The complexity of the metastatic process, and its attendant rate-limitation associated with disruption of any individual step, is at the basis of the efficacy of postsurgical adjuvant chemotherapy to prolong survival and reduce mortality in patients with early metastatic colorectal cancer, as determined by lymph node involvement in the absence of distant metastases.\textsuperscript{23}

Current pathologic assessment of regional colorectal cancer metastasis is performed by examining mesenteric nodes in surgical colectomy specimens with traditional hematoxylin-eosin staining.\textsuperscript{24} However, accuracy in estimating node tumor burden is hampered by the great variability in nodal harvest, as a result of interpatient anatomic
differences, extent of surgical lymphadenectomy and the ability to search for lymph nodes by the pathologist. The unclear number of lymph nodes to analyze and the imperfect sensitivity of histopathology represent other obstacles for appropriate metastatic disease evaluation. Although introduction of newer molecular-based methods of diagnosis selective for the metastatic process has been invoked, specific biological markers of colorectal cancer metastasis have not emerged. Here, node-positive disease always was associated with increased MMP-9 expression in tumor cell compartments compared to matched normal mucosa cells, indicating that relative increase of MMP-9 expression in colorectal tumor cells is a specific biomarker of metastatic dissemination. Importantly, MMP-9 overexpression in cells of the primary tumors could be detected by both RT-PCR and standard IHC techniques. The implications of these findings on patient management are important, because they suggest that pathological analysis of the primary tumor, regardless of lymph node status, may be sufficient for detecting early colorectal cancer metastasis.

Pathologic staging of patients remains the most important prognostic factor for colorectal cancer. Indeed, the survival rate of these patients is inversely correlated with the AJCC/TNM stage (Table II), and while more than 90% of patients with stage I survive 5 years from the initial diagnosis, less than 60% or 10% of them are still alive when metastasis has spread to regional (stage III) or distant (stage IV) locations, respectively. There is a considerable risk to understage patients with current histological methods of pathologic node evaluation. Specifically, a clear prognostic dis-homogeneity exists for the node-positive stage III group, and various permutations of the N category have been proposed to better stratify colorectal cancer patients with lymph
node involvement.\textsuperscript{24} Here, relative MMP-9 overexpression in tumor epithelial cells, compared to normal epithelial cells, predicted lymph node involvement and correlated with increased metastatic tumor burden. Importantly, the patient population exhibiting relative increase of tumor epithelial cell MMP-9 was significantly enriched in higher node-positive stages, suggesting that cancer cell MMP-9 overexpression is a novel prognostic factor in colon cancer. Moreover, colorectal cancer group staging is the fundamental predictive factor for patient management. Thus, pre-metastatic disease stages often require surgery alone with curative outcomes, while metastatic stages warrant postsurgical adjuvant chemotherapy with the intent to improve expectancy and quality of life.\textsuperscript{6,7} Careful stratification of node-positive stage III groups, in turn, may represent the key discriminator for directing patients to less (for stage IIIA), intermediate (for stage IIIB) or more (for stage IIIC) aggressive therapeutic regimens.\textsuperscript{24} Patients with relative increase of MMP-9 levels in tumor epithelial cells signified advanced disease stages, characterized by significantly higher proportion of regional lymph nodes harboring metastasis compared to patients with a relative decrease in MMP-9 expression. Thus, MMP-9 overexpression in primary tumor epithelial cells may represent a highly specific molecular marker for both the prognostic and predictive stratification of patients with colorectal cancer.

In colorectal tumors, fibroblasts and inflammatory cells of the stromal compartment are a principal source of MMP-9, which may confer metastatic abilities to neighboring cancer cells.\textsuperscript{17,18} However, examination of pure cell populations from tumors and adjacent normal mucosa of patients with colorectal cancer demonstrated that MMP-9 of epithelial, but not stromal, cells functions as a pathological marker of metastatic disease.
progression. Accordingly, cancer cell MMP-9 is a critical regulator of the pathobiology underlying colorectal metastasis, selectively promoting migration, invasion and seeding of target organs by tumor cells.\textsuperscript{8} Thus beyond stromal cell contribution, MMP-9 produced by tumor epithelial cells may control unique cellular functions mediating metastasis. Precise elucidations of these mechanisms underlying colorectal cancer invasion and metastasis may provide novel, highly selective and effective therapeutic strategies for targeted prevention and treatment of colorectal cancer metastasis.
CONCLUSION

Advanced, metastatic colorectal cancer remains incurable. Accurate patient stratification into defined prognostic and predictive disease stages is essential to improve quality of life, prolong survival and reduce mortality in colorectal cancer.\textsuperscript{5,7,25} MMP-9 overexpression in tumor epithelial cells is a novel, specific and selective marker for the prognosis and management of patients with colorectal cancer. Further, cancer cell MMP-9 represents a novel predictive factor which may indicate treatment response and targeted therapy selection to prevent and treat metastatic disease progression. If future studies will demonstrate that MMP-9 overexpression in cancer cells is an independent prognostic and predictive factor, translation of these previously undescribed MMP-9 functions into clinical practice have the potential to profoundly impact the management and cure of patients with colorectal cancer.
REFERENCES


**Table I. Clinicopathologic Parameters of Colorectal Cancer Patients**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y) Median (range)</td>
<td>61 (54-85)</td>
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<tr>
<td>Gender (%)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>7 (63.6)</td>
</tr>
<tr>
<td>Female</td>
<td>4 (36.3)</td>
</tr>
<tr>
<td>Tumor Site (%)</td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>10 (90.9)</td>
</tr>
<tr>
<td>Rectum</td>
<td>1 (9.1)</td>
</tr>
<tr>
<td>Dukes' Tumor Stage (%)</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1 (9.1)</td>
</tr>
<tr>
<td>B</td>
<td>3 (27.2)</td>
</tr>
<tr>
<td>C</td>
<td>5 (45.5)</td>
</tr>
<tr>
<td>D</td>
<td>2 (18.2)</td>
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<tr>
<td>Differentiation Grade (%)</td>
<td></td>
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<tr>
<td>Well</td>
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</tr>
<tr>
<td>Moderate</td>
<td>7 (63.6)</td>
</tr>
<tr>
<td>Poor</td>
<td>3 (27.2)</td>
</tr>
<tr>
<td>Tumor Depth* (%)</td>
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<tr>
<td>Tis</td>
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</tr>
<tr>
<td>T1</td>
<td>0 (0)</td>
</tr>
<tr>
<td>T2</td>
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<tr>
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<td>9 (81.8)</td>
</tr>
<tr>
<td>T4</td>
<td>1 (9.1)</td>
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<td>Lymph Node Metastasis (%)</td>
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<td>Yes</td>
<td>7 (63.6)</td>
</tr>
<tr>
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<tr>
<td>Distant Metastasis (%)</td>
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<td>2 (18.2)</td>
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<tr>
<td>Not determined</td>
<td>9 (81.8)</td>
</tr>
</tbody>
</table>

*Tis, limited to mucosa (carcinoma in situ); T1, limited to submucosa; T2, invading the muscularis propria; T3, invading the serosa; T4, invading adjacent organs.
<table>
<thead>
<tr>
<th>Stage Group</th>
<th>TNM*</th>
<th>Lymph Nodes Examined (Range)</th>
<th>Patient Number (%)</th>
<th>Score</th>
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<tbody>
<tr>
<td>0</td>
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<td>0 (0)</td>
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<tr>
<td>I</td>
<td>T1-2, N0, M0</td>
<td>27 (9.1)</td>
<td>2</td>
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<td>IIA</td>
<td>T3, N0, M0</td>
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<td>13-33 (27.3)</td>
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<tr>
<td>IV*</td>
<td>T1-4, N0-2, M1</td>
<td>0 (0)</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

*T categories are as described in Table 1; N0, no lymph nodes involvement; N1, metastasis in 1-3 lymph nodes; N2, metastasis in ≥4 lymph nodes; M0, no distant metastasis; M1, metastasis at distant organs.

*No patients were included in the stage IV group because almost universally the M category was not determined (Table 1).
FIGURE LEGENDS

Figure 1. Isolation of pure epithelial and stromal cell populations from primary tumors and matched normal mucosa of patients with colorectal cancer. Following collagenase digestion and MACS-sorting, epithelial (MACS elution) or stromal (MACS flow-through) cells of normal adjacent tissue (NAT) and colorectal tumors (Tumor) isolated from patients were subjected to FACS-sorting. Pure intestinal epithelial (red box for PE-conjugated anti-CD104) or stromal (green box for FITC-conjugated anti-CD45) cell populations were collected for further analysis.

Figure 2. Relative MMP-9 expression levels in colorectal tumor cell populations exhibit prognostic significance. (A) Stromal and epithelial cells from primary colorectal tumors (Tumor) and normal adjacent tissue (NAT) isolated by MACS- and FACS-sorting were subjected to RT-PCR. Sample levels of MMP-9 mRNA were normalized to respective β-actin mRNA using the formula $2^{[(MMP-9\text{ Ct})-(β\text{-actin\text{ Ct}})]}$, where Ct is the sample threshold cycle number. Lines connect data from matched specimens from the same patient. Box plots, median and values from the 25th-75th percentile; whiskers, range of values. (B) Lymph nodes involvement in the different sub-groups of patients as defined by the relative expression of tumor cell MMP-9 compared to the matched normal mucosa cell MMP-9. ↑MMP-9, MMP-9 overexpression; ↓MMP-9, MMP-9 downregulation; E, tumor epithelial cells; S, tumor stromal cells.

Figure 3. MMP-9 overexpression in tumor epithelial cells is a biological marker of colorectal cancer metastasis. Association between MMP-9 overexpression in tumor cell populations and lymph node involvement (left panels). Compared to those with tumor MMP-9 downregulation, patients with MMP-9 overexpression in tumor epithelial,
but not stromal, cells exhibit advanced disease stage (middle panels) and increased lymph node tumor burden (right panels). N categories and disease stage scores are specified in Table II and are based on the AJCC/TNM system.\textsuperscript{19} \(\uparrow\)MMP-9, MMP-9 overexpression; \(\downarrow\)MMP-9, MMP-9 downregulation. Values in middle and right panels are means ± SEM.*\textsuperscript{*}, p<0.05 by the Wilcoxon two-sample test.

**Figure 4. Overexpression of tumor epithelial cell MMP-9 in metastatic colorectal cancer is detected by immunostaining.** IHC (left panels) and immunofluorescence (right panels) analyses of primary tumors (Tumor) and matched normal adjacent tissues (NAT) from colorectal cancer patients with metastatic disease progression. For IHC (magnification, 40X), tissues were stained with specific goat polyclonal anti-MMP-9 (brown) and hematoxylin (blue, nuclei). For immunofluorescence, tissues were stained with DAPI (blue, nuclei) and specific antibodies against MMP-9 (red) and the epithelial-specific marker \(\beta\)-catenin (green), and subjected to confocal microscopy (magnification, 100X).
Zuzga et al., Figure 1

NAT

Tumor

Acquisition Dot Plot

MACS Elution

MACS Flow-through

CD104-PE

CD45-FITC
Zuzga et al., Figure 3

**Stromal Cells**

**Epithelial Cells**

**Lymph Node Involvement (%)**

- **MMP-9 upregulated**
- **MMP-9 downregulated**

**Disease Stage Score**

**Patient Number (%)**

- N0
- N1
- N2

**Lymph Node Involvement (%)**

- N0
- N1
- N2

*p = 0.74

*p = 0.02
Zuzga et al., Figure 4

NAT

MMP-9

Tumor

IHC

IHC

Immunofluorescence

DAPI

MMP-9

β-Catenin

Merge