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Predictive Biomarkers and Personalized Medicine

Clinical Cancer Research

Absence of MMP2 Expression Correlates with Poor Clinical Outcomes in Rectal Cancer, and Is Distinct from MMP1-Related Outcomes in Colon Cancer

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Abstract

Purpose: Treatments for colorectal cancer (CRC) are primarily disease stage based. However, heterogeneity in outcome within even a single stage highlights its limitations in predicting disease behavior. Recently, the role of gene expression as predictive and prognostic markers has been explored. Our objectives were to identify consistently differentially expressed genes through meta-analysis of high-throughput gene-expression studies, and evaluate their predictive and prognostic significance in colon (CC) and rectal (RC) cancers.

Experimental Design: Publications applying high-throughput gene- expression technologies to specific CRC stages were identified. A vote counting strategy was used to identify the most significant differentially expressed genes. Their predictive and prognostic values were independently assessed in a tissue microarray of 191 cases of stage II-IV CC/RC from two tertiary care centers. Their biological effects were also examined *in vitro*.

Results: MMP1 and MMP2 were identified as consistently underexpressed in liver metastasis compared with primary CRC. Shorter time to distant metastasis and overall survival occurred in stage III CC lacking MMP1 expression, and in stage III RC lacking MMP2. MMP1 levels in stage II and III CC were associated with increased likelihood of distant metastasis, whereas the risk of local recurrence in stage III RC could be stratified by MMP2. Promotion of cell invasion of CRC cell lines exposed to MMP1/2 inhibitors were confirmed *in vitro*.

Conclusions: MMP1 and MMP2 may be useful biomarkers that can help stratify patients at higher risk of developing recurrence in colorectal cancer, and guide individualized treatment decisions to achieve better outcomes. *Clin Cancer Res; 17(12); 4167–76. ©2011 AACR.*

Introduction

Recurrence of colon and rectal cancers, either locally-regionally at the site of original disease or distantly as metastases, after "curative" surgical resection represents a significant cause of morbidity and mortality. Risk reduction interventions have centered on the use of neoadjuvant and/or adjuvant chemotherapy and/or radiotherapy. In stage III colon cancers, whereas resection alone leaves a 50% to 60% risk of disease recurrence, the addition of adjuvant chemotherapy could improve the five year overall survival by greater than 10% (1). As a result, surgical tumor resection followed by adjuvant 5-fluorouracil (5-FU)–based chemotherapy represents the standard of care for stage III colon cancers (2). In stage II disease, however, the role of adjuvant chemotherapy is more controversial due to its minimal efficacy (maximal 5% improvement in five year overall survival) in exchange for potentially serious lifelong toxicities, and is therefore not recommended according to the most recent practice guidelines (3). However, it is recognized that 25% to 30% of stage II disease will experience recurrence after resection (3). This heterogeneity in disease behavior, even within a single stage, highlights the limitations of staging as the sole determinant of treatment strategies. This has led to searches for better predictive markers that could complement staging, ensuring those at the highest risk for recurrence are identified and receiving appropriate and timely treatment. Whereas stage II disease would seem to benefit most from such markers, their application to stage III disease may conversely identify those at lower risk of recurrence. Although the majority of stage III disease receive adjuvant chemotherapy, it is

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Translational Relevance

Currently, colorectal cancer treatment regimens are primarily dictated by tumor node metastasis (TNM) stage. However, variability in disease behavior even within a single disease stage reflects its limited specificity to predicting outcomes on a more individualized basis. The consequences are that some patients may be unnecessarily offered adjuvant chemotherapy, whereas others are not receiving it because of being in a perceived low-risk stage. There is a need to identify more specific predictive and prognostic markers. Our study first identified MMP1 and MMP2 as consistently downregulated proteins in liver metastases compared with primary colorectal cancer by meta-analysis of high-throughput gene-expression studies. Using tissue microarrays consisting of 191 cases of colon and rectal cancers, absence of MMP1 expression in stage III colon cancer was predictive of shorter time to distant metastasis and overall survival, whereas absence of MMP2 expression was associated with poorer outcomes in rectal cancers (higher local recurrence, distant metastasis, and death). Clinically, the expression of both MMP1 and MMP2 can be used to risk stratify stage III colon and rectal cancers, identifying those at highest risk who should be receiving more aggressive chemotherapy.

plausible that some individuals may remain disease-free even without this treatment. Consider the wide range in five year overall survival within stage III disease, from 83% with limited nodal disease to 44% when extensive nodal involvement is present (4). By identifying those at the lowest risk, some stage III individuals may delay starting or even forego chemotherapy and its considerable side effects.

Compared with colon cancers, disease relapse in rectal cancers is more likely from local recurrence. For locally advanced disease (T3/T4 lesions or those with regional lymph node involvement), numerous risk reduction strategies, including surgical resection, and both neoadjuvant and adjuvant chemoradiation, have been shown to be efficacious (5–9). The decision to implement these strategies however require accurate staging, which in Europe is more commonly provided by conventional MRI compared with in the United States, where endoscopic ultrasound (EUS) is more widely used (10). Although recent meta-analyses on the sensitivity and specificity of EUS for staging depth of invasion were reported to be between 80% and 96%, and 90% and 98%, and for assessing nodal metastases were 73% and 76%, respectively, inexperienced operators have been shown to provide less accurate staging (11–13). Overstaging, more common than understaging, has the consequence of subjecting patients to overtreatment. Once again, identification of markers of disease recurrence may complement existing staging techniques to individualize treatment regimen for patients.

In the last several years, the role of gene expression as possible predictive and prognostic markers in multiple types of malignancies including colorectal cancers (CRC) has been explored. Some studies have identified differences in gene expression between normal-colon mucosa, adenomas and CRCs, suggesting their potential role as diagnostic markers (14). Others have correlated gene expression with response to chemotherapy or overall survival, identifying them as putative predictive or prognostic biomarkers, respectively (14–15). Unfortunately, the biological significance and clinical application of such findings are often unclear due to the small sample sizes of the studies, and the lack of independent replicated studies. As a way of highlighting genes of greater clinical significance in the setting of CRC, Chan and colleagues recently completed a meta-analysis, reviewing 25 independently published cancer profiling studies comparing gene expression between CRC and normal colon, resulting in the successful identification of numerous genes that were consistently up or downregulated in CRC across multiple published studies (16).

The objectives of our study were to complete a similar meta-analysis, but of gene expression studies comparing expression profiles of liver metastases versus the primary CRC. These consistently identified differentially expressed genes would be considered as high-yield markers of metastatic disease. Because metastatic disease is associated with worse overall survival, the validity of these putative genes as predictive markers of disease recurrence and prognostic markers for survival in a much earlier stage of disease were evaluated by immunohistochemistry using a tissue microarray of stage II, III, and IV colon and rectal cancers. Their effects on CRC cell lines were also assessed *in vitro* by invasion assay.

Materials and Methods

Identifying high-throughput gene expression studies

We utilized PubMed to identify publications that applied transcript-based high-throughput gene expression technologies to the following comparisons: liver metastases versus corresponding primary CRCs (type one); primary CRCs that underwent liver metastasis versus those that did not (type two); primary CRCs that underwent lymph node metastasis versus those that did not (type three). We limited our search to studies published between 2001 and 2007, and utilized the key words "colorectal," "metastatic," "liver," and "lymph node." When possible, the given gene identifiers were mapped to the NCBI Entrez Gene ID using the Clone/Gene ID Converter tool (17).

Ranking differentially expressed genes and assessing significance

The meta-analysis method was previously developed by our group (16, 18). Briefly, the method involved a vote-counting strategy whereby differentially expressed genes reported in independently published studies were ranked according to 3 criteria in the following order of importance:

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(i) the number of studies reporting the differential expression of a gene (i.e., a gene's overlap), (ii) the number of tissue samples utilized in these studies, and (iii) the average fold change. To assess the statistical significance of the number of genes reported in at least two or more studies (i.e., multistudy genes), we developed Perl scripts that carried out Monte–Carlo simulations. This assessment for statistical significance was carried out to determine if the observed number of multistudy genes could be observed by chance alone. One can imagine randomly choosing genes from each expression-profiling platform of each study, randomly labeling them as up or downregulated, and observing some level of overlap due to chance alone. For each study in each comparison type, the same number of mapped up and downregulated genes was randomly chosen from the corresponding list of genes that were represented on each of the expression profiling platforms. Note that these genes represent those that could potentially be reported as differentially expressed by each study. Next, the number of genes with an overlap of two, three, four, etc., would be calculated. Ten thousand such permutations were executed. At the conclusion of the permutations, a distribution of overlap results from the simulations was determined and a *P*-value was estimated by comparing the overlap from the simulations to the level of overlap in the actual data. Statistical significance was defined at $P < 0.05$.

Note that for 10 microarray studies, we were unable to obtain the genes represented on the corresponding expression profiling platform (Supplementary References 2, 4, 5, 8–11, 14, 16, 18). Thus, an approximation approach was used in which the appropriate number of genes was randomly chosen from the combined gene lists from the other platforms. For the lone serial analysis of gene-expression (SAGE) study, the genes listed in the tag to gene-mapping data from SAGE Genie were used as genes that SAGE can potentially identify as differentially expressed (19).

Characteristics of patients represented in tissue microarray

191 patients with colon and rectal cancers distributed across stages II, III, and IV, diagnosed at Ajou University School of Medicine (Korea) from 1994 to 2002 and the British Columbia Cancer Agency (BCCA) from 2000 to 2008, consented to study participation and inclusion of their tissue in the tissue microarray (TMA). Ninty-seven patients had colon cancer with 23 at stage II, 37 at stage III, and 37 at stage IV. The stage distribution of the 94 rectal cancers were 29, 51, and 14 for stage II, III, and IV, respectively. One hundred were men and 84 were women, with the remaining 7 unknown due to lack of registry information. The median age at diagnosis was 60 years (range: 30 to 91) (Table 1). In the Korean cohort, stage II colorectal cancers underwent surgical resection followed by 12 months of adjuvant oral 5-FU based chemotherapy (doxifluridine, 900 mg/day). Stage III colorectal cancers postresection received the modified Mayo regimen (six cycles of continuous infusion of 5-FU 1000 mg/m^2) plus bolus injection of 30 mg of leucovorin for 5 consecutive days. For the BCCA cohort, first-line therapy for colon cancers was irinotecan alone or in combination with 5-FU for 50% of patients, oxaliplatin and 5-FU based therapy in 37%, and capecitabine alone among 7 (8%). The remaining 5% received second-line treatment. Among the 24 patients with rectal cancer, 4 received no radiation to the primary tumor, 16 received preoperative radiation, and 4 postoperative radiation. Local recurrence, distant metastasis, and death occurring during a 60-month follow up period were documented for each patient. Ethics approval was obtained from the institutional review boards.

Tissue microarray construction and immunohistochemistry

Two-formalin-fixed, paraffin-embedded cores were taken from representative areas of primary tumors from each patient and mounted onto the TMA block. 4 μm–thick sections were made from the TMA block and subsequently deparaffinized in xylene and rehydrated. Sections were heated in citrate buffer for 15 minutes in a cooker for antigen retrieval. Endogenous peroxidase activity was blocked using 0.3% H_2O_2 and washed with PBS for 10 minutes. Immunohistochemical staining with all primary antibodies was carried out using Ultravision LP detection kit (Thermo Fisher Scientific). Sections were treated with Ultra V Block for 5 minutes to prevent nonspecific reaction with primary antibodies, then incubated at 4°C for 24 hours with primary antibodies, followed by incubation with a primary antibody enhancer for 10 minutes at room temperature. Subsequently, sections were treated with horseradish peroxidase (HRP) polymer for 15 minutes and the reaction product was developed using 3,3-diaminobenzidine tetrahydrochloride (Zymed). The sections were counterstained with hematoxylin and mounted with Tissue–Tek Glas 6419 (Sakura Finetek). Negative controls consisted of omission of the primary antibodies. Primary antibodies used in this study were directed against MMP1 (Thermo Fisher Scientific, rabbit polyclonal, diluted 1:30), and MMP2 (Epitomics, rabbit polyclonal, diluted 1:50). Staining expression scores were based on the number of tumor cells with positive staining in the cytoplasm, and were categorized as follows: 0 or none (expression < 10%), 1+ or weak (10% to 50%), 2+ or strong (50% to 80%), and 3+ or intense (>80%), by two independent pathologists at each center (DFS, XS, KWS, YK) who were blinded to

Table 1. Characteristics of patients represented in the tissue microarray

	Colon cancer ($n = 97$)	Rectal cancer ($n = 94$)
Male:Female:Unknown	49:46:2	51:38:5
Median age at diagnosis (Range)	60 (30–85)	61 (35–91)

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clinicopathologic data. The two expression scores per sample were averaged, with the average representing the patient's final expression intensity staining.

Statistics

The χ^2 test was used to examine for associations between expression of putative genes with patient and tumor characteristics including patient's age, gender, and disease stage. Independently analyzing stage II and III colon and rectal cancers, the χ^2 test was used to correlate candidate gene expressions of the primary tumor at baseline to the development of local recurrence and distant metastasis during follow up. Disease-free (DFS) and overall survivals (OS) were separately computed for stage II, III, and IV colon and rectal cancers by Kaplan–Meier method and significance compared with log–rank test. All tests were two-tailed and considered significant at a $P < 0.05$. All calculations were carried out with SPSS version 16.0.

Cell culture

Human CRC cell line RKO was maintained in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% FBS, 1% penicillin/streptomycin and Kanamycin (Invitrogen Corp.) at 37°C and 5% CO_2. Drug resistant cells (RKO/5FU and RKO/CPT) were developed following long-term incubation with incremental concentration of 5-FU and CPT-11 for more than 3 months and maintained in the same medium as above with the addition of 5-FU (50 μmol/L) and CPT-11 (5 μmol/L), respectively. IC_{50} was at least 10-fold higher in the resistant cells than their sensitive parental cells.

Cell invasion

Invasion assay was determined using a Transwell system (6.4 mm diameter, 3-μm pore size with PET membrane; BD Biosciences). Cells were starved overnight in serum-free medium containing 0.1% FBS. Matrigel (40 μl) was coated and polymerized on the upper surface of the transwell membrane at 37°C for 3 hours. Cells (1×10^5) were added to the upper chamber containing 200 μl of serum-free medium (containing 0.1% FBS), in the presence/absence of MMP1 inhibitor (MMP1-I: 5 μmol/L, Cat# 444250, Calbiochem) or MMP2 inhibitor (MMP2-I: 8.5 μmol/L, Cat# 444244, Calbiochem). Six-hundred microliter medium was added to the lower well (+/− MMP1-I or MMP2-I). After 3 days of incubation at 37°C and 5% CO_2, cells remaining in the upper surface of the membrane were completely removed with cotton swabs. The medium in the lower chamber was removed to collect any cells that had invaded through the membrane. In addition, fresh medium (100 μl) was added to the lower well and the attached cells at the bottom of the well were counted under microscope. To collect the cells that had migrated to the undersurface of the membrane, the membranes were washed with PBS three times (all PBS washes were collected), and then trypsinized with 0.25% trypsin. All cells were collected following centrifugation at 500 g for 15 minutes. The cell pellet was resuspended and stained with trypan blue, and counted under the microscope. Data collected from triplicate experiments were analyzed by two-sample test of means using Smith's statistical package.

Results

Identification of consistently differentially expressed genes by meta-analysis

In total, 18 independent metastasis studies were included in the meta-analysis (Supplementary Table SI). These studies reported 1,341 differentially expressed genes (528 up and 813 downregulated), of which 1,232 (91.9%) were mapped successfully to an NCBI Entrez Gene ID (506 up and 726 downregulated). Supplementary Table SII summarizes the results of the analysis for gene overlap for each comparison. For each comparison type, the number of (a) independent studies included, (b) differentially expressed genes identified, and (c) multistudy genes identified (that is, genes reported in at least two independent studies with the same direction of differential expression) are detailed. Of note, five studies were included in comparison type one (liver metastases versus corresponding primary CRCs), which yielded six multistudy genes, which was significantly greater than the average of 1.264 (95% CI: 1.255 to 1.273) genes expected by Monte–Carlo simulations to have an overlap of two by chance alone ($P = 0.001$). The number of multistudy genes observed in all remaining comparisons (types two, three, combined liver studies, and combined all studies) was not statistically significant (Supplementary Tables SIII and SIV). Of the six multistudy genes identified in comparison type one, MMP1 and MMP2 were significantly underexpressed by an average 1.96 and 1.64 fold in liver metastasis, respectively (Table 2). Due to their role as putative markers of metastasis, their predictive and prognostic significance were independently assessed by a tissue microarray consisting of stage II–IV colon and rectal cancers, and by *in vitro* cell invasion studies.

Low MMP1 expression is associated with poor outcomes in Stage III colon cancer

Correlation with disease stage. MMP1 expression was downregulated with advancing disease stage for both colon and rectal cancers. However, the progressive effect was more pronounced in rectal cancers ($P = 0.002$) (Fig. 1A and B). Whereas less than 25% of stage II rectal cancers had no MMP1 expression and more than 70% had strong expression, by stage IV metastatic disease, the proportion with no MMP1 staining increased to almost 60%, with a remaining 20% having strong MMP1 expression. A representative staining pattern for MMP1 expression is shown in Fig. 2.

Correlation with disease recurrence, disease-free, and overall survivals. For both colon and rectal cancers, MMP1 expression in neither stage II nor III disease was associated with local recurrence. However, development of distant metastasis in stage II ($P = 0.008$) and stage III ($P = 0.01$) colon cancers during a five-year follow-up period

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Table 2. Six multistudy genes identified from comparison type one (liver metastasis vs. primary CRC). For each gene, a brief description, average and range of fold change observed in the listed studies are detailed

Gene name (Description)	References of studies (Total sample size)	Average fold change (Range of fold change)
ADFP (Adipose differentiation-related protein)	35–36 (37)	6.59 (2.50 to 10.67)
SLC22A1 (Solute carrier family 22/organic cation transporter 1)	35,37 (53)	3.35 (2.50 to 4.20)
CXCL12 (C-X-C motif ligand 12)	35,38–39 (163)	2.18 (1.25 to 3.70)
PDPN (Podoplanin)	35,38 (87)	− 2.21 (−3.16 to −1.27)
MMP1 (Matrix metallopeptidase 1/interstitial collagenase)	35,38 (87)	−1.96 (−2.65 to −1.26)
MMP2 (Matrix metallopeptidase 2/72kDatypeIV collagenase)	35,38 (87)	−1.64 (−1.69 to −1.58)

correlated to lower MMP1 staining in the primary malignancy (Figs. 1C and D). Of the 23 stage II–colon cancers represented in the TMA, 10 did not experience distant metastasis and within this group, 70% had strong MMP1 expression. In contrast, of the remaining 13 patients that did have distant metastasis, almost 70% had no MMP1 expression. Among the 37 stage III–colon cancers, 16 patients had distant metastasis during follow-up and almost 70% of this group had no MMP1 expression. Only one third of the remaining 21 patients that did not have recurrence had no MMP1 staining. MMP1 did not correlate to distant metastasis in rectal cancer. Interestingly, among stage II, III, and IV colon and rectal cancers, stage III colon cancer without MMP1 experienced both shorter time to distant metastasis ($P = 0.001$) and overall survival ($P = 0.007$) (Figs. 1E and F).

Low MMP2 expression is associated with poor outcomes in rectal cancer

Correlation with disease stage. Similar for MMP1, downregulation of MMP2 occurred with more advanced stages of colon and rectal cancers. Among stage II rectal cancers, 21% did not express any MMP2, whereas 31% showed strong expression. The proportion of stage IV disease without MMP2 expression and strong expression increased to 50%, and decreased to 7%, respectively (Figs. 3A and B).

Correlation with disease recurrence, disease-free, and overall survivals. The only association noted between MMP2 expression and recurrent disease was found in stage III rectal cancers where downregulation of MMP2 was correlated to local recurrence ($P = 0.002$) (Fig. 3C). Of interest, all 7 of the 51 stage III rectal cancers that experienced local recurrence did not have any MMP2 expression. This is compared with the group without local recurrence, of which only 30% had no MMP2 staining. Therefore, a MMP2 of 0 (if considered as the positive test result) had a negative predictive value of 100% and a positive predictive value of 35% for local recurrence in stage III rectal cancer. Finally, absent MMP2 was associated with a shorter time to local recurrence ($P = 0.001$), distant metastasis ($P = 0.02$), and death ($P = 0.008$) in stage III rectal cancers (Figs. 3D, E, and F). At the end of the five years follow-up, 80% of stage III rectal cancers with any expression of MMP2 survived compared with only 40% of no expressors. No correlations to disease-free or overall survivals were noted in stage II or IV rectal disease.

In vitro studies: inhibition of MMP1 and MMP2 promotes cell invasion. The effect of MMP1 and MMP2 inhibition on cell invasion was examined *in vitro*. Following incubation with either MMP1-I or MMP2-I, all cell lines examined (chemotherapy sensitive CRC cell lines RKO and HCT 116, as well as CPT-resistant RKO/CPT, and 5-FU-resistant RKO/5-FU colorectal cells) showed an increased number of cells that had invaded through the matrigel membrane compared with control cells not exposed to either MMP1-I or MMP2-I (Fig. 4). For sensitive cells, cell invasion increased from 27.0 + 7.1 cells to 51+ 5.6 cells ($P = 0.03$) and 58.0 + 1.4 cells ($P = 0.013$) following inhibition with MMP1-I or MMP2-I, respectively in RKO cells; and similarly, from 177.0 + 4.2 cells to 223.0 + 12.7 cells ($P = 0.0166$, MMP1-I) and 284.0+32.5 cells ($P = 0.028$, MMP2-I) in HCT116 cells. The most dramatic increase was observed in resistant RKO/CPT and RKO/5FU cells: incubation with inhibitors resulted in a 1.6- to 4-fold increase in cell invasion in RKO/CPT and RKO/5FU cells, respectively following incubation with MMP1-I, whereas MMP2-I induced a 0.5- to 2.9-fold increases in cell invasion in the same cell lines, respectively.

Discussion

In 2010, there were an estimated 142,570 new diagnoses of and 51,370 deaths in the United States due to colon and rectal cancers, the third most common cause of death among malignancies (20). Unfortunately, disease

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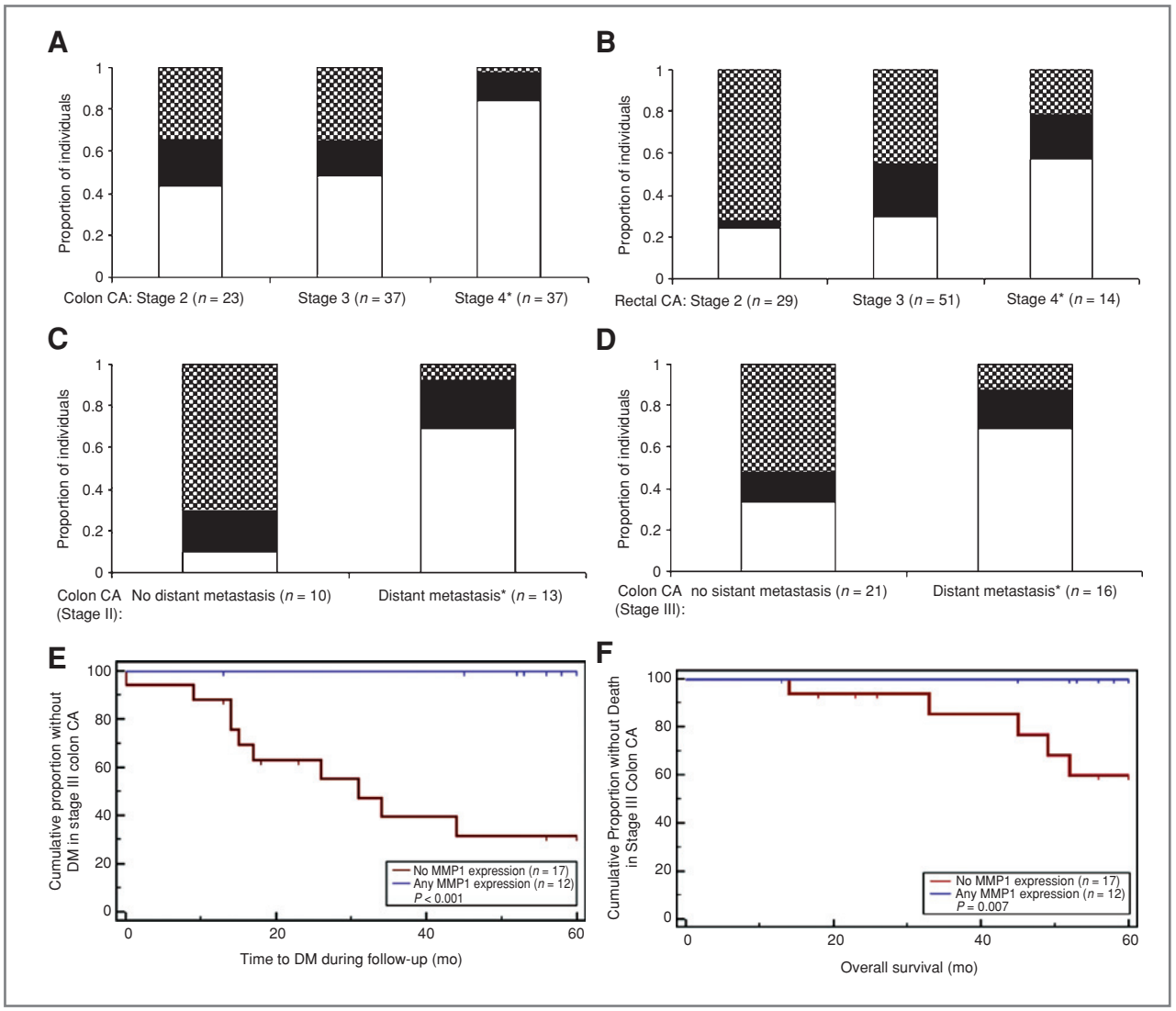

Figure 1. Downregulation of MMP1 expression significantly correlated to (A) more advanced colon cancer stage ($P = 0.001$); (B) more advanced rectal cancer stage ($P = 0.002$); Development of distant metastasis in (C) stage II ($P = 0.008$) and (D) stage III ($P = 0.01$) colon cancer; (E) shorter time to distant metastasis (DM) in stage III colon cancer; (F) shorter overall survival in stage III colon cancer (□ = No MMP1 expression, ■ = Weak MMP1 expression, ▚ = Strong MMP1 expression).

recurrence after "curative" surgical resection contributes to many of these mortalities. Neoadjuvant and/or adjuvant chemotherapy with/without radiation is frequently offered to select patients for risk reduction. For example, in stage II colon cancer, the presence of poor prognostic features such as a clinical presentation of bowel obstruction or perforation, a T4 primary, lymphovascular invasion, poorly differentiated histology, and a small number of lymph nodes sampled are used at some centers to justify adjuvant chemotherapy (21). Identifying biomarkers predictive of local recurrence or distant metastasis and their complementary applications to patients may permit more individually tailored treatments. Further, understanding their molecular roles may help to clarify the pathogenesis underlying tumor growth, local invasion, and distant metastasis.

Through our meta-analysis of high throughput expression studies comparing gene profiles of liver metastases versus primary CRC, six genes were identified as consistently differentially expressed across multiple published studies. Specifically, MMP1 and MMP2 were significantly underexpressed by 1.96- and 1.64-fold in liver metastasis, respectively. This downregulation was further confirmed in a recent reverse transcriptase PCR–based study not included in our meta-analysis (22). Briefly, MMP1 and MMP2 are two of more than 20 members of zinc-dependant matrix metalloproteinases that collectively function as the main extracellular matrix remodelling enzymes. They can be further subgrouped by their substrate specificity, with MMP1 classified as a collagenase and MMP2 as a gelatinase. There is however redundancy, with

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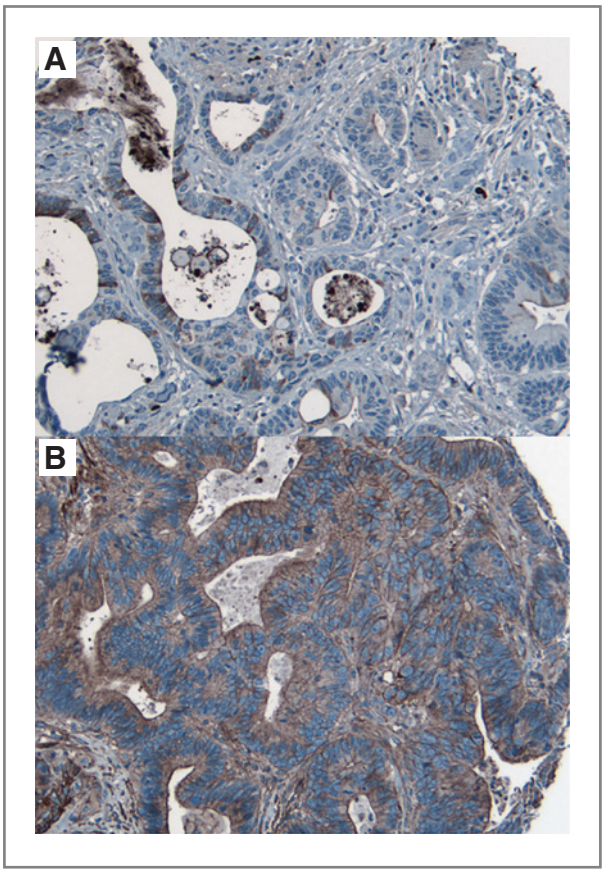

Figure 2. Representative images of (A) MMP1 expression in CRC scored as 1+ expression; and (B) MMP2 expression scored as 2+ expression. 40× magnification.

each metalloproteinase able to breakdown more than one extracellular matrix component (23). Their activity is tightly controlled (i) at the gene transcription level both positively and negatively by various oncogenes, cytokines and growth factors; (ii) via proteolytic activation of pro-MMPs; (iii) by endogenous tissue inhibitor of matrixproteinases (TIMPs) at varying affinity (24).

In this study, we identified stage specific MMP1 differences by noting downregulation of MMP1 with advancing colon and rectal cancer stages. Weaker MMP1 expression was also associated with development of distant metastasis in stage II and III colon cancers, suggesting its role as a possible predictive marker in identifying high-risk patients where adjuvant chemotherapy may be most beneficial. In earlier studies, it was stronger MMP1 expression that correlated to distant metastasis development, but those cohorts were mixtures of colon and rectal cancers of all stages (25–26). Because disease recurrence in colon cancer occurs more commonly via distant metastasis than local recurrence, the absence of any correlations to the local recurrence endpoint was not unexpected. Differences in dividing patient subgroups for analysis, and cohort compositions, may also explain inconsistent results in survival correlations. Whereas we report stage III colon cancer without MMP1 had earlier time to distant metastasis and death, making it a putative prognostic marker, Hilska and colleagues noted no such associations, whereas Murray and colleagues found it was stronger MMP1 which had worse prognosis (27–28). That correlations were noted in colon, but not rectal cancers, could be attributed to different pathogenesis and clinical behaviors of malignancies from the two sites.

We also noted downregulation of MMP2 with advancing stages in both colon and rectal cancers. Given that MMP2's upregulation is often associated with loss of the basement membrane type IV collagen (29), reflecting its role in degrading components of the extracellular matrix to facilitate local invasion and disease progression, and that cleavage of laminin-5 by MMP2 may expose a putative cryptic promigratory site on Ln-5, triggering cell motility (30), it was expected that MMP2 would be progressively overexpressed with advancing disease stage, as shown by Li and colleagues (31). However, in support of our observations, and in keeping with the results of our meta-analysis, Waas and colleagues similarly showed that low stage (I and II) disease showed higher MMP2 expression than more advanced stages (III and IV) (32). Interestingly, mRNA levels of MMP2's inhibitor, TIMP1, have been reported to be significantly upregulated in stage IV compared with stages I and II disease (33). At the time, it was postulated this was in response to high levels of MMP2 in the more advanced stages. Our results raise the possibility that progressively higher levels of TIMP1 may be responsible for, rather than a consequence of, progressively downregulated MMP2. In addition, TIMP1 has been shown to have growth promoting properties, and together with low MMP2 expression, may be potentiating the growth and metastatic potential of colon and rectal cancers, as observed in this study. Evaluation of TIMP1 in future studies would help test this hypothesis.

To understand how low expressions of MMP1 and MMP2 could be associated with shorter time to metastasis in both colon and rectal cancers, *in vitro* studies were conducted. Interestingly, the results showed that inhibition of MMP1 and MMP2 promoted cell invasion and in particular, that induction of cell invasion was most dramatic in chemoresistant cells. Our *in vitro* observations can help explain the clinical outcomes observed in this study. However, that low MMP1 expression may predict distant metastasis and poor survival in cases of colon cancers only, whereas MMP2's prognostic value is limited to rectal cancers, suggest differences in biologic behavior of cancers at these two distinct anatomic sites.

Another interesting observation from our TMA was that low MMP2 expression in stage III rectal cancers correlated with local recurrence, the first such finding to our knowledge. Within our TMA, absence of MMP2 expression in stage III rectal cancer had a negative predictive value of 100% and a positive predictive value of 35% for local recurrence. These findings highlight the predictive and prognostic value of MMP2 expression in rectal cancers and suggest that it could complement existing modalities,

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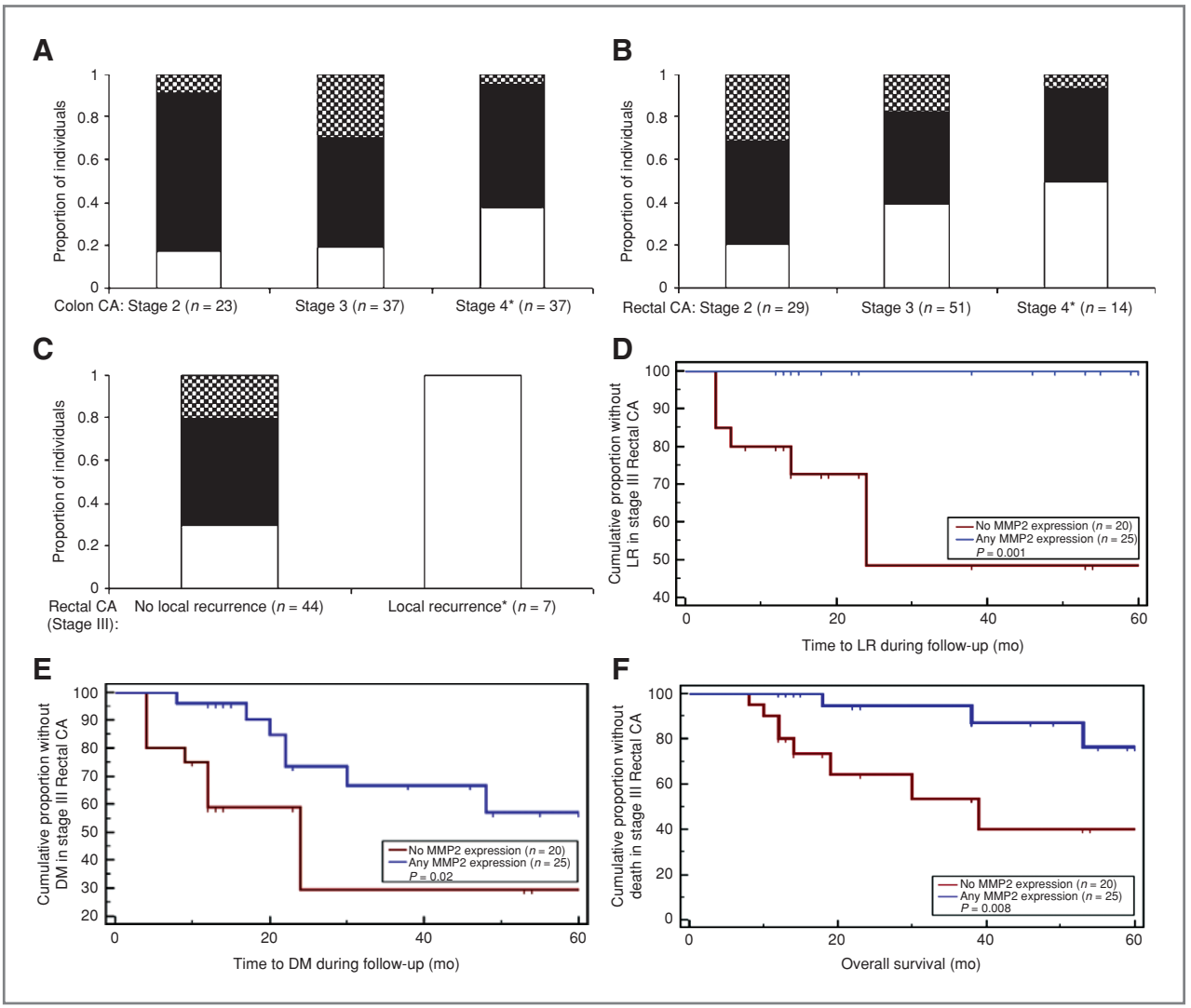

Figure 3. Downregulation of MMP2 expression correlated to (A) more advanced colon cancer stage ($P = 0.01$); (B) more advanced rectal cancer stage ($P = 0.05$); (C) development of local recurrence in stage III rectal cancer ($P = 0.002$); (D) shorter time to local recurrence (LR), (E) distant metastasis (DM) and (F) overall survival in stage III rectal cancer (□ = no MMP2 expression, ■ = weak MMP2 expression, ▚ = strong MMP2 expression).

such as EUS or MRI, in stratifying a stage III rectal cancer's risk of local recurrence, and therefore, influence treatment strategies for this disease. Stage III rectal cancers expressing no MMP2, not only had earlier local recurrence, but also shorter time to distant metastasis, and death, compared with higher MMP2 expressors. This is in contrast to findings that higher MMP2 was associated with shorter cumulative survival in colon cancers, in a recent study involving all stages, but predominantly stage III disease (27).

Tumor MMP2 was not associated with local recurrence or distant metastasis in either stage II colon or rectal cancers, suggesting its limited role as a predictive marker at this earlier disease stage. This was in an agreement with a recent study involving only stage II colon cancers, which interestingly showed it was stromal MMP2 expression that was an independent risk factor for recurrence and associated with shorter disease-free survival (34).

Of note, the current study was primarily limited by the absence of typical clinico-pathologic prognosticators such as differentiation, and lympho/vascular invasion, preventing multivariate analysis.

In summary, we completed a large meta-analysis of CRC high-throughput gene-expression studies, identifying six genes as differentially expressed in liver metastasis compared with primary CRC. Using an independent TMA, the expressions of two such genes, MMP1 and MMP2, were separately analyzed in stages II-IV colon and rectal cancers. We noted decreased MMP1 and MMP2 expressions correlated with advancing colon and rectal cancer stages. Interestingly, for stage III colon cancer, development of distant metastasis, and shorter

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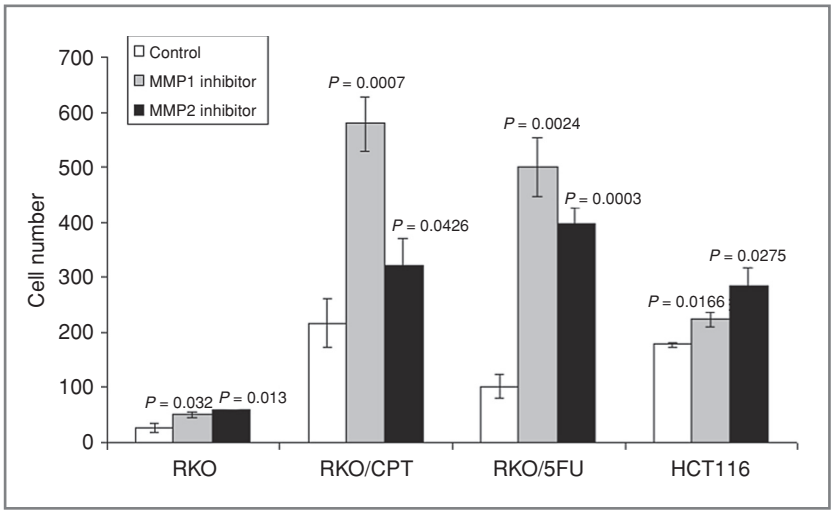

Figure 4. Effect of MMP1 and MMP2 inhibitors on cell invasion of RKO and its drug-resistant cell lines (RKO/5FU, RKO/CPT), and HCT116 CRC cells.

disease free and overall survivals were associated with the absence of MMP1 expression, whereas the lack of MMP2 expression was associated with poorer outcomes in stage III rectal cancers, including the risk of local recurrence. Overall, given our findings of MMP1 and MMP2's associations with earlier disease recurrence and death, they may be useful biomarkers, complementing existing staging techniques, to guide more individualized treatment decisions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Absence of MMP2 Expression Correlates with Poor Clinical Outcomes in Rectal Cancer, and Is Distinct from MMP1-Related Outcomes in Colon Cancer

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