Helicobacter pylori upregulates matrilysin (MMP-7) in epithelial cells in vivo and in vitro in a Cag dependent manner

J R Bebb, D P Letley, R J Thomas, F Aviles, H M Collins, S A Watson, N M Hand, A Zaitoun, J C Atherton

Background and aims: Matrix metalloproteinase-7 (MMP-7) is important in normal and pathological remodelling of epithelial-matrix interactions, and is upregulated in gastric cancer. Helicobacter pylori infection is the first stage in gastric carcinogenesis, and therefore our aim was to determine if H pylori upregulated gastric MMP-7 expression and if this was affected by strain virulence.

Methods: We took gastric biopsy specimens at endoscopy from H pylori infected (n = 17) and uninfected (n = 18) patients and assessed MMP-7 expression by ELISA, real time polymerase chain reaction (PCR), and immunohistochemistry (concentrating on epithelial cells in the proliferative zone). We PCR typed H pylori for cagE and vacA. We performed H pylori/cell line coculture studies with wild-type pathogenic and non-pathogenic H pylori strains and with CagE+ and VacA- isogenic mutants.

Results: Gastric biopsy specimens from H pylori+ patients expressed higher levels of MMP-7 at the protein and mRNA levels in the antrum and corpus (for example, by ELISA: H pylori+ 0.182 OD units v H pylori− 0.059; p = 0.009 antrum). Epithelial cells from H pylori+ patients stained more intensely for MMP-7 than those from uninfected patients, including in the proliferative zone containing pluripotent cells (p<0.03 antrum, p<0.04 body). Upregulation of MMP-7 in epithelial cells was confirmed at the protein and mRNA levels by H pylori/cell line coculture. These experiments also showed that MMP-7 upregulation was dependent on an intact H pylori cag pathogenicity island but not on the vacuolating cytotoxin.

Conclusion: We speculate that increased expression of MMP-7 in H pylori gastritis may contribute to gastric carcinogenesis.

The main cause of peptic ulceration and gastric MALT lymphoma is Helicobacter pylori, and H pylori is the strongest risk factor for the development of distal gastric adenocarcinoma. The outcome of H pylori infection is dependent on host, environmental, and bacterial factors. Strains possessing the cag pathogenicity island, encoding a type IV bacterial protein secretion system, are more strongly associated with increased levels of inflammation and disease, as are those producing an active form of VacA, a pore forming toxin that induces cytoplasmic vacuolation in vitro. Gastrointestinal mucosa is a complex, dynamic, and highly specialized tissue that provides a barrier to the penetration of dietary material and pathogens. Matrix metalloproteinases (MMPs) are a family of diverse zinc dependent proteolytic enzymes that are important in maintenance and remodelling of interactions between epithelial cells and basement membrane. They have many substrates, including collagen and elastin, and play an important role in promoting invasion and metastasis of cancer cells. MMPs also cleave cell surface bound substrates (“sheddase” activity) such as membrane bound cytokines, cytokine receptors, and adhesion molecules, releasing soluble or inactive forms. MMP-7 (matrilysin) is the smallest member of the MMP family and part of the stromelysin subclass. It is upregulated in epithelial cells in gastric cancer. MMP-7 degrades various matrix substrates, including proteoglycans, gelatin, and elastin, and cleaves from the cell surface non-matrix substrates, including E-cadherin, pro-tumour necrosis factor α (TNF-α), and Fas ligand. A growing body of evidence suggests that MMP-7 plays an early role in tumorigenesis in colorectal cancer. The MMP-7 null Min mouse exhibits much reduced colonic tumour formation, and MMP-7 transcripts are found in the tumour epithelium of over 90% of colonic adenomas in both human and mice models.

Here we have examined if MMP-7 is upregulated in H pylori gastritis, as this is the first stage in the progression to gastric carcinoma. We show that H pylori upregulates MMP-7 expression at the mRNA level, and that MMP-7 is upregulated in inflammatory and epithelial cells, and crucially in the latter in the proliferative zone containing pluripotent cells. We show that this upregulation is caused by pathogenic strains of H pylori, and is dependent on the cag pathogenicity island, but not on the toxin, VacA.

METHODS

In vivo studies

Patients and biopsy samples

Patients were recruited from routine day case endoscopy lists performed at University Hospital, Nottingham. Patients were not included if they had taken a proton pump inhibitor, antibiotics, or bismuth containing compounds in the previous four weeks. The study had full ethical approval from the University Hospital Nottingham Ethics Committee. Details of patient demographics are shown in table 1. Gastric biopsy specimens were taken from the antrum and corpus, and

Abbreviations: MMPs, matrix metalloproteinases; MMP-7, matrix metalloproteinase-7 (matrilysin); FCS, fetal calf serum; PCR, polymerase chain reaction; GAPDH, glyceroldehyde-3-phosphate dehydrogenase; Ct, threshold cycle; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; ELISA, enzyme linked immunosorbent assay; TNF-α, tumour necrosis factor α.
immediately snap frozen in liquid nitrogen for later RNA extraction and enzyme linked immunosorbent assay (ELISA) analysis. Adjacent biopsies were placed in formalin for routine histological analysis and for MPP-7 immunohistochemistry. Further adjacent biopsies from the antrum and body were used for the rapid urease test (Clotest; Ballard Medical Products, Draper, Utah, USA), and placed in ICSensitest media (Oxoid, Basingstoke, UK) supplemented with 5% fetal calf serum (FCS) for later H pylori culture.

**MMP-7 ELISA**

The Quantikine human MMP-7 (total) ELISA kit (R&D Systems, Abingdon, UK) was used to assess total MMP-7 in gastric antral biopsies from the first 10 H pylori positive patients and 11 H pylori negative patients. Biopsies, which had been snap frozen in liquid nitrogen, were homogenised using an Ultra-Turrax T50 homogeniser (Becton Dickenson, Oxford, UK) for 30 seconds on ice in 1 ml of 20 mM HEPES with added protease inhibitor cocktail (P8340, Sigma Aldrich, Dorset, UK). A standard curve was prepared using recombinant human MMP-7 according to the manufacturer’s instructions. A spiked assay performed prior to running samples suggested there was no significant interference at 1:800 (Oncogene Research Products, Cambridge, UK; cat No OM71). The sections were picked up on Dako ChemMate microscope slides (gap 75 µm) to allow immunoperoxidase staining on a Dako TechMate 500 Plus automated stainer (Dako, Ely, Cambs, UK). Staining was previously optimised (including pretreatment) for each antibody and a labelled streptavidin biotin technique employed to detect localisation of the antigens using diaminobenzine as the chromogen. An identical procedure (including pretreatment) was used for the negative control but the primary antibody was replaced with diluent only.

Briefly, the procedure required microwave antigen retrieval with 10 mM citrate buffer (pH 6.0). All staining was performed at room temperature with an incubation of one hour for the primary antibody.

**Assessment of staining**

Two observers (AZ, an experienced histopathologist, and JB) assessed MMP-7 staining intensity. Initial observations suggested staining intensity differed between areas of biopsies, dependent on depth from the surface epithelium. We were particularly interested in staining in the proliferative zone, so we graded staining intensity here and in superficial and deep zones. Zones were defined by splitting the area between superficial cells and stromal cells into thirds. Staining intensity was graded 0 (no staining) to 4 (intense staining). Both observers were blinded to the H pylori status of the individuals and they scored slides separately: 95% concordance was reached on initial scoring. Discordant results were resolved by joint review, again with both scorers blind to the H pylori status. Up to 200 inflammatory cells (where present) were also counted per field view (crossing all three zones), and the percentage expressing MMP-7 calculated.

**Table 1 Patient demographics**

<table>
<thead>
<tr>
<th>Diagnosis/reason</th>
<th>H pylori+</th>
<th>H pylori−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)</td>
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<td>8/10</td>
</tr>
<tr>
<td>Age (y) (median)</td>
<td>65</td>
<td>52.5</td>
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<tr>
<td>Diagnosis/reason for OGD</td>
<td>9 duodenal ulcer disease</td>
<td>6 GORD</td>
</tr>
<tr>
<td></td>
<td>4 non-ulcer dyspepsia</td>
<td>5 non-ulcer dyspepsia</td>
</tr>
<tr>
<td></td>
<td>4 iron deficiency anemia</td>
<td>2 duodenal ulcer disease</td>
</tr>
<tr>
<td></td>
<td>normal OGD</td>
<td>2 iron deficiency</td>
</tr>
<tr>
<td></td>
<td>anemia, normal OGD</td>
<td>2 coeliac follow up</td>
</tr>
<tr>
<td></td>
<td>1 cancer of oesophagus</td>
<td></td>
</tr>
</tbody>
</table>

OGD, oesophago-gastro-duodeno-scopy; GORD, gastro-oesophageal reflux disease.

**Real time semiquantitative PCR and analysis**

RNA was reverse transcribed using the Qiagen Omniscript Reverse Transcriptase kit with additional RNase inhibitor (10 units/reaction) and 1 µM pdN6 salt random hexamer primer (Pharmaclia, Amersham, UK). Reverse transcription was carried out at 37°C for one hour followed by five minutes at 95°C to inactivate the reverse transcriptase. A negative control (without Omniscript reverse transcriptase and random hexamer primer) was used for each sample. Real time polymerase chain reaction (PCR) was carried out using the QuantiTect SYBR Green PCR kit (Qiagen) using a GeneAmp 5700 Sequence Detection System (Applied Biosystems, California, USA) with 0.5 units of uracil-N-glycosylase per reaction and oligonucleotide primers specific for MPP-7 (5′ GGTTAAGGGTTCAGATCTCCTGAGGATGC3′ and 5′ ACTCTCCAGCCATCCTGGCTTGCTG3′) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5′ GGTGAGTGTCGTAGTCCTGGAAGA3′ and 5′ GAGGGATCTAGCGCTCCCTGGAAGA3′). PCR conditions were as follows: 50°C for two minutes, 95°C for 15 minutes followed by 40 cycles of 94°C (15 seconds), 56°C (30 seconds), and 72°C (one minute). Relative expression of MPP-7 cDNA amplification, ΔCt (v GAPDH) was calculated using the formula 2(ΔCt (GAPDH)−ΔCt (MPP-7)) where Ct (threshold cycle) represents the fractional cycle number at which the fluorescence passes the fixed threshold. The correct PCR product size was confirmed by electrophoresis on a 2% agarose gel.

**Immunohistochemistry**

Gastric biopsies were fixed in formol saline for 24 hours and subsequently processed and embedded in paraffin wax. Each biopsy was sectioned at 4 µM, with sufficient sections to permit one negative control, and stained with a specific monoclonal antibody to MPP-7 at a dilution of 1:800 (Oncogene Research Products, Cambridge, UK; cat No OM71). The sections were picked up on Dako ChemMate microscope slides (gap 75 µm) to allow immunoperoxidase staining. Staining was previously optimised (including pretreatment) for each antibody and a labelled streptavidin biotin technique employed to detect localisation of the antigens using diaminobenzine as the chromogen. An identical procedure (including pretreatment) was used for the negative control but the primary antibody was replaced with diluent only.

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**H pylori culture and PCR typing for cagE and vacA**

Gastring biopsies were plated out within one hour on 5% horse blood agar plates and left for five days in a 5% CO2 incubator at 37°C. Colonies of H pylori were identified by morphology, Gram stain, and urease testing, and clonally expanded before freezing at 80°C. Genomic DNA was extracted as previously described and strains were PCR typed for the presence of cagE, a reliable marker for the presence of the cag pathogenicity island (5′ AAGGTTAAGGATGGACTGAT CAT 3′ and 5′ GGAAAGTGATACAAAAGAACATG 3′, conditions 95°C for two minutes, 35 cycles of 95°C for one minute, 56°C for two minutes, 72°C for four minutes, and 72°C for five minutes) and vacA (signal and mid region, as described previously). All PCR reactions were carried out on a Hybaid thermal cycler.
Definition of *H pylori* status

For the purposes of this study, patients were considered to be infected with *H pylori* if culture was positive, or in the case of negative culture, if histology showed characteristic organisms and the Clotest rapid urease test was positive.

In vitro studies

**H pylori** strains

The following strains were used for coculture experiments: 60190 (ATCC 49503, cagPath+, vacA S1m1), Tx30a (ATCC 51392, cagPath−, vacA S2/m2), 60190CagE− (cagE− insertion isogenic mutant of 60190, vacA S1/m1), 60190VacA− (cagPath+, disrupted vacA− insertion mutant of 60190). The mutant strain 60190CagE− was derived from the cagE (pticB) mutant 84-183:pMT3:k/m. A 3.4 kb fragment containing the disrupted cagE gene was PCR amplified from 84-183:pMT3:k/m and cloned into pgEM T-Easy (obtained from Promega Corp., Southampton, UK) to create pRT1. The chromosomal cagE gene of strain 60190 was then replaced with the disrupted cagE gene by natural transformation with pRT1, followed by allelic exchange and kanamycin marker rescue. PCR analysis and Southern blotting confirmed replacement of cagE with the disrupted allele. We confirmed that the mutant strain was phenotypically null for the cag encoded type IV secretion system by showing that, compared with wild-type, it had a markedly reduced ability to stimulate epithelial cell lines to express interleukin-8 and that it did not induce CagA phosphorylation in epithelial cells (data not shown). The mutant strain 60190VacA− has been described previously.

**Bacterial coculture**

*H pylori* strains 60190, Tx30a, and CagE− and VacA− isogenic mutants of 60190 were grown on 5% horse blood agar plates (Oxoid) in a 5% CO2 incubator at 37°C. HT29 cells (a human colonic adenocarcinoma cell line, obtained from the European Tissue Culture collection) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% FCS. For coculture, 48 hour plates of *H pylori* were added to subconfluent HT29 (in the absence of FCS) in 75 cm2 tissue culture flasks such that the final viable bacteria/epithelial cell ratio was approximately 100:1. Tissue culture flasks were placed in a 5% CO2 incubator at 37°C for 24 hours. Supernatants were then aspirated and used for immunoblots and zymography, and cell pellets used for RNA extraction.

Immunoblot and zymographic analysis of supernatants

Equal volumes of untreated (media) and bacterial treated epithelial cell supernatants were analysed for the presence of MMP-7 by immunoblot and casein zymography. For immunoblots, samples were mixed with sodium dodecyl sulphate (SDS) running buffer, boiled, and centrifuged before application to 12.5% SDS-polycrylamide gel electrophoresis (PAGE). For blotting, SDS-PAGE gels were blotted for one hour onto PVDF membranes at 150 mA, blocked in phosphate buffered saline-0.5% Tween with 5% skimmed milk powder (Oxoid) for one hour, and then incubated with primary antibody (monoclonal anti-MMP-7; Oncogene Research Products, Nottingham, UK) overnight, washed and incubated with peroxidase conjugated goat antirabbit IgG antibodies (Sigma-Aldrich), washed, and then developed using ECL system (Amersham Pharmacia-Biotech). For casein zymography, samples were mixed with SDS sample buffer (Novex; Invitrogen, Paisley, UK), applied to a 4–16% blue casein zymogram gel (Invitrogen) and run using the manufacturer’s recommended buffers. After running, the gels were placed in renaturing buffer and incubated overnight in developing buffer (both buffers from Invitrogen).

Areas of protease activity appeared as white bands on a blue background and were photographed using a digital camera.

RNA extraction and real time PCR analysis

RNA extraction, quantification, and real time PCR analysis was carried out in an identical manner to the in vivo studies.

Statistical analysis

All statistical analyses used the GraphPad Prism statistical package. Mann-Whitney U tests were used to compare median scores for histological grading. T tests were used to compare ΔCt values for mRNA and for comparing ELISA values.

RESULTS

We first aimed to assess whether MMP-7 expression in the gastric mucosa was upregulated by *H pylori* infection. To do this we performed a specific ELISA for total MMP-7 on gastric biopsy specimen homogenates from *H pylori* infected patients and uninfected control patients. MMP-7 was expressed in infected and uninfected gastric mucosa, with a threefold increase observed in *H pylori* infected patients (mean ELISA score (SEM): *H pylori*+ (n = 10) 0.182 (0.045) v *H pylori*− (n = 11) 0.059 (0.016); p = 0.009, unpaired t test). To confirm these results and to assess whether *H pylori* infection upregulated MMP-7 expression at the transcriptional level, we extracted total RNA from biopsy specimens, reverse transcribed, and quantified levels using real time PCR. Biopsies from *H pylori* infected patients expressed higher levels of MMP-7 mRNA in both the antrum (ΔCt (SEM): *H pylori*+ 0.038 (0.01) (n = 12) v *H pylori*− 0.008 (0.005) (n = 13); p = 0.007, t test) and corpus (*H pylori*+ 0.044 (0.021) (n = 12) v *H pylori*− 0.005 (0.002) (n = 13); p = 0.07). We had more samples for RNA analysis than for ELISAs because RNA extraction was performed first, and where this was unsuccessful the biopsy intended originally for ELISA was used.

Our principal interest in MMP-7 upregulation was in its implications for the pathogenesis of gastric adenocarcinoma. Thus we aimed firstly to assess whether upregulation occurred in epithelial cells and secondly whether it occurred in the proliferative zone, which contains gastric stem cells. To do this, we performed immunohistochemistry on biopsy specimens from the gastric antrum and corpus for MMP-7 expression in epithelial cells in the proliferative zone. Staining intensity scores were significantly higher in epithelial cells in the proliferative zone in *H pylori* infected patients than in those from uninfected patients (median scores for histological grading. T tests were used to compare ΔCt values for mRNA and for comparing ELISA values).
H pylori upregulates MMP-7 in epithelial cells in a Cag dependent manner

Figure 1 Dot plot showing epithelial cell staining grade (0–4) for the proliferative zone in the antrum (A) and corpus (B) for Helicobacter pylori- and H pylori- patients. Grades were higher for H pylori infected patients in both the antrum (p = 0.03 Mann-Whitney U test) and corpus (p = 0.04).

$H$ pylori $^+ $ 87.4 (2.8) v $H$ pylori $^-$ 29.9 (4.1), p < 0.01 t test; corpus, $H$ pylori $^+ $ 80.4 (2.7) v $H$ pylori $^-$ 38.6 (4.4), p < 0.01).

Because of the variation in level of MMP-7 expression between $H$ pylori $^+$ biopsy specimens, especially with regard to epithelial cell expression, we next asked whether strains of differing virulence were associated with different levels of MMP-7 expression. To do this, we assessed cagE status and vacA type of $H$ pylori isolates cultured from gastric antral biopsy specimens and correlated these with MMP-7 expression. Unfortunately, only three strains were cagE negative, but nevertheless, by MMP-7 ELISA, cagE positive strains appeared to induce higher levels of MMP-7 expression than cagE negative strains (mean (SEM): cagE $^+$ 0.218 (0.06), n = 7; cagE $^-$ 0.099 (0.019), n = 3; p = 0.25, unpaired t test). No obvious differences were demonstrated between cagE $^+$ and cagE $^-$ strains by real time PCR or immunohistochemistry. vacA genotyping of strains revealed 2 s1/m1, 6 s1/m2, 1 s2/m2, and 1 s2/m1 (for ELISA and real time PCR). Not surprisingly, given the small numbers, no significant differences in MMP-7 expression could be demonstrated.

To define precisely the role of bacterial virulence factors in induction of MMP-7 expression and to confirm the effects of $H$ pylori on MMP-7 expression by epithelial cells, we next performed a series of coculture experiments using a panel of $H$ pylori isogenic mutant strains and the epithelial cell line HT29. We selected the HT29 cell line because it is known to express MMP-7 in response to appropriate stimuli.10,11 Firstly, we analysed the effect of a pathogenic and a non-pathogenic $H$ pylori strain on MMP-7 expression. Immunoblot analysis of HT29 supernatants after $H$ pylori coculture showed an immunoreactive 29 kDa band (the predicted size for pro-MMP-7) after coculture with the cag positive vacuolating strain 60190 but no band following coculture with the cag negative non-vacuolating strain Tx30a, or for control cells without bacterial coculture. Next we defined the contribution of the vacuolating cytotoxin VacA and the cag PAI encoded type IV secretion system by comparing the effect on MMP-7 expression by HT29 cells of wild-type 60190 with the effects of its VacA$^-$ isogenic mutant (does not express full length or truncated VacA) and its CagE$^-$ isogenic mutant (does not express a functional cag encoded type IV secretion system). We showed that the VacA$^-$ mutant had identical effects to the parent strain, indicating that VacA was not necessary for the effect. In contrast, coculture with the CagE$^-$ mutant induced no MMP-7 expression, indicating that the effect was dependent on the cag PAI encoded type IV secretion system (fig 3A). To confirm these results, and to show that the 29 kDa proMMP-7 band had MMP-7 activity, we performed casein zymography with the same samples. Zymograms demonstrated caseinolytic activity at the same protein size, and increased activity was noted for strain 60190 and its VacA$^-$ isogenic mutant compared with the two strains lacking an intact cag pathogenicity island (Tx30a and 60190CagE$^-$) (fig 3B). Finally, to further confirm our results and to assess whether MMP-7 was upregulated at the level of transcription (as in our in vivo studies) we performed real time PCR on RNA preparations from cocultured HT29 cells. As expected, highest levels of MMP-7 mRNA expression were observed after coculture with the strains possessing an intact cag PAI, 60190, and 60190VacA$^-$ (fig 4).
DISCUSSION

We have demonstrated that *H pylori* infection causes upregulation of MMP-7 in epithelial cells, in vivo and in vitro, and that this is dependent on an intact *cag* pathogenicity island. MMP-7 is an important metalloproteinase enzyme that is upregulated in gastric cancer. It promotes tissue invasion and metastasis in various cancers through degradation of extracellular matrix. Interestingly, it has also recently been shown to have potential pro-oncogenic effects through its “sheddase” activity which may predispose to malignant transformation. Recently described substrates of MMP-7 sheddase activity include E-cadherin, Fas ligand, and pro-TNF-α. E-cadherin is an important cell adhesion molecule which forms a key part of the adherens junctions between epithelial cells. Changes in E-cadherin at the germline and somatic levels are associated with both intestinal and diffuse gastric cancer. Fas ligand has an important role in apoptosis, and one of the mechanisms by which *H pylori* is thought to predispose to gastric carcinogenesis is through disruption of the balance between cell proliferation and apoptosis. TNF-α is a proinflammatory cytokine that is induced in vivo by *H pylori*. Induction of a chronic inflammatory state predisposes to gastric atrophy and precancerous changes in the gastric epithelium. Activation or alteration of any of these important oncogenic or inflammatory molecules by *H pylori* potentially could be important in gastric carcinogenesis.

Our work using isogenic mutants clearly demonstrates a role for an intact *cag* pathogenicity island in the upregulation of MMP-7. The mechanism of upregulation of MMP-7 gene expression is likely to be complex as the promoter region is susceptible to a number of controls; MMP-7 transcription has been shown to be dependent on various signalling pathways, including β-catenin signalling and activation of the PEA3 member of the Ets transcription factors. Translocation and phosphorylation of CagA stimulates MAP kinases and the *cag* encoded type IV secretion system itself stimulates nuclear factor κB activation and interleukin 8 transcription. Further work is needed to define which pathways are responsible for *H pylori cag* mediated MMP-7 induction. Most of our patient strains were *cagE−* by PCR analysis, yet there was considerable heterogeneity in MMP-7 expression in biopsies, and so clearly factors other than *cag* status may be important in MMP-7 upregulation, and the in vivo situation may be different from that in vitro.

Several groups have examined MMP-7 expression in gastric cancer and reported conflictingly on expression of MMP-7 in normal (non-malignant) tissue. Using a well-defined monoclonal antibody raised against recombinant human MMP-7, we demonstrated clearly that MMP-7 is expressed in inflamed and to a lesser extent in non-inflamed non-malignant gastric mucosa, and we confirmed these findings by mRNA analysis. Importantly, for a potential mechanistic role in carcinogenesis, we have also shown that major differences exist in the proliferative zone between *H pylori* infected and uninfected patients. Effects on cells within this pluripotent area are likely to be necessary for carcinogenesis.

There is considerable evidence to support a role for MMP-7 in the very early stages of colorectal cancer. Induction of MMP-7 in *H pylori* induced gastritis, the initial stage in the proposed pathway to gastric adenocarcinoma, may be of pathogenic importance. Future strategies for treatment, and prevention, of gastric cancer may include use of inhibitors of metalloproteinase enzymes. Understanding what stimulates these oncogenic proinflammatory proteins adds to our current knowledge in this field. *H pylori* mediated stimulation of MMP-7 is a potentially important carcinogenic effect of the bacterium.

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