Comprehensive Analysis of Matrix Metalloproteinase and Tissue Inhibitor Expression in Pancreatic Cancer: Increased Expression of Matrix Metalloproteinase-7 Predicts Poor Survival

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ABSTRACT

Purpose: To enable the design of improved inhibitors of matrix metalloproteinases (MMPs) for the treatment of pancreatic cancer, the expression profiles of a range of MMPs and tissue inhibitors of MMPs (TIMPs) were determined.

Experimental Design: Nine MMPs (MMPs 1–3, 7–9, 11, 12, and 14) and three TIMPs (TIMPs 1–3) were examined in up to 75 pancreatic ductal adenocarcinomas and 10 normal pancreata by immunohistochemistry. Eighteen additional pancreatic ductal adenocarcinomas and an additional eight normal pancreata were also analyzed by real-time reverse transcription-PCR and additionally for MMP-15.

Results: There was increased expression by immunohistochemistry for MMPs 7, 8, 9, and 11 and TIMP-3 in pancreatic cancer compared with normal pancreas (P < 0.0001, 0.04, 0.0009, 0.005, and 0.0001, respectively). Real-time reverse transcription-PCR showed a significant increase in mRNA levels for MMP-11 in tumor tissue compared with normal pancreatic tissue (P = 0.0005) and also significantly reduced levels of MMP-15 (P = 0.0026). Univariate analysis revealed that survival was reduced by lymph node involvement (P = 0.0007) and increased expression of MMP-7 (P = 0.005) and (for the first time) MMP-11 (P = 0.02) but not reduced by tumor grade, tumor diameter, positive resection margins, adjuvant treatment, or expression of the remaining MMPs and TIMPs. On multivariate analysis, only MMP-7 predicted shortened survival (P < 0.05); however, increased MMP-11 expression was strongly associated with lymph node involvement (P = 0.0073).

Conclusions: We propose that the principle specificity for effective inhibitors of MMPs in pancreatic cancer should be for MMP-7 with secondary specificity against MMP-11. Moreover, these studies indicate that MMP-7 expression is a powerful independent prognostic indicator and potentially of considerable clinical value.

INTRODUCTION

Pancreatic cancer is a major cause of cancer death in North America and Europe, and its incidence has appeared to increase dramatically in the last 70 years (1–4). Disappointingly, the 5-year survival rate of <5% has shown little improvement in the last 30 years (2, 5, 6). In those patients with resectable tumors, the 5-year survival is of the order of 10–24% and may be improved by adjuvant therapies (7–9). The low survival rates are attributed to an aggressive biological phenotype that is characterized by early local invasion and metastasis (10). At a molecular level, pancreatic cancer is characterized by a high frequency of KRAS mutations in combination with the loss of function of the TP53, p16INK4A, and SMAD4 tumor suppressor genes (11–17).

Pancreatic cancer progression is associated with increased expression of growth factor receptors and their ligands (18–21), alterations in the level of expression of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs), which may result in an imbalance between these (22–24), alterations in antiapoptotic pathways (25–27), the loss of additional tumor suppressor gene function (28–30), and the acquisition of neoangiogenic properties (31, 32).

Early invasion means that most patients have lymph node metastases and both neural and vascular invasion at the time of presentation (33–35). Indeed, it seems very likely that the cancer is disseminated at the time of diagnosis because virtually all patients die of pancreatic cancer irrespective of their treatment (2, 5–7, 36, 37).

Given the limited response to standard forms of chemotherapy, there has been considerable interest in treatments based on biologically relevant target molecules (38–40). Synthetically developed inhibitors of MMPs (MPIs) have been of particular interest (41–45).

The MMPs are a family of zinc-containing proteolytic enzymes that break down proteins in the extracellular matrix in both physiological and pathological conditions (46–49). The four main subgroups, organized in part according to in vitro substrate specificity and in part by association with the cell membrane, are the collagenases (MMPs 1, 8, and 13), the gelatinases (MMPs 2 and 9), stromelysins (MMPs 3, 10, 11, and 18), and the membrane-bound MMPs (MMPs 14–17; Ref. 40). MMPs can regulate the activity of several classes of...
molecules including cytokines, growth factors, and growth factor receptors such as interleukin-1β, insulin growth factor-binding proteins, heparin-binding epidermal growth factor-like growth factor, and fibroblast growth factor receptor-1 (reviewed in Ref. 50). In addition proteolytic modification of extracellular matrix components such as laminin-5, decorin, entactin, and fibronectin by MMPs have been connected to alterations in a broad range of cellular events including cell migration, apoptosis, and proliferation as well as growth factor sequestration. Loss of the tight control of MMP activity in cancer is thought to contribute to excessive destruction of the extracellular matrix, neo-vascularization, tumor spread, and metastases.

There have been reports of increased MMP expression in several human malignancies with reported prognostic significance. In gastric cancer, the levels of MMPs 2 and 9 have been found to be elevated along with MMPs 7 and MMP 14 (51–53). The expression of MMP-2 was higher in patients with gastric cancer that had a poor prognosis, although the difference was not significant (54). Murray et al. (55) showed that MMP-1 expression was associated with a poor prognosis in colorectal cancer. Increased expression of MMPs 2 and 9 and TIMPs 1 and 2 has also been shown to correlate with a poor prognosis in renal cell carcinoma (56). Other studies using various techniques have reported a prognostic significance of increased MMP expression in carcinomas of the colon (57), ovary (58), breast (59), prostate (60), and lung (61). Although early studies suggested that TIMPs may have an antitumor or antimetastatic activity, it is now thought that they may have a more complex function. For example there is a positive correlation between increased TIMP-2 levels and poor outcome in breast cancer (62).

In pancreatic cancer, there have been several studies of MMP and TIMP expression, and these have often involved either limited numbers of patient samples (22, 63) and/or the analysis of only a few MMPs or TIMPs (64, 65). Perhaps not surprisingly, given the variety of techniques used in these studies, some conflicting results have been generated. More recently studies by Yamamoto et al. (66) have suggested that MMP-7 might be an independent prognostic indicator and the potential involvement of MMP-7 has been strongly supported by another recent investigation (67). To further investigate the involvement of MMPs in pancreatic cancer and to expand the range of individual MMPs and TIMPs studied for this disease, we have sought to perform the most comprehensive evaluation of MMPs and TIMPs that has been performed to date. This is a high priority because the poor or inappropriate specificity of therapeutic MPIs tested thus far may account for the rather disappointing results in recent pancreatic cancer trials (44, 45, 68).

We have used immunohistochemistry and real-time reverse transcription-PCR analysis of pancreatic ductal adenocarcinoma and normal tissue to examine the level and pattern of expression of MMPs and TIMPs. Using this data, we then determined whether any of these molecules may be implicated in the progression of pancreatic cancer and also whether any of these molecules might act as prognostic indicators.

### MATERIALS AND METHODS

#### Patients and Specimens.

The pancreatic cancer and control tissue samples (described in Table 1) were obtained from the Pancreas Tissue Bank of the Department of Surgery, Liverpool Candi Tissue Bank and the Department of pathology, Royal Liverpool University Hospital. Formalin-fixed, paraffin-embedded material was available for immunohistochemistry from between 45 and 75 (depending on the MMP or TIMP studied) consecutive tissue sections from patients who had undergone pancreas resection and for whom clinical and follow-up data were available (note however that two of these patients were lost to follow-up). Tissue from 18 additional freshly collected patient samples were rapidly frozen and stored in liquid nitrogen for reverse transcription (RT)-PCR analysis. All of the tissues were examined by an experienced pancreas pathologist (F.C.) using H&E-stained slides, and only pancreatic ductal carcinomas (ICD-0 C25.0–2, 8) were included in the study. Adenocarcinomas arising from the intra-pancreatic bile duct, ampulla of Vater, or duodenum and other malignant neoplasms such as acinar cell carcinomas or cystadenocarcinoma were specifically excluded. Pathological staging was undertaken according to the Union Internationale Contre le Cancer TNM classification (69). Clinical and staging details are described in Table 1. The ten “normal pancreas” tissue specimens were derived from (a) four patients with common bile duct tumors with histologically normal pancreas removed, (b) two patients with pancreatic cancer with normal pancreas removed distant from between 45 and 75 (depending on the MMP or TIMP

### Table 1 Details of the 75 patients with pancreatic ductal adenocarcinoma used for immunohistochemistry

<table>
<thead>
<tr>
<th>Sex</th>
<th>Number (total = 75)</th>
<th>Prognostic significance</th>
<th>Correlation with MMP expression</th>
</tr>
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<tbody>
<tr>
<td>Men</td>
<td>43</td>
<td>NS</td>
<td>Nil</td>
</tr>
<tr>
<td>Women</td>
<td>32</td>
<td>NS</td>
<td>Nil</td>
</tr>
<tr>
<td>Median age (range) (40–77) years</td>
<td>65</td>
<td>NS</td>
<td>Nil</td>
</tr>
<tr>
<td>Tumor grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well differentiated</td>
<td>8</td>
<td>NS</td>
<td>Nil</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>30</td>
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<td></td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT1</td>
<td>5</td>
<td>NS</td>
<td>Nil</td>
</tr>
<tr>
<td>pT2</td>
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<td>Nil</td>
</tr>
<tr>
<td>pT3</td>
<td>39</td>
<td>NS</td>
<td>Nil</td>
</tr>
<tr>
<td>pT4</td>
<td>3</td>
<td>NS</td>
<td>Nil</td>
</tr>
<tr>
<td>Lymph node status</td>
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<td>NS</td>
<td>Nil</td>
</tr>
<tr>
<td>Positive</td>
<td>53</td>
<td>NS</td>
<td>Nil</td>
</tr>
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<td>Resection margins</td>
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<tr>
<td>Unknown</td>
<td>2</td>
<td>NS</td>
<td>Nil</td>
</tr>
<tr>
<td>pT4</td>
<td>36</td>
<td>NS</td>
<td>Nil</td>
</tr>
<tr>
<td>Rejection margins</td>
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<td>NS</td>
<td>Nil</td>
</tr>
<tr>
<td>Positive (R1)</td>
<td>40</td>
<td>NS</td>
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<td>Adjuvant therapy</td>
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<tr>
<td>Yes</td>
<td>18</td>
<td>NS</td>
<td>Nil</td>
</tr>
<tr>
<td>No</td>
<td>57</td>
<td>NS</td>
<td>Nil</td>
</tr>
</tbody>
</table>

* MMP, matrix metalloproteinases; NS, not significant.

+ As part of the European Study Group for Pancreatic Cancer-1 trial (8, 9).
from the tumor, (c) two patients with focal pancreatitis with adjacent normal pancreas, (d) one patient with duodenal tumor, and (e) one patient with a benign pancreatic cyst.

For RNA analysis, there were 18 pancreatic ductal adenocarcinomas, and 8 "normal" pancreatic tissues were obtained from histologically normal areas of the parenchyma from two patients with focal chronic pancreatitis, four patients with pancreatic tumors, and two transplant donors.

This study was undertaken with the formal approval of the Liverpool Research Ethics Committee and written informed consent was obtained for all samples.

Antibodies. Mono- and polyclonal antibodies were supplied by the research and development section of British Biotech (Oxford, UK) and are not commercially available. These included antibodies to MMP-1 (interstitial collagenase; 36632), MMP-2 (gelatinase A; 36033, 1A10, SE592), MMP-3 (stromelysin 1; 10D6, RP58), MMP-7 (matrilysin; RP21), MMP-8 (neutrophil collagenase; SE603), MMP-9 (gelatinase B; 36074, 4H3, 2C10, SE601), MMP-12 (metalloellastase; 10D10, RP54), and MMP-14 (MT1-MMP; RP64, 5H2). Antibodies from British Biotech were later validated by comparing the staining pattern to that obtained with commercially available antibodies on a subset of samples and examining these for consistency. In addition, the Ab-1 antibody to MMP-7 and Ab-5 antibody to MMP-11 were obtained from Labvision (Newmarket, UK). In all cases, antibodies recognize both latent zymogens and active species. Monoclonal antibodies to Timp-1 (147-6D11), Timp-2 (67-4H11) and Timp-3 (136-13H4) were obtained from Chemicon Europe Ltd. (Chandlers Ford, UK). We observed no evidence of cross-reactivity between antibodies to different MMPs or TIMPs.

Immunohistochemical Staining. Tissue specimens were fixed for 48 h in 10% formalin and embedded in paraffin wax before sectioning. Four-μm sections of each specimen were prepared on poly- L -lysine-coated slides and kept in an oven at 37°C overnight before further processing. Positive controls for each antibody were obtained from a bank of control slides in use in the Immunohistochemistry Laboratory, Department of Pathology, Royal Liverpool University Hospital. To establish which controls were used to confirm the staining patterns observed using the British Biotech antibodies. For trypsinization pretreatment, slides were soaked in 0.05% trypsin, in Tris-buffered saline [TBS; 50 mm Tris-HCl, 150 mm NaCl (pH 7.6)] for 20 min at 37°C. For microwave pretreatment, slides were immersed in 10 mm EDTA and irradiated with 850 joules/second⁻¹ for 15 min. Slides were washed with TBS and then blocked with 5% BSA in TBS. Primary antibodies were diluted in 5% BSA in TBS, and 100 μl of each antibody solution was added to each slide for 1 h at room temperature. Slides were washed twice in TBS for 4 min. Secondary antibody staining was performed using the Dako EnVision+ kit (DAKO, Ely, UK) according to the manufacturers instructions. Slides were then washed twice with TBS for 4 min, incubated with 3,3-diaminobenzidine HCl solution for a further 20 min, finally rinsed with distilled water, and counterstained with hematoxylin. Sections were dehydrated with ethanol followed by xylene and mounted using DPX (VWR International Ltd., Poole, UK) mountant. Immunostaining specificity was established using a negative control for each section, in which the primary antibody was omitted. Also included in each batch of samples was a known positive control.

Assessment of Immunohistochemical Staining. Two observers scored all sections independently. The intensity of the immunostaining of the tumor epithelium, tumor stroma, and pancreatitis (if present) and of normal ducts, acini, and islets was graded as negative (0), weak (1), moderate (2), and strong (3). To facilitate interpretation and comparison of heterogeneous tissue staining, whole sections were assigned scores according to the strongest intensity staining on a section, providing that ≥10% of the specified cell type (i.e., adenocarcinoma/stroma/epithelia) on the section stained. In cases where <10% of cells of a specific type were stained the section was assigned a score of 0. Note that there were no cases in which <10% of cells stained predominantly strongly or moderately. Differences in assigned scores were resolved by simultaneous re-examination by both scorers to achieve a consensus. There were no cases where the scores assigned differed by >1 grade.

RNA Isolation. Total RNA was isolated from approximately 100 mg of tissue using RNAzol-B (Biogenesis, Poole, MA).

Table 2 Details of the antibodies used

<table>
<thead>
<tr>
<th>MMP¹</th>
<th>Antibody name</th>
<th>Pretreatment</th>
<th>Dilution</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP1: 36632 monoclonal</td>
<td>Interstitial collagenase</td>
<td>None</td>
<td>1:100</td>
<td>Duodenum</td>
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<tr>
<td>MMP2: 36033 monoclonal</td>
<td>Gelatinase A</td>
<td>None</td>
<td>1:100</td>
<td>Smooth muscle</td>
</tr>
<tr>
<td>MMP3: RP58 monoclonal</td>
<td>Stromelysin 1</td>
<td>None</td>
<td>1:200</td>
<td>Kidney</td>
</tr>
<tr>
<td>MMP7: RP21 monoclonal</td>
<td>Matrilysin</td>
<td>None</td>
<td>1:200</td>
<td>Skeletal muscle</td>
</tr>
<tr>
<td>MMP8: SE603 monoclonal</td>
<td>Neutrophil collagenase</td>
<td>None</td>
<td>1:200</td>
<td>Skeletal muscle</td>
</tr>
<tr>
<td>MMP9: 4H3 monoclonal</td>
<td>Gelatinase B</td>
<td>Trypsin (20’)</td>
<td>1:100</td>
<td>Appendix</td>
</tr>
<tr>
<td>MMP11 monoclonal</td>
<td>Stromelysin 3</td>
<td>None</td>
<td>1:100</td>
<td>Breast cancer</td>
</tr>
<tr>
<td>MMP12: RP54 monoclonal</td>
<td>Metalloellastase</td>
<td>None</td>
<td>1:400</td>
<td>Kidney</td>
</tr>
<tr>
<td>MMP14: RP63 monoclonal</td>
<td>MT-MMP1</td>
<td>None</td>
<td>1:200</td>
<td>Smooth muscle</td>
</tr>
<tr>
<td>TIMP 1 monoclonal</td>
<td>147-6D11</td>
<td>Microwave</td>
<td>1:200</td>
<td>Pancreas</td>
</tr>
<tr>
<td>TIMP 2 monoclonal</td>
<td>67-4H11</td>
<td>Trypsin (20’)</td>
<td>1:200</td>
<td>Colon</td>
</tr>
<tr>
<td>TIMP 3 monoclonal</td>
<td>136-13H4</td>
<td>Microwave</td>
<td>1:1000</td>
<td>Colon</td>
</tr>
</tbody>
</table>

¹ MMP, matrix metalloproteinases; TIMP, tissue inhibitor of the metalloproteinases.
UK) as described by the manufacturer. RNA was quantified by spectrometry at 260 nm and integrity was determined by running 500 ng of total RNA on a 1% Tris-acetate-EDTA gel. To remove any contaminating DNA, the samples were treated with DNase I. Approximately 10 μg of total RNA was digested with DNase I (RNase free; Sigma, Poole, UK) in the presence of 40 units RNasin (Promega, Southampont, UK), in 100 mM sodium acetate (pH 5) and 5 mM MgCl₂ at 25°C for 1 h. The integrity of the RNA was checked before and after digestion by gel electrophoresis. DNase was then removed by phenol/chloroform/isooamyl alcohol extraction, and RNA was precipitated with absolute ethanol.

**cDNA Synthesis.** cDNA was synthesized from 5 μg of total RNA using 200 ng of oligonucleotide-dT primer (Life Technologies, Inc., Paisley, UK) annealed in 10 mM HEPES (pH 7.0), 1 mM EDTA in a final volume of 10 μl, heated to 90°C for 2 min, and cooled on ice. First-stand synthesis was performed by adding 1× first-strand buffer (5× buffer 250 mM Tris-HCl pH 8.3), 375 mM KCl, 15 mM MgCl₂, 1 mM DTT and 0.5 mM dNTPs, and 300 units Moloney murine leukemia virus reverse transcriptase (all from Life Technologies, Inc.) to a final volume of 20 μl and incubating at 37°C for 90 min. Control samples were prepared without reverse transcriptase. To verify the cDNA and the absence of contaminating DNA, PCR was performed on both test and control samples for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The PCR reaction contained 0.5 μl of cDNA product, 200 μM dNTPs, 0.5 μM of each primer forward 5’TGGCTGTCTAGAAAAACCTGC 3’ and reverse 5’ACCCTGTTGCTGTAGCCAAA 3’. The PCR cycles were 95°C for 1 min. This produced a 200-bp product. As the efficiency of PCR amplification from the plasmid was comparable for each product (with detection of each product occurring within one cycle of all of the other products), all MMPs and TIMPs were quantified against the internal GAPDH standard curve. The quantity of each MMP and TIMP was then expressed as a fraction of GAPDH level for each sample. The efficacy of all primer pairs for MMPs and TIMPs was validated on RNA obtained from appropriate positive control tissues. The primers were as follows: (5’-3’) MMP-1 forward AATGTGCTACAGG-GATACCC reverse CTTTGTGCCAATTCAGGA; MMP-2 forward CGGCCTTTAAGTGGCAAAC, reverse TTTGGTT- CTCCAGCTTCCAG; MMP-3 forward GAGGAAAATCGAT- GCAGCCA, reverse CTCCAACTGTAAGATCCCA; MMP-7 forward TTTGTGGGGCAGAAACAC, reverse GGGG-ATCTCCATTTCCCATAG; MMP-9 forward GAAGATGCT- GGTGGTCAGGC, reverse ACTTGTGCAAACCTGTTCA; MMP-11 forward AGATCTAATCTTCCAGGCG, reverse TTTCCAGGCCCTCACCCTA3; MMP-13 forward ATAGGCCTAGACTCTCCTGT; reverse CGCAACAATACGGG- TTACTCC; MMP-14 forward CAGAAGCTGAGGTAGACC, reverse CATTGCGTGCTGAAGAAGAAG; MMP-15 forward GAGACAGCCAGAGGGC, reverse TTGCGTA- AAGCCGACAG; MMP-16 forward CATTGGGTAGCCACAC; TIMP-1 forward CCGGGGCTCTCC; TIMP-2 forward GAAGAAGGCCCCTGACAACA, reverse GTCCTG- GTACGTGAAACTC; TIMP-3 forward TCGGTATACCTC- GGTTGTA, reverse GTCGTTGGGATCTGATGTC. Prim- ers were supplied by either Roche or Helena Biosciences (Tye and Wear, UK).

**Statistical Analysis.** Categorical data were analyzed by the χ² test and Fisher’s exact probability test. Continuous data were analyzed by the Mann-Whitney U test, and correlation analysis was by Spearman’s rank correlation. Survival curves were calculated using the Kaplan-Meier method. Cox proportional hazards modeling was used for multivariate analysis. The level of significance was set at 0.05.

**RESULTS.**

For immunohistochemical analysis, either 45 or, for some MMPs and TIMPs depending on availability of the tissue and/or antibody, 75 cancer and 10 normal specimens were stained with each antibody to assess patterns in the differential expression of all of the MMPs and TIMPs. Where results suggested significant differences, antibody staining was confirmed with an additional antibody or antiserum to the MMP or TIMP on a subset of these samples. These results are summarized in Table 3. For the measurement of mRNA levels, real-time RT-PCR was performed on 18 cancer and 8 normal specimens. These results are summarized in Table 4.

**MMP-1.** MMP-1 was expressed in 29 of 45 (64%) cancers and in 3 of 10 (30%) normals. Immunostaining was weak to moderate in the majority of cancers with only 3 to 29 (10%) cases staining strongly. Staining in all cancers was restricted to the tumor cells, with no staining of the stroma. Staining occurred in the acini, ducts, and islets of adjacent pancreatitis in 7 of 45 (16%) cancers. Weak staining was observed in the acini of 3 to 10 normals. There was no significant difference in the expression of MMP-1 between the cancer and normal samples. There was no difference in survival between the two groups. Also, no correlation was observed between the intensity of MMP-1 staining and tumor differentiation, tumor size, lymph node status, or patient survival. Real-time RT-PCR demonstrated that MMP-1 mRNA was expressed at significantly higher levels (P = 0.006) in tumor tissue than in normal tissue, and this is compatible with the immunohistochemical staining pattern albeit that the latter did not reach statistical significance.
<table>
<thead>
<tr>
<th>Antibody for:</th>
<th>Staining intensity</th>
<th>Pancreatic ductal adenocarcinoma</th>
<th>Normal pancreas</th>
<th>Prognostic significance</th>
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<tr>
<td></td>
<td></td>
<td>Carcinoma</td>
<td>Epithelia</td>
<td>Stroma</td>
</tr>
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<td></td>
<td></td>
<td>Stroma</td>
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<td>Stroma</td>
</tr>
<tr>
<td>MMP-1</td>
<td>0</td>
<td>16 (36%)</td>
<td>Nil</td>
<td>38 (85%)</td>
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<tr>
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<td>1</td>
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<tr>
<td>N = 10 normal</td>
<td>2</td>
<td>15 (33%)</td>
<td>5 (11%)</td>
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</tr>
<tr>
<td>3</td>
<td>3 (7%)</td>
<td>3 (7%)</td>
<td>3 (7%)</td>
<td>3 (7%)</td>
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<td>35 (47%)</td>
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<td>9 (12%)</td>
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<td>9 (36%)</td>
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<tr>
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<td>23 (31%)</td>
<td>53 (71%)</td>
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<tr>
<td>N = 75 PDAC,</td>
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<td>18 (24%)</td>
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<tr>
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<tr>
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<tr>
<td>TIMP-3</td>
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<td>7 (9%)</td>
<td>21 (28%)</td>
<td>8 (11%)</td>
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<tr>
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<td>29 (39%)</td>
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</table>

* MMP, matrix metalloproteinases; TIMP, tissue inhibitor of the metalloproteinases; NS, not significant; RT, reverse transcription; PDAC, pancreatic ductal adenocarcinoma.

b MMP-2 correlated with MMP-14 (P < 0.0001).
c MMP-7 correlated with MMP-14 (P = 0.0005).
d MMP-8 correlated with MMP-14 (P < 0.0001). NS = not significant.
e Not all tumor sections had adjacent pancreatitis, percentage expressed as fraction of scored adjacent pancreatitis.
MMP-2. MMP-2 was expressed in 50 of 75 (66%) cancers and in 7 of 10 (70%) normals. Staining was moderate to strong in 29 of 50 (58%) cancers and was weak in 21 of 50 (42%). Staining in all cancers was restricted to the tumor cells, with no staining of the stroma. Adjacent pancreatitis similarly expressed MMP-2. Expression in the normals was weak or moderate predominantly in acini and islets. There was no significant difference in the intensity of staining between the cancer and normal specimens. There was no correlation among MMP-2 staining and tumor differentiation, tumor size, tumor lymph node status, or patient survival. Nevertheless, there was a correlation between the staining of MMPs 2 and 14 (P < 0.0001). Real-time RT-PCR demonstrated that MMP-2 mRNA was expressed to comparable levels in both tumor and normal tissues, and this is compatible with the immunohistochemistry.

MMP-3. MMP-3 was expressed in 40 of 45 (89%) cancers and in 10 of 10 (100%) normals. In cancers, staining was generally weak or moderate in both carcinoma and stroma, although staining was strong in 12 of 40 (30%) cancers. Normal stroma stained strongly in 10 of 10 samples, including the periductal and inter-lobular connective tissue, and also the walls of vessels. Interestingly, the staining within the normal stroma was significantly stronger than the staining of the malignant stroma (P < 0.0001). Islets, ducts, and acinar cells stained weakly in all normal pancreas and weak to moderately in adjacent pancreatitis. There was no correlation between the tumor cell expression and tumor stroma expression. Nor was a correlation observed among MMP-3 expression and tumor differentiation, tumor size, lymph node status, or patient survival. Real-time RT-PCR demonstrated that MMP-3 mRNA was expressed to comparable levels in both tumor and normal tissues, and this is compatible with the immunohistochemistry.

MMP-7. MMP-7 was expressed in 41 of 45 (91%) of cancers and in 2 of 10 (20%) normals (P < 0.0001). Tumor cells in 28 of 41 (68%) cancers stained moderately to strongly. The proportion of positive cells in tumors was variable; in some cases, focal staining was observed. Expression in adjacent pancreatitis was predominantly moderate with 26 of 45 (58%) staining positively. Staining in the two normals was weak with only a few islets or ducts being positive. There was no staining in the stroma of cancers or normals. Fig. 1A illustrates a typical staining pattern in pancreatic adenocarcinoma and, for comparison, in normal pancreas. The identity of the staining pattern was confirmed using a commercial antibody for MMP-7. No correlation was observed among MMP-7 expression and tumor differentiation, tumor size, or lymph node status. However, there was a correlation between MMP-7 expression and the expression of MMP-14 (P = 0.0005). Most significantly tumors strongly positive for MMP-7 had a significantly worse prognosis than MMP-7 negative/weakly staining tumors (P = 0.005) as shown in Fig. 2A. Real-time RT-PCR demonstrated that MMP-7 mRNA was expressed to comparable levels.

MMP-8. MMP-8 was expressed in 33 of 45 (73%) of the cancers and in 6 of 10 (60%) of normals. Expression in tumor cells was moderate to strong in 15 of 33 (45%) cancers. There was no staining of the tumor stroma. Expression in the normal pancreas was weak within ducts, and occasionally islet staining was seen. The intensity of staining was increased in cancers compared with normals and was significant (P = 0.04). There was no correlation among MMP-8 staining and tumor differentiation, tumor size, tumor lymph node status, or patient survival. Real-time RT-PCR failed to detect the mRNA for MMP-8.

MMP-9. MMP-9 was expressed by 19 of 45 (43%) of cancers and was not detectable in normals (P = 0.0009). Six of 19 (32%) cancers stained moderately or strongly, and this staining was focal. There was no staining of the adjacent pancreatitis and no staining of stroma in either cancers or normals. Neutrophils stained positively in both the cancer and normals. No correlation was observed between MMP-9 expression and tumor differentiation, tumor size, lymph node status, or patient survival. Real-time RT-PCR detected low-level expression of MMP-9 in normal and cancer samples at comparable levels.

MMP-11. MMP-11 was expressed by 52 of 75 (69%) of cancer cases and in 2 of 10 (20%) normals (P = 0.004). Fig. 1B illustrates a typical staining pattern for pancreatic adenocarcinoma and for comparison, normal pancreas. There was staining of stroma in 22 of 75 (29%) cancers, but only one of these stained strongly. Only stromal cells adjacent to cancer stained positively, and the staining was weaker or not detectable in stromal cells further from the primary tumor. Forty of 75 (53%) of adjacent pancreatitis stained for MMP-11 with 31 of 40 (78%) staining moderate to strong. The two normals that were positive had weak staining in the acini, islets, and ducts. The identity of the staining pattern was confirmed using a commer-
cial antibody for MMP-11. There was no correlation among MMP-11 expression, tumor differentiation, and tumor size. However, there was a correlation between MMP-11 expression and lymph node status, with those tumors having a higher level of expression correlating with positive lymph nodes ($P = 0.0073$). In one case in which there was an adjacent lymph node, there was staining of MMP-11 in the metastatic deposit in the lymph node. Patients with MMP-11-positive carcinoma had a significantly shorter overall survival time than did those with MMP-11-negative carcinoma ($P = 0.02$) as shown in Fig. 2B. Real-time RT-PCR demonstrated that MMP-11 mRNA was expressed at significantly higher levels ($P = 0.0005$) in tumor tissue than in normal tissue, and this is compatible with the immunohistochemical staining pattern.

MMP-12. MMP-12 was expressed by 20 of 45 (44%) cancers and in 10 of 10 (100%) normals. Nineteen of 45 (42%) cancers stained weakly. There was no stromal staining. There was weak to moderate staining in 25 of 45 (55%) adjacent pancreatitis. In normals ducts and islets stained more strongly than acini, there was no significant difference in the expression of MMP-12 between cancer and normal and no correlation was observed among MMP-12 expression and tumor differentiation, tumor size, lymph node status, or patient survival. Real-time RT-PCR failed to detect the mRNA of MMP-12.

MMP-14. MMP-14 was expressed by 44 of 75 (59%) of cancers and 4 of 10 (40%) normals. Thirty-eight of 44 (86%) cancers stained weakly to moderately and only 6 of 44 (14%)
stained strongly. There was no staining of stroma. Thirty-seven of 75 (49%) adjacent pancreatitis stained weak to moderate. In normals, occasional acini and ducts stained weakly. There was no significant difference in expression of MMP-14 in the cancers compared with normals. There was no staining of any stroma. No correlation was observed among MMP-14 expression and tumor differentiation, tumor size, or lymph node status. However, there was a strong correlation with MMP-14 expression and MMP-2 (P < 0.0001), MMP-7 (P = 0.005), and also MMP-8 (P < 0.0001) expression.

MMP-15. In the absence of an antibody at the time of the study, MMP-15 was only studied by real-time RT-PCR. Nevertheless, MMP-15 expression was found to be significantly lower (P = 0.0026) in tumor tissue compared with normal tissue by real-time RT-PCR (see Table 4).

TIMP-1. TIMP-1 was expressed in 3 of 45 (7%) cancer cases and in 0 of 10 (0%) normals. In the three cancer cases, there were only occasional foci of TIMP-1 staining. There was no staining of adjacent pancreatitis or stroma. No correlation was observed among TIMP-1 expression and tumor differentiation, tumor size, lymph node status, or patient survival. RT-PCR demonstrated that TIMP-1 mRNA was expressed at higher levels in cancers than in normals, and this difference was significant (P = 0.03).

TIMP-2. TIMP-2 was expressed by 20 of 45 (44%) cancers and in 8 of 10 (80%) normals. The staining was weak in 17 of 20 (85%) positive cancers with only 2 of 20 (10%) staining strongly. Adjacent pancreatitis stained moderately or weakly in 18 of 35 (51%) cases, and this did not correlate with the level of tumor staining. There was no stromal staining. Eight of 10 normals stained weakly for some positivity in ducts and the occasional acinar cell. There was no significant difference in expression of TIMP-2 between cancers and normals. No correlation was observed among TIMP-2 expression and tumor differentiation, tumor size, lymph node status, or patient survival. Real-time RT-PCR detected low levels of TIMP-2 mRNA in both cancers and normals.

TIMP-3. TIMP-3 was expressed by 68 of 75 (91%) cancers and in 8 of 10 (100%) normals. Staining was moderate to strong in 55 of 68 (81%) cancers. There was moderate to strong staining of stroma in 45 of 75 (60%) cancers. Adjacent pancreatitis stained in 67 of 75 cancers and 54 of 67 (81%) of these were stained moderate to strong. Eight of 10 (80%) normals stained weakly for TIMP-3 in ductal elements. However, acini and islets stained more strongly. There was no stromal staining in normals. There was an increased intensity and statistically significant difference in expression of TIMP-3 between cancers and normals (P < 0.0001). There was also a
significant increase in TIMP-3 expression in cancer stroma versus normals ($P < 0.0003$). No correlation was observed among TIMP-3 expression and tumor differentiation, tumor size, lymph node status. Real-time RT-PCR demonstrated that TIMP-3 mRNA was expressed to comparable levels in tumor and normal tissues.

**Survival Analysis.** Of the tumor staging characteristics, tumor grade, diameter, and differentiation were not significant as summarized in Table 1. Lymph node status correlated to survival ($P = 0.007$). Univariate analysis revealed a relationship between overexpression of MMPs 7 and 11 ($P = 0.005$ and $P = 0.02$, respectively) and an unfavourable outcome in operable pancreatic carcinoma. Kaplan-Meier plots for MMPs 7 and 11 are shown in Fig. 2. Multivariate analysis was then conducted that included all significant and marginally significant values from the univariate analysis to identify any independent predictors of survival. Cox proportional hazard modeling demonstrated that MMP-7 overexpression was the only significant independent indicator for survival ($P < 0.05$).

**DISCUSSION**

We have found that several MMPs and TIMPs may have a role in the evolution of human pancreatic adenocarcinoma. In particular, MMPs 7, 8, 9, 11 and TIMPs 1 and 3 were expressed at significantly higher levels in the tumor as shown by immunohistochemistry and/or real-time RT-PCR. Also, we found that MMP-15 was expressed at significantly lower levels in the tumor by real-time RT-PCR, but we were unable to confirm this by immunohistochemistry. Of most significance, both MMPs 7 and 11 predicted survival. In the case of MMP-7, this was of independent prognostic significance.

Studies of MMP-7 in pancreatic cancer have shown that it is present at higher levels in cancers than in the normal pancreas (24, 66, 67), and our results support this conclusion. Yamamoto et al. (66) found that MMP-7 expression in pancreatic adenocarcinoma correlated with a poor prognosis and was a significant independent prognostic factor for overall survival. Our data also support this finding.

High levels of MMP-7 expression have been associated with a poor prognosis in other tumors: for example, colon and gastric (70, 71), pancreas (66, 67), prostate (72), and brain (73). MMP-7 up-regulation is often identified as an early event in tumor development (66, 74). Although the mechanisms underlying these observations are likely to involve a number of molecules and pathways, several important parts of the jigsaw puzzle may connect the activity of MMPs with expression and feedback regulation of specific adhesion molecules.

**FasL**

FasL has been most clearly implicated in events likely to lead to pancreatic ductal adenocarcinoma. Crawford et al. (67) demonstrated that FasL was cleaved to a soluble form, soluble (s)FasL, in an MMP-7-dependent manner during acinar to ductal metaplasia in a mouse model. One explanation for the metaplasia could be that there is a selective advantage as a result of insensitivity to apoptosis in the emerging ductal population (67, 85). This interpretation requires either that ductal cells no longer respond to proapoptotic signaling by sFasL or that sFasL has more potent antiapoptotic activity on the trans-differentiated ductal cells. Which of these possibilities is correct is unclear. Certainly, sFasL has been shown to exhibit both pro- and antiapoptotic activities (81, 86–88), and this might reflect different cleavage (producing different peptides with various activities) or differential activity of sFasL on cells at different stages of either tumor development or differentiation, perhaps as a result of changes in the protein composition of the extracellular milieu (86).

Osteopontin has been identified as a target of MMP-7 (and of MMP-3; Ref. 82) and has been implicated in wound healing, inflammation, and tumorigenesis. In vitro, cleavage of osteopontin by MMP-7 was observed to lead to an increase in the migratory and adhesive activity of osteopontin in an integrin-dependent manner (82), although whether this contributes to tumor invasiveness in vivo is not known.

MMP-7 expression is regulated by the wnt/β-catenin/tcf signaling pathway, and it is therefore not surprising to find other connections between MMP-7 and this pathway, as can be illustrated by β4-integrin (79) and E-cadherin (89). Interestingly, β4-integrin has been implicated recently in pancreatic ductal adenocarcinoma by virtue of its expression being up-regulated (90). Several members of the integrin family have been shown to contribute to repression of metastasis by promoting adhesion to the extracellular matrix. Thus cleavage by MMPs may compromise the activity of other β4-integrin-containing complexes, albeit this does not explain why there appears to be a selective advantage during tumor evolution for up-regulation of expression of β4-integrin. One potential explanation for this may be that the proteolytic release of the integrin ectodomains leads to these domains competing with membrane-associated integrins for target binding, thus reducing the strength of interaction of the latter with the extracellular matrix. It is noteworthy that in studies of an oral carcinoma cell line, it was found that in addition to E and N-cadherins, β1 integrins also played a role in activating expression of MMP-7 (89). This pathway that leads to amplification of the effects of increased MMP-7 expression and activity may connect the activity of MMPs with expression and feedback regulation of specific adhesion molecules.

MMP-7 has been shown to cleave E-cadherin at the cell surface and release soluble E-cadherin (80, 91). The soluble E-cadherin fragment thus released has been shown to inhibit E-cadherin function in a paracrine fashion (80, 91). Loss of E-cadherin expression in a transgenic mouse model of pancreatic β-cell carcinogenesis (Rip1Tag2) coincides with the transition from well-differentiated adenoma to invasive carcinoma (92). Curiously, it has also been demonstrated that E-cadherin stimulates expression of MMP-7 through the wnt-signaling pathway (93).

Our study is the first to identify a correlation between...
MMP-11 expression and outcome. MMP-11 was found previously to be expressed more highly in pancreatic ductal adenocarcinoma than in normal pancreata by immunohistochemistry with the highest expression observed in stromal regions adjacent to the tumor (64). MMP-11 expression has been observed in breast tumors and tumors of the head and neck (94, 95). In these reports, MMP-11 has also been detected only in stromal fibroblasts surrounding the malignant cells and not in the malignant cells themselves or in fibroblasts distant from the tumor (94). Others have also found that the expression of MMP-11 was limited to stromal fibroblasts adjacent to breast cancer cells (96). This is not always the case however. In one series, it was reported that in 15 of 54 (28%) breast cancer samples, the tumor cells themselves were positive, and also in the adjacent stroma, 34 of 54 (63%) samples exhibited positive staining (97). In another study, it was reported that 6 of 21 (29%) cases of pancreatic cancer expressed MMP-11 in the epithelial tumor cells with 17 of 21 (81%) cases displaying expression in the adjacent stromal cells (64). Data from our study suggests that the majority of MMP-11 expression is derived from the tumor epithelial cells themselves, with 52 of 75 (69%) cases staining these cells. We have also observed adjacent staining of malignant stromal cells in 22 of 75 (29%) of cases. In vitro studies in fibroblasts have demonstrated that basic fibroblast growth factor and epidermal growth factor can act as potent inducers of MMP-11 expression (18, 97). Pancreatic cancer cells are known to over-express these growth factors; therefore, these may stimulate the expression of MMP-11 in pancreatic cancer (18, 19).

In breast carcinoma, the level of MMP-11 mRNA has been found to be highly predictive for the presence or absence of distant metastases (98). This has also been related to invasiveness, with only one in ten ductal-carcinoma in situ cases expressing MMP-11 compared with 62% of invasive cancers (99). A correlation has been also shown in breast cancer between expression of MMP-11 and survival, with those tumors expressing a high level of the enzyme being associated with fatal metastatic disease (98). Multivariate analysis has identified that MMP-11 may be a strong independent prognostic parameter for disease-free survival ($P = 0.005$; Ref. 96). Those patients with a moderate to strong MMP-11 level had significantly shorter disease-free survival than those with no or low expression. In our study, MMP-11 expression in pancreatic cancer also appeared to correlate with invasive potential, with high levels of expression being related to the extent of nodal disease ($P = 0.0073$). Also in our study, those patients with moderate or high MMP-11 expression had a significantly shorter survival than those patients with negative or low expression ($P = 0.02$). As has been found previously in breast and head and neck cancer, we found that there was no obvious correlation between the degree of pancreatic tumor differentiation and the extent of MMP-11 expression (95, 98).

Previous studies have suggested that MMP-9 expression correlates with increased metastatic potential in colorectal cancer (100). It has been demonstrated that latent MMP-9 was present in both normal pancreas and pancreatic adenocarcinoma (101). However, there was an increase in activity shown by zymography, with 21.2% of tumors having MMP-9 activity in comparison to 15.4% of normal pancreas specimens. Neutrophil staining was a consistent feature, and this was supported by other studies (63). Forty-two percent of our cancer cases stained positively for MMP-9 with individual cells and cellular clusters staining, whereas the adjacent stroma did not. In situ hybridization studies have also detected higher levels of MMP-9 expression in tumor epithelium than in the adjacent stroma (63, 65).

Membrane-bound MMP-14 has previously been found expressed exclusively localized in the tumor epithelial cells (24). In contrast to our study, studies using in situ hybridization found that MMP-14 mRNA was not localized to the tumor epithelial cells, but was present in cellular elements within the tumor stroma (102, 103). It has been suggested that MMP-14 may activate pro-MMP-2 (104). Interestingly, we have found that the pancreatic cancers that expressed MMP-14 also expressed MMPs 2 and 7. Others have found that the presence of MMP-14 mRNA correlated with that of MMP-2 mRNA (24). Although it is clear that the activation and inhibition of MMPs and TIMPs is a complex issue, it has been suggested previously that there is a link between MMP-14 and pro-MMP-2 activation (24). The correlation that we and others have found may reflect such a link.

There are currently only two studies in which expression of MMP-15 in cancer has been described, and no doubt this reflects the past shortage of available reagents. In recent studies, MMP-15 mRNA was found at higher levels in hepatocellular carcinoma cell lines (105) and by microarray analysis in lung cancer tissues from patients who had received chemotherapy (106). By contrast, we have found that MMP-15 mRNA levels were significantly lower ($P = 0.0026$) in tumor tissue compared with normal tissue. Clearly, none of these studies determine the source of the expressing cell type or even whether the changes in expression are the result of changes in tissue composition. Nonetheless, the loss of expression in the tumor that we have observed warrants additional investigation.

Early studies suggested that the TIMPs exerted an antitumor or antimetastatic effect, and this was supported by in vitro studies which showed that increased TIMP expression was associated with a decrease in tumor cell growth and spread (107, 108). However, more recent work has suggested a positive correlation between TIMP levels and poor outcome. High expression levels of TIMP-1 have been linked with a poor prognosis in gastric tumors, with those tumors that expressed the highest levels of TIMP1 having a poorer prognosis (57). Higher levels of TIMP mRNA have also been shown to correlate with lymph node metastases and reduced 5-year survival in patients with colorectal carcinoma (109). In breast carcinoma TIMP mRNA expression was found to be higher in the carcinoma than the surrounding normal breast tissue (24). In this latter study, the TIMP-1 signal was substantially lower in the tumor cells than in the stroma. Our own data show that TIMP-1 expression can only be detected by immunohistochemistry in 7% (3 of 45) of pancreatic adenocarcinoma samples. In these three cases, the staining was confined to individual tumor cell clusters, and there was no staining of the stroma. This contrasts with the study by Bramhall et al. (24) where by Northern blot analysis, seven (7 of 7) pancreatic cancer cell lines and all (17 of 17) of the pancreatic tumor samples expressed detectable TIMP-1 mRNA. However, using in situ hybridization, these authors found that the expression was epithelial in origin.

TIMP-3 has been less well studied than TIMP-1 and unlike
the soluble TIMPs 1 and 2, TIMP-3 is unusual because it is a component of the extracellular matrix (110). A study of breast carcinoma and malignant melanoma cell lines showed that increased expression of TIMP-3 resulted in a significant reduction in tumor cell growth. Adenovirus-mediated gene delivery of TIMPs-1, -2, and -3 revealed that the overproduction of TIMP-3 inhibited invasion of malignant melanoma cells (111). In human pancreatic cancer, TIMP-3 expression has not been investigated previously. Our findings suggest that increased TIMP-3 expression could be associated with a survival disadvantage although this link did not attain statistical significance ($P = 0.06$). This observation may warrant additional investigation.

Ultimately, the expression profiles of MMPs and TIMPs are of interest for two reasons. First, they may inform us about the changes that take place in the cells (and also in the tissues) that ultimately become tumors. Second, this information can be used to guide the design of novel therapeutics.

The potential for MPI therapy in pancreatic cancer was demonstrated recently in a phase III trial of 414 patients with advanced disease who were randomized to an MPI (marimastat at 5, 10, or 25 mg twice daily) or gemcitabine (1000 mg/m²; Ref. 44). There appeared to be a dose response in terms of patient survival for marimastat. The survival rate of patients treated with gemcitabine was significantly greater than for patients who received 5 and 10 mg of marimastat b.i.d but was not different from the survival of patients who received 25 mg of marimastat b.i.d. The major toxicity of marimastat was musculoskeletal affecting 44% of the patients so treated (44). Moreover, the combination of gemcitabine and marimastat was found not to be superior to gemcitabine and placebo in a phase III of advanced pancreatic cancer (45). In the dose-determining phase II trial, prolonged usage of the higher dose of 25 mg of marimastat at b.i.d caused musculoskeletal side effects in 5% of patients (43). Thus the efficacy of MPI therapy could be improved by higher dosing and by using more selective inhibitors that minimize the musculoskeletal side effects.

Marimastat is a powerful general MPI with an IC_{50} to MMPs 1, 2, 3, 7, 8, 9, and 14 of 5, 6, 200, 20, 2, 3, and 1.8 nM, respectively (40). We would conclude from our study that the principle specificity for an effective MPI in pancreatic cancer should be for MMP-7, but MMP-7 is also heavily expressed by skeletal muscle (Table 2). Whether the musculoskeletal side effects of marimastat are attributable to specific MMP inhibition or whether there is another mechanism responsible is unclear. A recent phase I study of the MPI inhibitor MMI270 resulted in musculoskeletal toxicity in 42% of patients (112). Of interest is that this MPI has IC_{50} to MMPs 1, 2, 3, 7, 9, and 13 of 33–43, 11, 13–50, 8, and 6 nM, respectively (40). The agent BAY 12-9566 is an example of a “deep-pocket” MPI inhibitor with $K_i$ to MMPs 1, 2, 3, 9, and 13 of $>5000$, 11, 143, 301, and 1470 nM, respectively (40), that has no musculoskeletal side effects (113) but also lacks clinical efficacy (114). It has been proposed that dose-limiting musculoskeletal toxicity may result from the inhibition of MMP-1 or of sheddases, which are closely related metalloproteases, but at present there are insufficient clinical data for a clear conclusion (68, 114).

We believe that the findings of this investigation may aid in the development of MPIs that are more specifically targeted toward pancreatic cancer than heretofore possible.

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REFERENCES


Comprehensive Analysis of Matrix Metalloproteinase and Tissue Inhibitor Expression in Pancreatic Cancer: Increased Expression of Matrix Metalloproteinase-7 Predicts Poor Survival

Lucie E. Jones, Michelle J. Humphreys, Fiona Campbell, et al.


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