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Clinical evidence for a protective role of lipocalin-2 against MMP-9 autodegradation and the impact for gastric cancer

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A B S T R A C T

Recently, complexes of matrix metalloproteinase matrix metalloproteinase-9 (MMP-9) with lipocalin-2 (neutrophil gelatinase-associated lipocalin) were found in the urine obtained from breast cancer patients, while these were completely absent in that obtained from healthy controls. *In vitro* data suggested a possible role for lipocalin-2 in the protection of MMP-9 against autolysis. To establish this effect *in vivo*, we determined the presence of MMP-9, lipocalin-2 and their complex in tumour tissue from 81 gastric cancer patients. The effect of the presence of the individual parameters, the complexes, and the inhibitors TIMP-1 and TIMP-2 on MMP-9 activity was evaluated with a bioactivity assay. Immuno-histochemical (double) staining identified epithelial cells as the most likely cellular source. Finally, evaluation of all these parameters with clinicopathological scores revealed that tumour MMP-9/lipocalin-2 complexes were significantly related with the classifications of Laurén and WHO, and highly associated with worse survival in Cox's univariate (HR 2.087, $P = 0.006$) and multivariate analyses (HR 2.095, $P = 0.025$).

1. Introduction

Lipocalin-2 (also known as neutrophil gelatinase-associated lipocalin) is a member of the highly heterogeneous family of lipocalins, sharing a common tertiary structure.[1,2] Lipocalin-2 has initially been discovered in specific granules of human neutrophils[3] and was later shown to be expressed also by certain epithelial cells, in particular during inflammatory or cancerous circumstances.[4–10] There is little information about the physiological functions of lipocalins, but lipocalin-2 has been associated with cellular iron uptake, antibacterial activity and epithelial cell differentiation.[2,9]

Enhanced tissue, blood and urine levels of matrix metalloproteinase-9 (MMP-9) have been associated with the malignancy of various tumour types.[11–14] Using quantitative zymography and immunoassays, we have previously shown that MMP-9 as well as MMP-2 are enhanced in gastric cancer tissue and that high levels are associated with worse survival of the patients.[15,16] Next to MMP-9 and MMP-2, the zymo-

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grams revealed extra bands, particularly between 125 and 135 kDa. These bands have been described before in the urine obtained from cancer patients, and are most likely complexes of MMP-9 with lipocalin.[17,18] *In vitro* experiments suggested a role for lipocalin-2 in the protection of MMP-9 against autolysis.[17]

To investigate the suggested relevance of MMP-9/lipocalin-2 complexes *in vivo*, we determined the levels of MMP-9, lipocalin-2 and their complex in tissue homogenates from 81 gastric carcinomas in comparison with adjacent normal mucosa from the same patients. We used immunohistochemical staining of paraffin-embedded tissue sections to establish the cellular origin of MMP-9 and lipocalin-2. To confirm the histological findings, the levels of MMP-9, lipocalin-2 and the MMP-9/lipocalin-2 complexes in the homogenates were compared with markers for neutrophils, a known source of MMP-9 and lipocalin-2. The effect of complex formation between MMP-9 and lipocalin-2 on the MMP-9 activity state was evaluated using a specific MMP-9 bioactivity assay. Finally, the possible clinical consequence of the presence of MMP-9/lipocalin-2 complexes in gastric tumours was evaluated by examining for correlations with established clinico-pathological parameters of the carcinoma patients, including univariate and multivariate Cox proportional hazard survival analyses.

2. Materials and methods

2.1. *Patients and study design*

Fresh tissue specimens from 81 patients (21 females and 60 males, mean age 65.9 years, range 35.1–91.3) who underwent resection for primary gastric adenocarcinoma between 1984 and 1996 at the Department of Oncologic Surgery, Leiden University Medical Centre were collected prospectively. Samples from the mid-central non-necrotic part of the carcinoma and from normal mucosa, taken approximately 10 cm from the tumour, were snap-frozen and stored at −70 °C until extraction. All carcinomas were classified according to the TNM classification (UICC 1992), and localisation as well as diameter of the tumour was registered. Microscopical histological parameters, including differentiation-grade, WHO-, Borrmann- and Laurén-classification, as well as the presence of intestinal metaplasia in the normal gastric mucosa, were revised by a gastroenterologist and a pathologist. All patients entered the study at operation date, and the patient's time experience ended in the event of death or, when still alive, at the common closing date. The minimal follow-up was 33 months with a decreasing overall survival according to TNM stage, i.e. from TNM I (52.2%, $n = 23$), to TNM II (26.9%, $n = 26$), to TNM III (28%, $n = 25$) and to TNM IV (0%, $n = 7$). The study was performed according to the instructions and guidelines of the LUMC medical ethics committee.

2.2. *Tissue preparation and protein concentration*

Homogenisation of tissue specimens and determination of protein concentrations were performed as described previously.[15]

2.3. *MMP-9/lipocalin-2 complex zymography*

Quantitative gelatin zymography for MMP-9/lipocalin-2 complexes was performed as described before,[15] using an Ultroscan XL Laser Densitometer (LKB) for quantification. The MMP-9/lipocalin-2 complex levels in tissue homogenates were expressed in arbitrary units (AU) per mg protein.

2.4. *Enzyme-linked immunosorbent assays (ELISAs) for MMP-9, lipocalin-2, MMP-9/lipocalin-2-complexes, MMP-8 and TIMPs*

Total antigen levels of MMP-9, lipocalin-2 and MMP-8 were determined using the previously described ELISAs.[19–22] The concentrations of MMP-9/lipocalin-2 complexes, TIMP-1 and TIMP-2 were measured using commercial ELISAs according to the manufacturer's instructions (R&D Systems Europe, Abingdon, UK). The MMP-9/lipocalin-2 ELISA immobilises complexes via anti-MMP-9 antibodies followed by detection using anti-lipocalin-2 antibodies and does not detect MMP-9 or lipocalin-2 in their free forms.

2.5. *MMP-9 activity assay*

The bioactivity assay (BIAs) for MMP-9 was done as described previously.[14,19,22] This assay detects active MMP-9 and total MMP-9 levels in parallel in 96-wells plates coated with MMP-9 specific antibodies and using modified MMP-sensitive pro-urokinase as substrate. The fraction of the latent MMP-9 proform is calculated by subtraction of active from total MMP-9.

2.6. *Myeloperoxidase (MPO) activity assay*

MPO activity was measured as described previously.[23] In short, tissue homogenates were incubated with 0.5% hexadecyl-trimethylammonium bromide in 50 mM potassium phosphate buffer (pH 5.5), plus 0.026% *ortho*-dianisidine dihydrochloride substrate and 0.018% H_2O_2. The reaction kinetics were followed for 30 min at 450 nm in 96-well plates. The specificity of the reaction was checked with sodium azide (0.1 mM). All samples were analysed in duplicate and standardised using a homogenate of pooled human neutrophils, and MPO activity was expressed in arbitrary units.

2.7. *Immunohistochemistry and immunofluorescence double staining*

Paraffin sections (5 μm) from the same tumours as used for the homogenates were deparaffinised and stained for the localisation of MMP-9 and lipocalin-2. Antigen retrieval was performed through boiling in a 0.01 M citrate solution (pH 6.0) for 12 min in a microwave oven. After being rinsed in phosphate-buffered saline (PBS) and incubated with 10% of normal goat serum (Dako) for 30 min, the sections were incubated with the primary antibody polyclonal rabbit anti-lipocalin-2 (1:100, from Drs. H. Tschesche and O. Hiller) or polyclonal rabbit anti-MMP-9 (1:400, TNO, Leiden, The Netherlands) overnight at 4 °C. After washing, the sections were incubated with biotinylated goat anti-rabbit 1:400 (Dako) for

30 min, followed by washing and incubation with Streptavidin/ABCcomplex/HRP (DakoCytomation) for 30 min. The brown colour was developed by 0.004% H_2O_2 (Merck) and 0.05% diaminobenzidine tetrahydrochloride (Sigma) in 0.01 M Tris–HCl, pH 6.0, for 10 min. The slides were counterstained with Mayer's haematoxylin (Merck). For specific cell recognition, i.e. epithelial cells, (myo)fibroblasts, neutrophils and endothelial cells, sequential tissue sections were stained with mouse anti-pan-cytokeratin (1:1000, clone C11, Santa Cruz biotechnologies, Santa Cruz, USA), mouse anti-vimentin (1:400, clone V9 Santa Cruz), mouse anti-smooth muscle actin (1:1000, clone ASM-1, Progen Heidelberg, Germany), rabbit anti-myeloperoxidase (1:1000, Dako) and mouse anti-CD31 (1:400, clone JC70A, Dako) followed by appropriate second antibodies and staining procedures. Immunofluorescence double staining was performed as described before.[24] In short, sections were incubated for 1 h with rabbit polyclonal anti-lipocalin-2 and mouse monoclonal anti-MMP-9 (clone GE-213, 1:400, NeoMarkers, Fremont, CA) antibodies, appropriately diluted in PBS with 1% bovine serum albumin (BSA), washed and incubated with, respectively, Alexa Fluor 488- and 546-conjugated anti-rabbit and anti-mouse antibodies (Molecular Probes, Leiden, The Netherlands) diluted in PBS-BSA. After incubation and washing, the sections were mounted in Mowiol. A Zeiss LSM 510 confocal microscope equipped with argon and He/Ne lasers and a 20× objective were used to obtain the images.

2.8. *Statistical analysis*

Differences between normal and tumour values for all parameters were calculated using the Wilcoxon signed ranks test and visualised by Box–Whisker graphs using lower and upper margins of 5%. Correlations between parameters were determined according to Spearman's ρ test. For the survival analyses the clinico-pathological parameters were dichotomised as described previously,[15] unless indicated. Optimised cut off values or median values for MMPs and related factors were used. Survival analyses were performed with the Cox proportional hazards model using the SPSS Windows Release 12.0.1. Statistical Package (2004, SPSS Inc., Chicago, Illinois,USA). Multivariate survival analyses were performed using the Cox proportional hazards method by separately adding the significant MMP variables to the dichotomised clinico-pathological parameters. Survival curves were constructed using the method of Kaplan and Meier, including the Log-rank test. Differences were considered significant when $P \leqslant 0.05$.

3. Results

3.1. *Quantification of MMP-9/lipocalin-2 complexes in gastric cancer tissue homogenates*

The presence of MMP-9/lipocalin-2 complexes in tissue homogenates from gastric cancer patients was determined using zymography and ELISA. Fig. 1 shows a typical gastric cancer homogenate within the zymogram abundant MMP-9 mediated lysis and a smaller band at molecular weight 135 kDa, corresponding with standard MMP-9/lipocalin-2 complex. The nature of this band was further verified using immunoblots for, respectively, MMP-9 and lipocalin-2 under normal (Fig. 1) and reduced conditions (not shown). The amount of the MMP-9/lipocalin-2 complexes was quantified

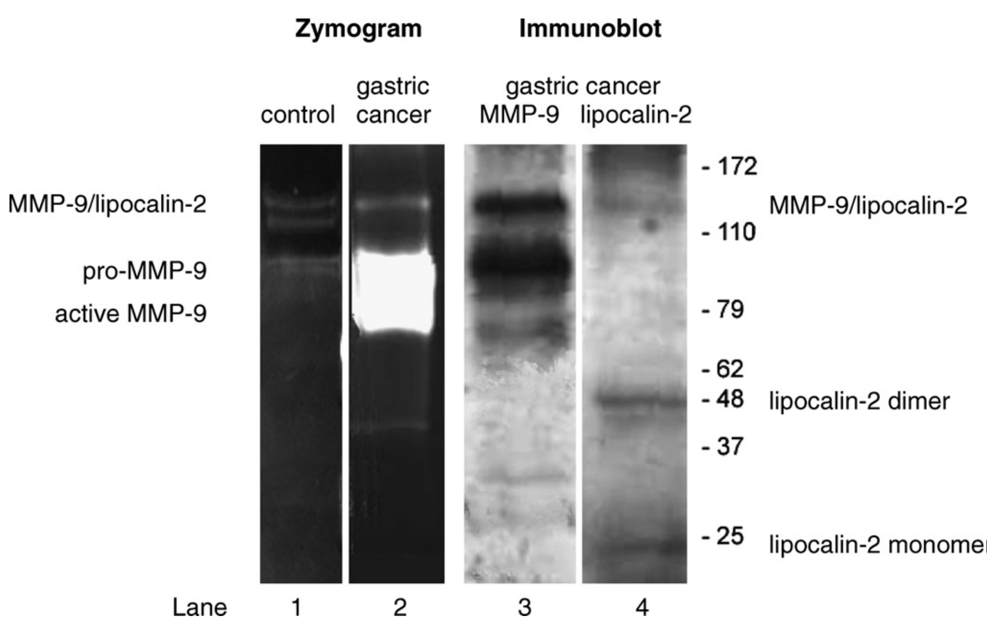

Fig. 1 – Zymogram and immunoblot showing MMP-9, MMP-9/lipocalin-2 complexes and lipocalin-2 in a representative gastric cancer tissue homogenate (lanes 2–4). MMP-9 activity is located in the zymograms (lane 2) between 70 and 92 kDa, representing active MMP-9 and pro-MMP-9, and at 135 kDa corresponding with MMP-9/lipocalin-2 complex standard (lane 1). The immunoblots show corresponding complex bands for MMP-9 (lane 3) and lipocalin-2 (lane 4) with extra bands at approximately 25 and 50 kDa representing, respectively, the monomer and homodimer forms of lipocalin-2. Lane 1 contains 20 μl standard from the MMP-9/lipocalin-2 ELISA (≈0.8 ng).

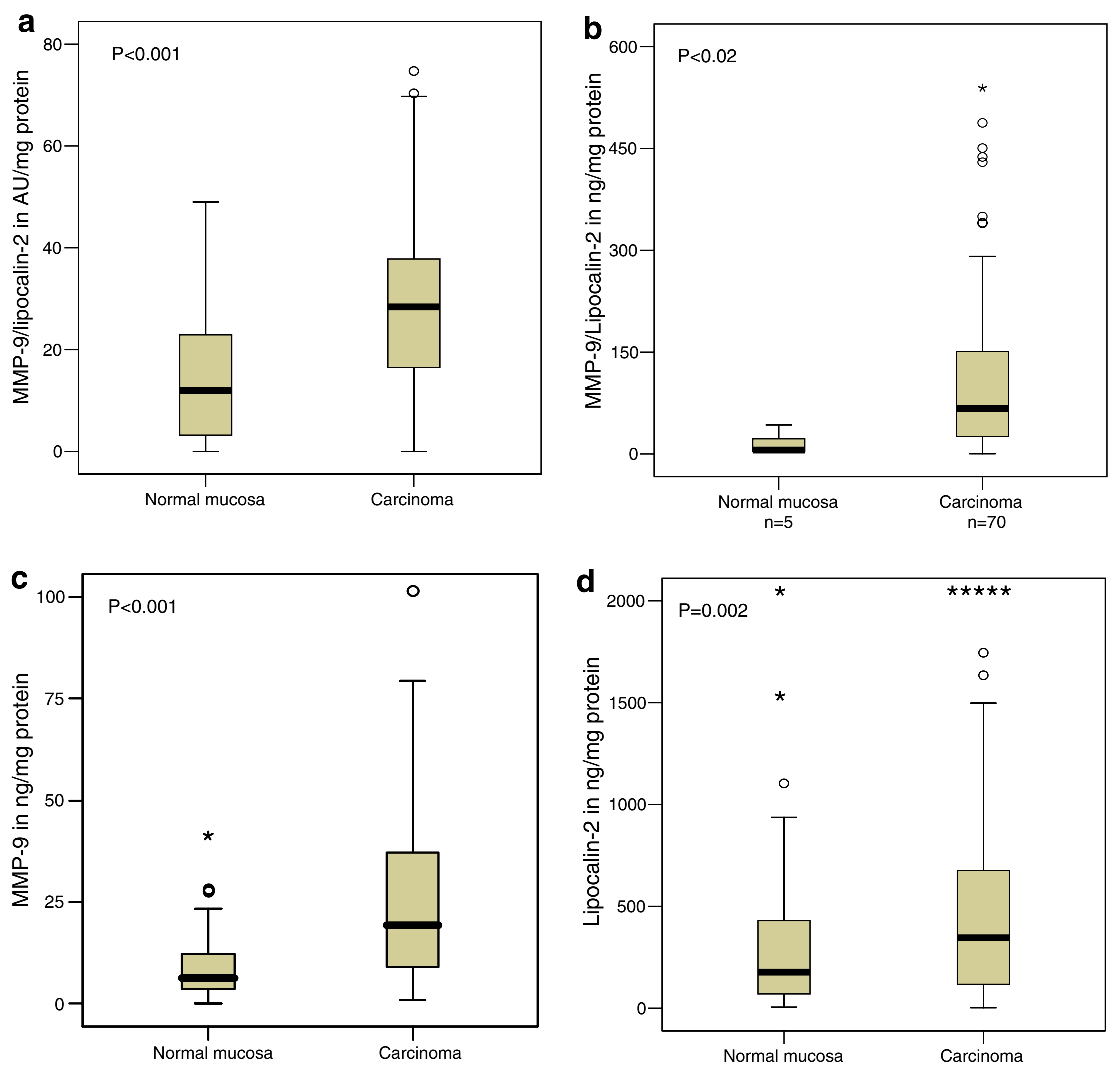

Fig. 2 – Levels of (a) MMP-9/lipocalin-2 complex in AU/mg protein, (b) MMP-9/lipocalin-2 complex in ng/mg protein, (c) MMP-9 in ng/mg protein, and (d) lipocalin-2 in ng/mg protein in carcinoma tissue and adjacent normal mucosa from 81 gastric cancer patients. *n* = 81 unless indicated.

from the zymograms, using laser densitometry (Fig. 2a). MMP-9/lipocalin-2 complexes were significantly enhanced in cancer tissue compared with control mucosa (27.3 ± 2.0 versus 14.5 ± 1.4 AU/mg protein, $P < 0.001$, $n = 81$). The data from this semi-quantitative assay were compared with the results obtained with a commercial ELISA (Fig. 2b). The correlation between both assays was highly significant (rho = 0.488, $P < 0.0001$, $n = 75$, i.e. 5 normal mucosa and 70 carcinoma homogenates).

3.2. *Levels of MMP-9 and lipocalin-2 in gastric cancer tissue homogenates*

The tissue levels of MMP-9 and lipocalin-2 are shown in Fig. 2c and d. The gastric carcinomas contained significant higher concentrations of MMP-9 ($P < 0.001$) and lipocalin-2 ($P = 0.002$) than adjacent normal tissues. In general, lipocalin-2 was more abundantly present than MMP-9, in specific cases even more than 100 times higher.

3.3. *Correlation between MMP-9 and MMP-9/lipocalin-2 with MMP-9 activity state*

The correlation of MMP-9, lipocalin-2 and MMP-9/lipocalin-2-complex with MMP-9 activity in tissue homogenates of gastric cancer patients is shown in Table 1. Active MMP-9 levels correlated significantly with the total antigen level of MMP-9, but more interestingly also with the MMP-9/lipocalin-2 concentration ($P = 0.038$), suggesting a protective role for lipocalin-2-complex formation in MMP-9 (auto)activation. The tissue concentration of TIMP-1, the most relevant tissue inhibitor of MMP-9, was equally correlated with the levels of MMP-9 and lipocalin-2, but not with MMP-9 activity.

3.4. *Immunohistochemical staining for MMP-9 and lipocalin-2*

To establish the cellular source of the MMP-9/lipocalin-2 complexes, sequential paraffin sections adjacent to the tissue

Table 1 – Correlation coefficients (ρ plus P-values) for MMP-9, lipocalin-2 and MMP-9/lipocalin-2 complexes in relation to myeloperoxidase (MPO), MMP-8 and TIMP-1 in 162 gastric cancer tissue homogenates (81 normal/81 cancer)

	MMP-9	NGAL	MMP-9/Lipocalin-2 complex	MMP-9 active	MMP-9 latent
MMP-9 (ng/mg protein)		0.438 (0.000)	0.641 (0.000)	0.240 (0.003)	0.817 (0.000)
Lipocalin-2 (ng/mg protein)			0.273 (0.001)	−0.121 ns	0.443 (0.000)
MMP-9/Lipoc-2 (AU/mg protein)				0.166 (0.038)	0.586 (0.000)
MMP-9 active (U/mg protein)					0.263 (0.001)
MPO (AU/mg protein)	0.486 (0.000)	0.280 (0.000)	0.332 (0.000)	0.073 (ns)	0.462 (0.000)
MMP-8 (ng/mg protein)	0.810 (0.000)	0.482 (0.000)	0.578 (0.000)	0.128 ns	0.734 (0.000)
TIMP-1 (ng/mg protein)	0.358 (0.000)	0.363 (0.000)	0.315 (0.000)	−0.097 (ns)	0.240 (0.004)

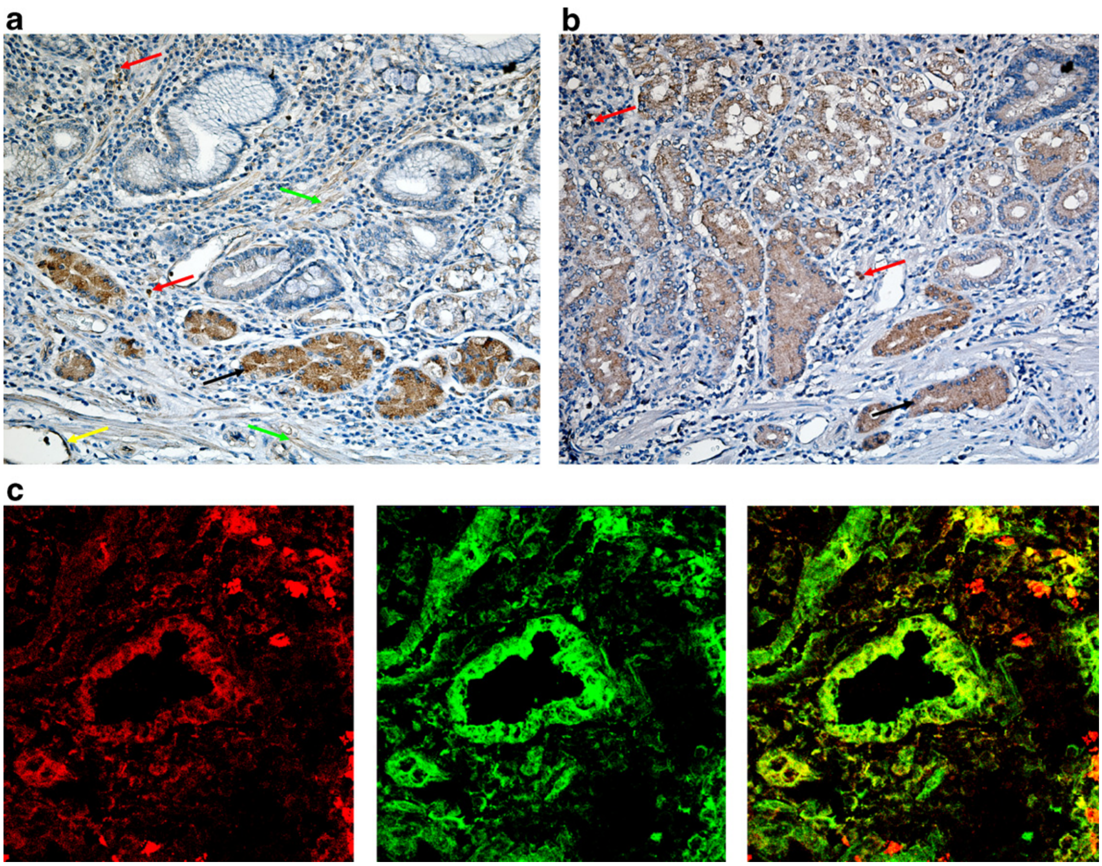

Fig. 3 – Typical immunohistochemical staining of a human gastric intestinal type carcinoma for: (a) MMP-9 (200×) and (b) lipocalin-2 (200×). Black, red, green and yellow arrows indicate, respectively, epithelial cells, neutrophil-like cells, (myo)fibroblast like cells and endothelial cells. Protein levels in corresponding homogenate for MMP-9, lipocalin-2 and complex are, respectively, 29 ng/mg, 4928 ng/mg and 17 AU/mg protein. (c) Immunofluorescence double staining (400×) for MMP-9 (red) and Lipocalin-2 (green). Yellow colour suggests complex formation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

used for homogenates were stained for MMP-9 and lipocalin-2. Normal mucosa showed barely any staining for MMP-9 nor lipocalin-2 (not shown). In carcinoma tissues staining for MMP-9 was found in neutrophils and a substantial part of the epithelial cells, occasionally in endothelial cells, and incidentally in muscle cells, macrophages and fibroblasts (Fig. 3a). In neutrophils and epithelial cells lipocalin-2 was similarly distributed compared with MMP-9, but lipocalin-2 was additionally present in tumour epithelial subgroups which lacked MMP-9 staining (Fig. 3b). Endothelial cells and fibroblasts showed little or no staining for lipocalin-2. Immunofluorescence double staining confirmed that particular epithelial cells stained for lipocalin-2 but not for MMP-9 (Fig. 3c, red versus green[d]). Furthermore, this staining revealed that only a fraction of MMP-9 and lipocalin-2 was actually in close proximity (Fig. 3c, yellow versus green[d]). Yellow staining was found in particular at the periphery of cells, suggesting that the majority of both proteins is uncomplexed and presumably still compartmentalised within the cells, as suggested by zymographic analysis.

[d] For interpretation of the references to colour in Fig. 3, the reader is referred to the web version of this article.

3.5. *Correlations between MMP-9, lipocalin-2, MMP-9/lipocalin-2, MMP-8 and MPO*

To confirm the similarities and the apparent difference between MMP-9 and lipocalin-2 in cellular origin, as found by immunohistochemistry, the concentrations of MMP-9, lipocalin-2 and MMP-9/lipocalin-2-complex in the tissue homogenates were evaluated for correlations with the levels of MPO and MMP-8 (Table 1). MPO, a commonly used cell marker for neutrophils, correlated strongly with MMP-8, a collagenase abundantly present in neutrophils (0.445, $P < 0.0005$) as well as with MMP-9, but the correlation with lipocalin-2 was considerably less, suggesting a possible other source of lipocalin-2 than neutrophils only.

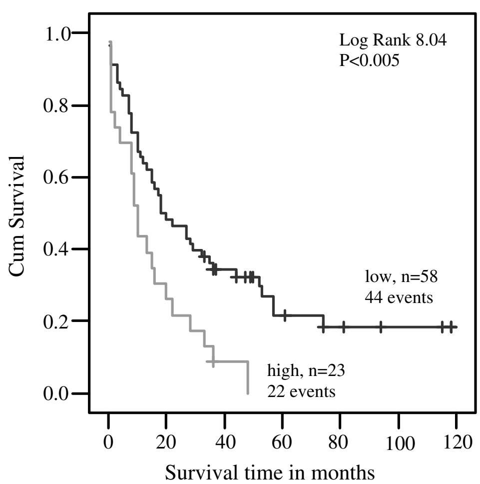

Fig. 4 – Kaplan–Meier survival curve for a cohort of gastric cancer patients subdivided by low (⩽36 AU/mg protein) or high (>36 AU/mg protein) levels of MMP-9/lipocalin-2 complex in their tumour tissue homogenate.

3.6. *Relation between MMP-9/lipocalin-2 complexes and clinico-pathological parameters*

The MMP-9/lipocalin-2 levels were significantly enhanced in differentiated tumours according to the WHO classification (30.9 ± 2.5 versus 19.6 ± 2.8 AU/mg protein, $P \leqslant 0.006$) and in tumours of the intestinal type (30.5 ± 2.6 versus 21.9 ± 2.7 AU/mg protein, $P \leqslant 0.04$). MMP-9/lipocalin-2 levels showed a trend to increase with higher TNM stages. Dichotomisation of the patients, based on low (AU < 36) or high (AU > 36) MMP-9/lipocalin-2 complex values in their tumour, showed a significant correlation with overall survival (Log Rank 8.04, $P < 0.005$, $n = 81$), as shown in Fig. 4. Analysis of the MMP-9/lipocalin-2 complex ELISA data showed a similar trend, but did not reach statistical significance (Log Rank, 3.04, $P = 0.0815$, $n = 70$).

3.7. *Survival analyses*

The relation of MMP-9/lipocalin-2 complexes with survival was further characterised with Cox's univariate and multivariate analyses against the clinico-pathological parameters (Table 2). The level of MMP-9/lipocalin-2 was significantly associated with worse survival and kept its significance in multivariate analyses, indicating its value as an independent prognostic factor.

4. Discussion

High levels of lipocalin-2 have been reported in various types of cancers.[6–10] Our study shows that lipocalin-2 levels are indeed significantly enhanced in gastric carcinomas compared to adjacent control tissue. Moreover and more interestingly, our data show that the complexes of lipocalin-2 with MMP-9 are also significantly enhanced in human gastric tumours.

In vitro experiments showed that lipocalin-2 is able to induce the expression of E-cadherin, to promote the formation of polarised epithelia, and to diminish the invasiveness and

Table 2 – Univariate and multivariate Cox proportional hazard overall survival analyses for low or high levels of MMP-9/lipocalin-2 in tissue homogenates of gastric cancer versus different clinico-pathological parameters

		n	Univariate			Multivariate		
			HR	CI 95%	*P*	HR	CI 95%	*P*
Gender	F/M	21/60	1.247	0.730–2.131	ns	1.622	0.900–2.923	ns
Age	⟨Median⟩	40/41	1.323	0.815–2.149	ns	1.504	0.860–2.629	ns
TNM	1	23/81	1	–	–	1	–	–
	2	26/81	1.984	1.033–3.813	0.040	2.133	1.009–4.639	0.047
	3	25/81	1.586	0.804–3.130	ns	1.623	0.737–3.704	ns
	4	7/81	3.248	1.261–8.366	0.015	6.027	1.876–20.46	0.003
Laurén	dif/mx versus inte	30/50	1.103	0.671–1.816	ns	1.125	0.402–3.137	ns
WHO	diff versus undiff	54/26	0.881	0.525–1.480	ns	0.874	0.289–2.609	ns
Borrmann	fung. versus infiltr.	55/24	1.025	0.591–1.778	ns	0.846	0.457–1.567	ns
Localisation	Cardia versus rest	36/45	0.603	0.368–0.989	0.045	0.419	0.223–0.764	0.005
Diameter	⩽5 versus >5 cm	47/34	1.062	0.652–1.729	ns	0.695	0.403–1.195	ns
Eosinophils	Few versus many	56/24	1.220	0.725–2.053	ns	1.846	1.023–3.544	0.042
Intestinal metaplasia	Not versus present	39/42	0.551	0.334–0.909	0.020	0.651	0.365–1.151	ns
MMP-9 antigen	⟨Median⟩	40/40	1.143	0.701–1.863	ns	1.336	0.756–2.363	ns
Lipocalin-2	⟨Median⟩	40/39	1.029	0.632–1.674	ns	0.772	0.422–1.413	ns
MMP-9/lipocalin-2	⩽36 versus >36 AU	58/23	2.087	1.229–3.544	0.006	2.095	1.099–4.031	0.025

metastasis of Ras-transformed cells,[25] suggesting a protective role against cancer. Other studies reported a positive correlation between lipocalin-2 expression levels and the growth rate of lipocalin-2 transfected MCF-7 human breast carcinoma cells, which were subcutaneously implanted in immuno-deficient mice.[18] Immunohistochemical analyses of these xenografted tumours showed that the over-expression of lipocalin-2 was accompanied by enhanced levels of MMP-9, suggesting the formation of complexes between MMP-9 and lipocalin-2. The formation of MMP-9/lipocalin-2 complexes has previously been shown to protect MMP-9 from auto-degradation *in vitro*.[17,18] MMP-9/lipocalin-2 complex formation could result in increased extracellular, tumour-associated MMP-9, and hence in enhanced tumour growth as recently suggested by Fernández et al.[18] We found that in gastric cancer tissue lipocalin-2 levels are in general 30 times higher than corresponding MMP-9 levels, presumably leading to MMP-9/lipocalin-2 complex formation of a substantial part of the MMP-9 fraction after it has been released from the cells. These complexes were significantly correlated with the active, as well as the latent fraction of MMP-9. Therefore, our data support the hypothesis that enhanced production of lipocalin-2 in cancerous tissue stimulates the formation of a complex with MMP-9, playing a role in the maintenance of an extracellular pool of a latent form of this powerful proteinase, by prevention from auto-degradation. This latent pool of secreted, lipocalin-2-bound MMP-9 has previously been shown to be important for the spatial control of VEGF release from the ECM and hence for enhanced angiogenesis.[26] Our study does not provide information about the presence and/or role of MMP-9/lipocalin-2/TIMP-1 complexes. These ternary complexes have previously been isolated from phorbol myristate acetate stimulated neutrophils and showed low gelatinase activity, as expected.[27] In our study, total TIMP-1 levels correlated significantly with all the forms of MMP-9, except for the active form of MMP-9, suggesting that other factors are involved in regulating the activity of MMP-9, besides the ratio between MMP-9 and TIMP-1. TIMP-2 levels were weakly inversely correlated with MMP-9 antigen levels, suggesting little or no mutual interaction (data not shown).

The quantitative determination of MMP-9 and lipocalin-2 in tissue homogenates, as performed in this study, has several advantages compared to semi-quantitative immunohistological detection methods but obviously does not provide information about the localisation of the proteins. Our immunohistochemical data revealed that lipocalin-2 as well as MMP-9 in gastric cancers is mainly present in neutrophils and epithelial cells, but that epithelial expression of MMP-9 is depending on the individual cancer and on the location within the tumour. MMP-9 was furthermore found in (myo)fibroblast-like cells and endothelial cells. These data are in accordance with what has been found previously in colonic cancer.[6,28] Our fluorescent double-staining data suggest that, although MMP-9 and lipocalin-2 seem to be present in close proximity especially within the cells, overlap of green and red colours, presumably representing extra-cellular complex formation, is limited and mainly restricted to peri-cellular areas. Whether the enhancement of MMP-9/lipocalin-2 complexes in gastric cancer compared with adjacent normal mucosa was caused by the influx of neutrophils or alternatively by upregulated expression in malignant epithelial cells could not be established in this study. The finding that high numbers of intra-tumoural neutrophils are associated with better survival of patients with gastric cancer[29] would suggest the latter.

From this study, the clinical relevance of MMP-9/lipocalin-2 complex formation appears most obvious from the correlation with overall survival of the patients. Enhanced levels of these complexes were highly prognostic for worse survival, whereas the levels of single MMP-9 and lipocalin-2 were not. The finding that MMP-9/lipocalin-2 levels are increased in gastric cancer tissue and that enhancement might be associated with clinical outcome of the patients is supported by a recent study reporting that similar complexes were present in approximately 90% of the urines obtained from breast cancer patients, but not in those from healthy controls.[18] The prognostic value of MMP-9/lipocalin-2 complexes is in accordance with the presumed role of lipocalin-2 in the protection of secreted MMP-9 against auto-degradation, which contributes to an enhanced pool of potentially active MMP-9, a proteolytic enzyme associated with angiogenesis and tumour growth. High total MMP-9 levels were not associated with survival in the present study. This is not in agreement with what we have published previously,[15] but those earlier data were based on a smaller group of patients and on detection of MMP-9 activity instead of total antigen level. The different outcome between both studies indicates the delicacy of the use of proteinase levels as prognostic indicators, as discussed before.[16,30] Apparently not just the enhanced presence, but more the (potential) activation state of the proteinase, i.e. the result of, respectively, production, release, activation, and the inactivation by inhibitors, seems to be crucial, similar to what has been described for other enzymes playing a role in gastric cancer like urokinase and MMP-2.[16,31] Additionally, our data indicate that prevention of auto-degradation of MMP-9 by lipocalin-2 might play an important role too.

In conclusion, we have shown for the first time that complexes between MMP-9 and lipocalin-2 are present in enhanced levels in gastric cancer tissue and that high levels are associated with worse survival of the patients. The potential clinical value of our findings should be confirmed in larger groups of cancer patients. Recently the enzymatic activity of MMP-9/lipocalin-2 complex has indeed been found to correlate significantly with the depth of tumour invasion in oesophageal squamous cell carcinomas.[32]

Conflict of interest statement

None declared.

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