ORIGINAL ARTICLE

MMP-2 but not MMP-9 associated with COX-2 and survival in gastric cancer

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Background and aim: Matrix metalloproteinases (MMPs) MMP-2 and MMP-9 can degrade type IV collagen of extracellular matrix and basal membranes. As cyclo-oxygenase-2 (COX-2) has been shown to activate MMPs, creating one of the COX-2-promoted pathways of tumour growth and metastasis, the prognostic role of MMP-2 and MMP-9 in gastric cancer was assessed and their association with COX-2 expression was evaluated.

Materials and methods: Samples were collected from 342 consecutive patients operated on for gastric cancer, of which 315 were acceptable for MMP-2, MMP-9 and COX-2 immunohistochemistry. Specimens were stained with specific antibodies, evaluated and categorised by two interpreters, and then correlated with clinical data and survival.

Results: Epithelial MMP-2 immunoreactivity was associated with male sex, high stage, advanced penetration depth, non-curative surgery, high COX-2 expression and poor survival. Stromal MMP-2 expression correlated with high stage, intestinal type and non-curative surgery whereas MMP-9 correlated only with intestinal type. Stage, intent of surgery and COX-2 were independent prognostic factors.

Conclusions: Epithelial MMP-2 expression in gastric cancer is associated with aggressive forms, COX-2 and poor survival, although MMP-2 was not an independent prognostic factor. In gastric cancer tumour growth is apparently induced by COX-2, and invasion is mediated by MMP-2.

Degradation of extracellular matrix is a critical part of growth, invasion, angiogenesis and metastasis of malignant tumours. This is orchestrated by matrix metalloproteinases (MMPs), zinc-dependent proteolytic enzymes that cooperate and activate each other. A basic component of basal membranes and extracellular matrix, type IV collagen, is degraded by gelatinases A (MMP-2) and B (MMP-9).¹ MMP-2, in particular, is considered to have an important role in the initial steps of tumour invasion.² In various solid cancers MMPs are associated with grade, stage and survival.³ In gastric cancer MMP-2 and MMP-9 are linked to tumour invasion and metastasis as well as poor prognosis.^{4–6}

Cyclo-oxygenase-2 (COX-2) is an inducible enzyme associated with inflammation and carcinogenesis.^{7–8} Animal studies have shown that pharmacological inhibition or genetic deletion of COX-2 leads to reduced tumour formation in the rodent gastrointestinal tract,^{9–12} whereas expression of COX-2 directly induces tumour growth.¹³ In humans, COX-2 is overexpressed in non-invasive gastric dysplasias^{14–16} and in gastric cancer tissue.¹⁷ Moreover, several clinical studies show that COX-2 is associated with tumour invasion and metastasis.^{18–28}

All the downstream cellular effects of COX-2 are not yet established. COX-2-derived prostanoids may, however, induce production of MMPs, and in tissue culture experiments COX-2 inhibitors may reduce synthesis of active MMPs.^{29–31} In addition, inhibition of COX-2 causes reduction in pro-MMP-2 expression in ovarian carcinoma cells,³² and MMP-9 expression is reduced in gastric cancer cells by the blocking of COX-2 with NS-398.³³ We reported that COX-2 expression is associated with MMP-2 expression in thyroid³⁴ and breast³⁵ cancers, suggesting that MMPs are linked to COX-2-mediated carcinogenesis. No association between COX-2 and MMP-2 or MMP-9 has, however, been reported in gastric cancer materials.

We have shown COX-2 to be an independent prognostic factor in gastric cancer.³⁶ In this study, we evaluated the prognostic significance of MMP-2 and MMP-9 expression in gastric cancer and also investigated by immunohistochemistry

whether any link exists between MMP-2 or MMP-9 and COX-2 expression in gastric cancer.

MATERIALS AND METHODS

Patients

The study includes 342 consecutive patients operated on for histologically confirmed gastric adenocarcinoma at the Department of Surgery, Helsinki University Central Hospital, between 1983 and 1999. Diagnosis and staging were performed according to the Union Internationale Contre Cancer classification (Tumour-Node-Metastasis, 1992). The local ethics committee gave its approval. Clinical and survival data were collected from patient records, the Finnish Cancer Registry and the Population Register Centre of Finland until February 2004, and entered into the database. Four patients were excluded owing to incomplete information: missing clinical data and unclear cause of death (one patient each), and missing follow-up (two patients). From the remaining 338 cases, we obtained 329 representative sections of tumour tissue for MMP-2 immunohistochemistry, 330 for MMP-9 and 318 for COX-2. All stainings were successful in 315 cases. The median age of patients was 66. Stages I and II were found in 137 (41%) patients, and III and IV in 196 (59%). Surgery for cure (R0 resection) was performed in 172 (52%) cases; the remaining operations were palliative. No patient received neoadjuvant treatment. The median follow-up time was 12.7 (range 4.7–20.8) years.

Immunohistochemistry

Immunostaining was performed separately for each antibody by a protocol similar to that described earlier.³⁴ In this study, we used MMP-2 mouse monoclonal antibody (A-Gel VC2, NeoMarkers, Lab Vision Corporation, Fremont, California, USA) at a dilution of 1:200, MMP-9 rabbit polyclonal

Abbreviations: COX-2, cyclo-oxygenase-2; MMP, matrix metalloproteinase

antibody (NeoMarkers) at a dilution of 1:5000 and COX-2 mouse monoclonal antibody (160 112, Cayman Chemical, Ann Arbor, Michigan, USA) at a dilution of 1:200.

For antigen retrieval, the sections were heated in a microwave oven in 0.3% citrate buffer (pH 6.0) for 4×5 min and washed with phosphate-buffered saline for 2×5 min. To block non-specific binding sites, the sections were treated with normal horse serum for 15 min. After inactivation of endogenous peroxidase activity by methanol containing 1.6% hydrogen peroxide and phosphate-buffered saline wash, primary antigens were inserted topically into each section at these dilutions and incubated overnight. For intensification of the primary antigen signals, the sections were first treated with biotinylated secondary antibody and then with avidin-biotinylated peroxidase complex for 30 min (Vectastain ABC Kits; Vector Laboratories, Burlingame, California, USA). Staining was visualised with 3-amino-9-ethyl-carbazole (A5754; Sigma, St Louis, Missouri, USA). Colorectal specimens served as positive controls, and sections incubated with phosphate-buffered saline instead of primary antibody as negative controls.

Scoring

MMP-2 immunoreactivity was scored separately for expression of epithelial and stromal cells in cancer tissue. The score was determined by the percentage of area positive for tumour. Absence of immunoreactivity was scored as 0, <10% positivity was scored 1, 10–70% was scored 2 and 70–100% was scored 3. The groups were classified as MMP-2-negative (score 0) and MMP-2-positive (scores 1–3). MMP-9 immunoreactivity was detected in the cytoplasm of the tumour cells and scored identically. Scoring of COX-2 was based on intensity and percentage positivity area. Two groups were created with low and high COX-2 staining.³⁶ Observers blinded to the study, with no information on clinicopathological characteristics, observed the staining. Two interpreters (JM and SN) scored MMPs by consensus and (JM and AR) scored COX-2 independently.

Statistical analysis

Fisher's exact test or the χ^2 test was used for calculating correlations between MMP-2, MMP-9 and COX-2 scores, and for clinicopathological factors. Univariate survival analysis

Table 1 Association of MMP-2 with clinicopathological variables in 329 patients with gastric adenocarcinoma

Clinicopathological variable	n	Epithelial MMP-2 immunoreactivity n (%)	p Value	Stromal MMP-2 immunoreactivity n (%)	p Value
Age (years)			0.10		0.73
<50	45	7 (16)		36 (80)	
50–59	63	13 (21)		45 (71)	
60–69	88	24 (27)		63 (72)	
≥70	133	43 (32)		98 (74)	
Sex			0.028		0.76
Female	158	33 (21)		115 (73)	
Male	171	54 (32)		127 (74)	
Stage			0.034		0.027
IA	50	4 (8)		32 (64)	
IB	44	10 (23)		29 (66)	
II	39	13 (33)		28 (72)	
IIIA	67	20 (30)		56 (84)	
IIIB	29	10 (34)		17 (59)	
IV	100	30 (30)		80 (80)	
Penetration depth			0.002		0.055
T1	56	4 (7)		36 (64)	
T2	57	13 (23)		40 (70)	
T3	153	50 (33)		112 (73)	
T4	63	20 (32)		54 (86)	
Lymph node metastases			0.061		0.31
N0	145	33 (23)		101 (70)	
N1	94	22 (23)		70 (74)	
N2	89	32 (36)		70 (79)	
Data not available	1				
Distant metastases			0.34		0.20
M0	236	59 (25)		169 (72)	
M1	93	28 (30)		73 (78)	
Tumour location			0.40		0.65
Upper 1/3	65	19 (29)		49 (75)	
Middle 1/3	110	34 (31)		75 (68)	
Lower 1/3	126	28 (22)		96 (76)	
Diffuse	20	3 (15)		15 (75)	
Stump	6	2 (33)		5 (83)	
Data not available	2				
Laurén classification			0.10		0.002
Intestinal	142	44 (31)		92 (65)	
Diffuse	187	34 (23)		150 (80)	
Tumour size median (cm)			0.080		0.60
≤5	178	41 (23)		128 (72)	
>5	145	46 (32)		108 (74)	
Data not available	6				
Resectability			0.011		0.002
Intent to cure	168	35 (21)		112 (67)	
Non-curative	143	48 (34)		117 (81)	
Data not available	18				
COX-2			0.0001		0.70
Low	142	22 (15)		106 (75)	
High	176	61 (35)		128 (73)	
Data not available	11				

COX-2, cyclo-oxygenase-2; MMP, matrix metalloproteinases.

Table 2 Association of MMP-9 with clinicopathological variables in 330 patients with gastric adenocarcinoma

Clinicopathological variable	n	Epithelial MMP-9 immunoreactivity n (%)	p Value
Age (years)			0.17
<50	46	9 (20)	
50–59	62	8 (13)	
60–69	89	17 (19)	
≥70	133	13 (10)	
Sex			0.84
Female	159	22 (14)	
Male	171	25 (15)	
Stage			0.80
IA	51	7 (14)	
IB	46	9 (20)	
II	39	6 (15)	
IIIA	67	7 (10)	
IIIB	28	5 (18)	
IV	99	13 (13)	
Penetration depth			0.67
T1	58	7 (12)	
T2	57	11 (19)	
T3	152	21 (14)	
T4	63	8 (13)	
Lymph node metastases			0.077
N0	148	26 (18)	
N1	94	7 (7)	
N2	87	14 (16)	
Data not available	1		
Distant metastases			0.97
M0	238	34 (14)	
M1	92	13 (14)	
Tumour location			0.67
Upper 1/3	69	10 (14)	
Middle 1/3	108	16 (15)	
Lower 1/3	125	16 (13)	
Diffuse	20	2 (10)	
Stump	6	2 (33)	
Data not available	2		
Laurén classification			0.005
Intestinal	142	29 (20)	
Diffuse	188	18 (10)	
Median tumour size (cm)			0.43
≤5	180	28 (16)	
>5	144	18 (12)	
Data not available	6		
Resectability			0.91
Intent to cure	171	25 (15)	
Non-curative	141	20 (14)	
Data not available	18		
COX-2			0.75
Low	142	20 (14)	
High	176	27 (15)	
Data not available	12		

COX-2, cyclo-oxygenase-2; MMP, matrix metalloproteinase.

was assessed by the Kaplan–Meier method; and significance of difference between groups was analysed using log rank test or log rank test for trend. Gastric-cancer-specific survival was calculated from the date of diagnosis to death from gastric cancer, or until the last day of follow-up. Deaths from causes other than gastric cancer were censored. Stepwise multivariate survival analysis was performed by the Cox proportional hazards model. Covariates assessed in the model were stage, resectability, epithelial MMP-2, stromal MMP-2, MMP-9, COX-2, age, sex, Laurén classification and tumour size and location. A p value <0.05 was considered significant and all p values were two sided.

RESULTS

Immunoreactivity

Of the 329 specimens, epithelial MMP-2 immunoreactivity was positive in 87 (26%) specimens and negative in 242 (74%). The corresponding figures for stromal MMP-2 immunoreactivity were 242 (74%) and 87 (26%). Of 330

Figure 1 Positive epithelial matrix metalloproteinases-2 immunoreactivity associated with poor survival (n=329, p=0.008).

Table 3 Univariate analysis of the relationship between preoperative characteristics and survival in 333 patients with gastric cancer

Clinicopathological variable	Patients	Cumulative 5-year survival (%)	χ^2	p Value
Epithelial MMP-2			7.05	0.008
Negative	242	37		
Positive	87	27		
Data not available	4			
Stromal MMP-2			1.55	0.21
Negative	87	40		
Positive	242	33		
Data not available	4			
Epithelial MMP-9			0.03	0.87
Negative	283	35		
Positive	47	32		
Data not available	3			
Age (years)			9.06	0.029
<50	46	27		
50–59	63	50		
60–69	89	37		
≥70	135	27		
Sex			1.41	0.23
Female	161	38		
Male	172	31		
TNM stage			218.45	<0.0001
IA	51	96		
IB	46	73		
II	40	38		
IIIA	67	15		
IIIB	29	7		
IV	100	3		
Penetration depth			137.13	<0.0001
T1	58	86		
T2	58	60		
T3	154	18		
T4	63	2		
Lymph node metastases			172.35	<0.0001
N0	148	66		
N1	95	14		
N2	89	4		
Data not available	1			
Distant metastases			133.31	<0.0001
M0	240	46		
M1	93	4		
Tumour location			28.30	<0.0001
Upper 1/3	69	22		
Middle 1/3	110	47		
Lower 1/3	126	37		
Diffuse	20	0		
Stump	6	0		
Data not available	2			
Laurén classification			0.04	0.84
Intestinal type	142	34		
Diffuse type	191	34		
Median tumour size (cm)			63.36	<0.0001
≤5	181	52		
>5	146	12		
Data not available	6			
Resectability			189.21	<0.0001
Intent to cure	172	60		
Non-curative	143	3		
Data not available	18			
COX-2			53.04	<0.0001
Low	139	54		
High	176	16		
Data not available	18			

COX-2, cyclo-oxygenase-2; MMP, matrix metalloproteinase; TNM, tumour node metastasis.
 χ^2 and corresponding p values calculated by log rank test or log rank test for trend.

specimens, MMP-9 staining was positive in 47 (14%) and negative in 283 (86%).

Associations of MMP-2 and MMP-9 with clinicopathological characteristics and COX-2

Epithelial MMP-2 expression was associated with male sex, high stage, advanced penetration depth and non-curative surgery, whereas stromal MMP-2 expression was associated with advanced stage, diffuse-type and non-curative surgery

(table 1). MMP-9 immunoreactivity correlated only with intestinal type of cancer (table 2). A positive correlation appeared between epithelial MMP-2 expression and high COX-2 expression ($p<0.0001$). No other correlations existed between the tested markers ($p>0.26$).

Survival analysis

Epithelial MMP-2 expression was associated with poor prognosis ($p = 0.008$). Stromal MMP-2 expression or positive

Table 4 Stepwise multivariate analysis of prognostic covariates of survival in 304 patients with gastric cancer

Covariate	p Value	HR	95% CI
TNM stage	<0.0001		
IA		1.00	
IB	0.0065	7.92	1.79 to 35.15
II	0.0002	16.52	3.84 to 71.00
IIIA	<0.0001	28.72	6.82 to 120.93
IIIB	<0.0001	38.90	8.52 to 177.67
IV	<0.0001	47.82	10.82 to 211.33
Resectability	0.0042	1.96	1.24 to 3.22
COX-2	0.0031	1.60	1.17 to 2.18
Epithelial MMP-2	NS		
Stromal MMP-2	NS		
Epithelial MMP-9	NS		
Age	NS		
Sex	NS		
Laurén classification	NS		
Tumour size	NS		
Tumour location	NS		

COX-2, cyclo-oxygenase-2; HR, hazard ratio; NS, non-significant; MMP, matrix metalloproteinases; TNM, tumour node metastasis.
Age and tumour size analysed as continuous variables. Other variables are coded as in table 3.

MMP-9 expression did not correlate with survival (fig 1). Other prognostic factors were old age, high stage, advanced penetration depth, presence of nodal or distant metastases, large tumour size, non-curative surgery and high COX-2 expression (table 3). In multivariate analysis, stage, intent of surgery and COX-2 were independent prognostic factors (table 4).

DISCUSSION

We report that in gastric cancer epithelial MMP-2 expression was associated with high COX-2 expression, but no correlation appeared between stromal MMP-2 or MMP-9 and COX-2. Our earlier data showed COX-2 to be an independent prognostic factor in gastric cancer and to be associated with several clinicopathological factors.³⁶ A substantial quantity of in vitro data suggest that COX-2 activates MMPs, and that the tumour growth-, invasion- and metastasis-enhancing properties of COX-2 are partially mediated by MMPs.^{29–33} In renal cell carcinoma, MMP-2 expression and COX-2 expression show a positive correlation.³⁷ We previously reported COX-2 to be associated with MMP-2 in thyroid³⁴ and breast³⁵ cancers. To our knowledge, this is the first report of a correlation between MMP-2 and COX-2 in clinical gastric cancer. Thus, enhancement of tumour growth and infiltration may be the result of activation of MMP-2 by COX-2 also in gastric cancer. We failed to show any association between COX-2 and MMP-9, although a combination of MMP-9 and COX-2 expression has shown prognostic value in adenocarcinoma of the lung.³⁸

We also report that epithelial MMP-2 expression is associated with several clinicopathological factors and poor survival, although multivariate analysis showed no independent prognostic value. Stromal MMP-2 correlated with stage, diffuse type and resectability, whereas MMP-9 correlated only with intestinal type of the tumour. The first reports of MMP expression in gastric cancer detected by immunohistochemistry showed that MMP-2 expression was more frequent in patients having advanced penetration depth, or dying of gastric cancer.⁴ Sier *et al*,³⁹ measuring MMP-2 and MMP-9 levels in gastric cancer by zymography, found no correlations with clinical parameters, but both MMP-2 and MMP-9 were independent prognostic factors in their 50-patient material. In concordance with our results, Murray *et al*⁴⁰ reported that MMP-9 is correlated with the intestinal type of tumour, but, they found no correlation of any MMP with stage or survival.

In our study, epithelial MMP-2 expression was positive in 59% of nodal-positive (N1+N2) patients and in 23% of nodal-negative patients (N0). Although no significant difference was found ($p = 0.061$), a trend was apparent. No correlation existed between MMPs and distant metastases. In other clinical studies, MMP-2 and MMP-9 expression has been linked with stage and metastasis,^{41–42} or even with poor survival, but in a non-independent manner, if tested multivariately.^{43–45} These studies support our findings that epithelial MMP-2 expression is more frequent in aggressive gastric cancers, whereas the clinical relevance of MMP-9 or stromal MMP-2 expression is less evident. In multivariate analysis, the independent prognostic factors were stage, intent of surgery and COX-2, but neither of the MMPs, thus proving the prognostic impact of MMPs in gastric cancer to be modest.

In conclusion, because epithelial MMP-2 expression was associated with advanced stage and poor survival in gastric cancer and epithelial MMP-2 expression was linked to high COX-2 expression, we suggest that in gastric cancer, COX-2-induced enhancement of tumour growth and infiltration is partly mediated by MMP-2.

Take-home messages

- We have previously shown that COX-2 is an independent prognostic factor in gastric cancer. In this study, we detected immunohistochemical expression of MMP-2 and MMP-9. Epithelial expression of MMP-2 is associated with poor survival and several clinicopathological parameters. MMP-9 or stromal MMP-2 expression showed no prognostic value.
- In multivariate survival analysis, none of the MMPs except stage, intent of surgery and COX-2 was an independent prognostic factor, revealing that the prognostic role of MMPs in gastric cancer is modest.
- Epithelial MMP-2 expression correlated with high COX-2 expression ($p < 0.0001$), suggesting that COX-2 facilitates tumour growth partly via MMP-2 in gastric cancer.

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