Increased expression of matrix metalloproteinase-2 (MMP-2) predicts tumour recurrence and unfavourable outcome in non-small cell lung cancer

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Summary. The purpose of this study was to analyse the expression of matrix metalloproteinase-2 (MMP-2) and its extracellular matrix metalloproteinase inducer (EMMPRIN) in non-small cell lung cancer (NSCLC), and to evaluate their significance to predict tumour behaviour.

The study consists of 212 patients treated by the resection of the tumour. Tumour samples were stained immunohistochemically, and the expression of MMP-2 and EMMPRIN was evaluated both in tumour cells and in peritumoural stromal tissue. The results were compared with clinicopathological factors and survival of the patients.

High expression of MMP-2 in tumour cells was found in 83 out of 191 cases (44%). Adenocarcinomas showed more often high expression of MMP-2 as compared with squamous cell or large cell carcinomas (p=0.001). High cancer cell associated MMP-2 expression was associated with increased tumour recurrence (p=0.001). Tumour stroma showed positive staining in 162 (98%) cases and was considered highly stained in 120 (72%) cases. The high stromal MMP-2 expression was noticed more often among large cell carcinomas as compared with other histological types (p=0.007). High cancer cell associated EMMPRIN expression was found in 115 (61%) cases and was associated only with high MMP-2 expression in tumour cells (p=0.006).

In overall survival (OS) and disease free survival (DFS) analyses, type of tumour (p=0.001 and p=0.0004), advanced stage (p=0.001 and p=0.013) and high MMP-2 expression in tumour cells (p=0.018 and p=0.001) were associated with poor survival. Also, high stromal MMP-2 expression was related to poor outcome in both OS and DFS analyses (p=0.010 and 0.045, respectively). In multivariate analysis, stromal MMP-2 expression retained its prognostic value to predict OS and DFS (p=0.028 and p=0.039, respectively), together with tumour type and stage (p=0.017, p=0.001 and p=0.021, p=0.008, respectively).

The present study shows the significant prognostic value of MMP-2 in NSCLC suggesting that the use of MMP-2 is valuable in determining the patients with more aggressive disease.

Key words: MMP-2, EMMPRIN, Lung cancer, Survival, Prognosis

Introduction

The importance of tumor-stroma interactions regulating cancer development has been fully pointed out in recent years (Polette et al., 2004; Turpeenniemi-Hujanen, 2005). Matrix metalloproteinases (MMPs) are proteolytic enzymes which are proved to have a significant role in the degradation of the extracellular matrix (Mott and Werb, 2004), thus enhancing tumour invasion and metastasis formation (Egeblad and Werb, 2002; Polette et al., 2004). The function of MMPs is adjusted by the action of both different inducers and inhibitors, which regulate their tissue spesific expression (Zucker et al., 2001; Turpeenniemi-Hujanen, 2005).

Matrix metalloproteinase-2 (MMP-2, gelatinase A) is secreted as an inactive form (proMMP-2), before transmembrane bound MMPs (MT-MMPs) capture and activate it to a catalytically active form of MMP-2 (Visse and Nagase, 2003). The expression of MMP-2 has been noted to be concentrated in cancer cells (Thomas et al., 2000; Gaiotto et al., 2004; Liu et al., 2005), and also in the peritumoural stromal cells, or both (Passlick et al.,

2000; Pellikainen et al., 2004; Ishikawa et al., 2004; Sier et al., 2006). The increased expression of MMP-2 among tumour cells and in the tumour stroma has been related to more aggressive disease and unfavourable outcome in many carcinomas from different sites (Trudel et al., 2003; Pellikainen et al., 2004; Li et al., 2005; Kubben et al., 2006; Liu et al., 2006; Mrena et al., 2006; Ruokolainen et al., 2006; Sier et al., 2006). Also, in lung carcinomas either increased stromal or cancer cell associated MMP-2 expression has been found to correlate with poor outcome (Passlick et al., 2000; Kumaki et al., 2001; Ishikawa et al., 2004), but the relationship with other clinicopathological parameters predicting tumour aggressiveness has remained controversial (Passlick et al., 2000; Schutz et al., 2002; Hoikkala et al., 2006).

MMP-2 expression is regulated by EMMPRIN (extracellular matrix metalloproteinase inducer, CD147), enriched on the surface of tumour cells (Biswas et al., 1995; Gabison et al., 2005). By interacting with neighbouring fibroblasts, EMMPRIN stimulates expression of several MMPs (Zucker et al., 2001; Gabison et al., 2005). Similarly with MMP-2, increased expression of EMMPRIN has been related to more aggressive behaviour in many tumours (Zucker et al., 2001; Kanekura et al., 2002; Reimers et al., 2004). In lung carcinomas the prognostic role of EMMPRIN has not been studied, but the expression of EMMPRIN has been found to be located in invasive areas as well as in the normal lung tissue (Caudroy et al., 1999).

We have previously shown that the increased expression of another gelatinase (gelatinase B, MMP-9) is associated with more aggressive tumour behaviour in non-small cell lung cancer (NSCLC) (Leinonen et al., 2006). In the present study we analyzed the expression of MMP-2 and its inducer EMMPRIN both in tumour and stromal cells in NSCLC. The results were compared with clinicopathological characteristics and survival of the patients.

Materials and methods

Patients and clinicopathological data

All clinicopathological data were available from the previous studies of the same clinical material (Pirinen et al., 2001). Together, 212 patients were treated by surgical resection of the tumour between the years 1978-1995. Mean and median age of the patients was 63 years (range 42-78 years). Exact TNM classification (UICC 2002) and the stage of tumour were stated by reviewing both the clinical, radiological and histopathological statements from the patients' files. The follow-up was carried out until death or July 2002.

Histology

The histological type and grade of the tumours were confirmed by two experienced histopathologists, who re-evaluated all cases according to the WHO classification (Travis et al., 2004). The representative samples were cut to five-µm thick sections, which were used in immunohistochemical analyses.

Immunohistochemistry for EMMPRIN and MMP-2

Briefly, the slides were deparaffinized in xylene, rehydrated with graded alcohols and washed with 0.1 M sodium phosphate buffer [PB (pH 7.4)]. The antigen retrieval was processed with a microwave oven in 1 mM EDTA buffer (pH 8.0) for 3x5 min. Endogenous peroxidase was blocked with 5% hydrogen peroxide for 5 min, and non-specific binding was prevented with 1.5% normal horse serum (NHS) in PBS. The primary antibody for EMMPRIN (Mouse monoclonal antibody, clone sc-21746; Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) was used in dilution of 1:50 and for MMP-2 (Mouse anti-mmp-2, clone MAB13431, Chemicon internationals Inc. Temecula, CA, USA), which recognizes both the pro- and active forms of MMP-2, in dilution of 1:100. The slides were incubated overnight at 4°C and washed with PBS for 2x5 min, and incubated with the biotinylated secondary antibody (anti-mouse IgG; ABC Vectastain Elite kit, Vector Laboratories, Burlingame, CA, USA) for 35 min at room temperature. After that, the slides were washed with PBS for 2x5 min, incubated for 45 min in avidin-conjugated peroxidase complex (Vector, USA) and washed twice for 5 min with PBS. DAB (0.05% diaminobentzine tetrahydrocloride) was used as a chromogen. Finally, the slides were counterstained with Mayer's haematoxylin, washed, dehydrated and mounted. Lung and tonsil samples with known positivity in tumour cells served as positive controls for EMMPRIN, respectively. In MMP-2 stainings ovarian tissue sample was used as a positive control. The negative control was processed in PBS without the primary antibody and showed no positivity.

Evaluation of stainings

Stainings were analysed by two observers (T.L., R.P.), who were unaware of the clinicopathological data. In MMP-2 stainings the positive staining signal was found in the cell cytoplasm. The staining intensity was graded as follows: 0= negative, 1=weak, 2=moderate, 3=strong. The strong intensity corresponded to that in the control samples used as standards. The percentage of the positively stained tumour cells in the section was evaluated using a continuous scale (0-100%). The stromal staining was also evaluated by using the same protocol. For statistical purposes, stainings in cancer cells and stroma were further divided into two groups (low and high) according to 5% and 60% of the stained tumoural or stromal area, respectively. Based on the frequency distributions (median was 0% with cancer cells), this cut-off level allowed the most clear-cut separation between the high and low expressions of MMP-2.

The staining signal of EMMPRIN on tumour cell membranes was considered positive, whereas cytoplasmic staining seen in some cases was graded as negative, together with slides showing no staining signal at all. The percentage of the stained tumoural area and intensity of the staining was graded similarly as with MMP-2. The stainings were further divided as high or low according to 60% value as a cut-off. Stromal staining was recorded positive if any positivity was seen.

Statistical analyses

SPSS for Windows was used in statistical calculations. The frequency distributions of studied variables were compared by χ^2-test. In univariate survival analyses the Kaplan-Meier method was used and the log-rank test was used to examine the significance of the difference between drawn survival curves. Multivariate survival analysis was performed by using Cox's multivariate hazards model.

Ethics

This study was a cohort from a study protocol previously approved by the ethical committee of the Kuopio University Hospital and the Finnish Ministry of Social affairs and Health (Pirinen et al., 2001).

Results

The final material consisted of either 191 or 190 cases with properly stained tissue slides for MMP-2 and EMMPRIN, respectively. This population was comparable to the original cohort (n=212) based on the clinicopathological characteristics (histological type of tumour, histological grade and stage).
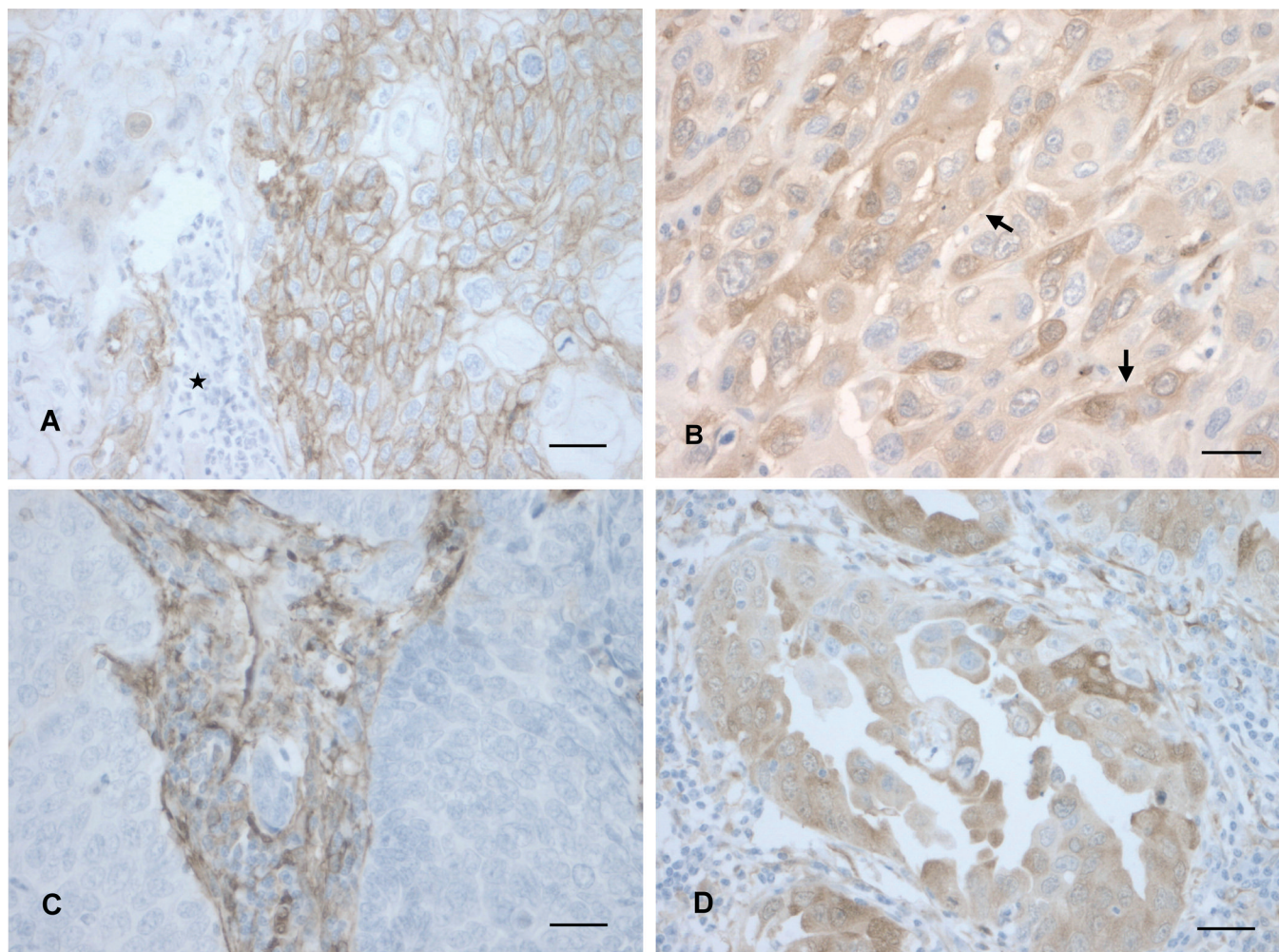

Fig. 1. A. High cell membrane associated EMMPRIN staining in squamous cell carcinoma. Tumour stroma is negative (star). **B.** Cytoplasmic expression (arrows) of MMP-2 in squamous cell carcinoma. **C.** High stromal associated MMP-2 staining in squamous cell carcinoma. Tumour cells are negative. **D.** Adenocarcinoma showing high MMP-2 staining in tumour cells. Bar: 300 μm.

MMP-2 expression in normal lung and dysplastic epithelium

The pseudostratified bronchial epithelium was intensely MMP-2 positive, as well as the epithelium of benign peribronchial glands. Dysplastic epithelium in 6 slides adjacent to normal bronchial epithelium was MMP-2 negative. Alveolar walls were always MMP-2 positive and normal connective tissue showed variable staining reaction being, however, usually MMP-2 positive.

MMP-2 expression in cancer cells and tumour stroma

A high expression of MMP-2 in tumour cells was found in 83 out of 191 cases (44%). Adenocarcinomas showed more often high expression of MMP-2 (35/47, 75%) as compared to squamous cell or large cell carcinomas (37/120, 31% and 11/24, 46%, respectively, Fig. 1) (p=0.001). A high expression of MMP-2 in tumour cells was correlated with an increased risk for tumour recurrence (p=0.001), which was also noticed in the subgroup of squamous cell carcinomas (p=0.004). However, no association was found between the expression of MMP-2 in tumour cells and tumour size (p=0.931), stage (p=0.163) or tumour differentiation (p=0.750) (Table 1).

There were 166 cases with enough representative stromal tissue in the slide to be analysed. The stromal staining was localized in peritumoural fibroblasts and inflammatory cells. Tumour stroma was stained positively in 162 (98%) cases and was considered highly stained in 120 (72%) cases. The high stromal MMP-2 expression was found significantly more often in large cell carcinomas (17/17, 100%) than in adenocarcinomas (29/40, 73%) or squamous cell carcinomas (74/109, 68%) (p=0.007) (Table 1). The stromal expression of MMP-2 was not related to tumour recurrence (p=0.364), size (p=0.847), stage (p=0.142) or histological grade (p=0.470), but was associated with lymph node status (p=0.037).

EMMPRIN expression in normal lung

Normal bronchial epithelium was EMMPRIN positive, as well as normal epithelium of peribronchial glands, which showed a weak staining reaction along cell membranes. Dysplastic epithelium (6 cases) was mostly negative, but a weak staining signal was present in the most superficial cell layers. Alveolar walls and blood vessels were EMMPRIN negative.

EMMPRIN expression in cancer cells and tumour stroma

The positive staining in cancer cells was found in 177 (93%) cases and was considered high in 115 (61%) cases. Weak stromal staining was noticed in 76 (40%) cases, usually only a small percent (<10%) of the stromal area showed a positive staining signal. No relationship was found between the expression of EMMPRIN in tumour cells or stroma and clinicopathological factors. However, high EMMPRIN expression in tumour cells was related to high cancer cell associated MMP-2 expression (p=0.006).

Survival analyses

During the follow up, a recurrent disease was noted in 102 (48%) patients and 118 (62%) patients died because of lung cancer. In overall survival (OS) analysis

Table 1. Expression of MMP-2 in tumour cells and tumour stroma and clinicopathological factors. The numbers identified (percentages) in each group.

	MMP-2 in tumour cells		p-value	MMP-2 in tumour stroma		p-value
	<5%	≥ 5%		<60%	≥ 60%	
Tumour type						
- squamous cell carcinoma	83 (69)	37 (31)	0.001	35 (32)	74 (68)	0.007
- adenocarcinoma	12 (26)	35 (74)		11 (27)	29 (73)	
- large cell carcinoma	13 (54)	11 (46)		0 (0)	17 (100)	
Histological grade						
- I-II	49 (56)	39 (44)	0.750	27 (35)	51 (65)	0.470
- III	43 (59)	30 (41)		18 (28)	47 (72)	
Tumour size						
- T1	25 (50)	25 (50)	0.931	12 (26)	34 (74)	0.847
- T2-4	82 (59)	56 (41)		34 (29)	83 (71)	
Nodal status						
- N0	80 (61)	51 (39)	0.109	26 (23)	87 (77)	0.037
- N1-3	27 (47)	30 (53)		20 (40)	30 (60)	
Stage						
- I	76 (60)	50 (40)	0.163	26 (24)	83 (76)	0.142
- II-IV	31 (49)	32 (51)		20 (36)	36 (64)	
Recurrence						
- yes	35 (43)	46 (57)	0.001	25 (33)	50 (67)	0.364
- no	61 (70)	26 (30)		18 (25)	53 (75)	

tumour type (p=0.001) and stage (p=0.001) were associated with survival. The high expression of MMP-2 in tumour cells was also associated with poor survival (p=0.018). Similarly, in disease free survival (DFS) analysis, tumour type (p=0.0004), advanced stage (p=0.013) as well as high MMP-2 expression in cancer cells (p=0.001) predicted poor survival. Also, high stromal MMP-2 expression was related to poor outcome in both OS and DFS analyses (p=0.010 and 0.045, respectively) (Fig. 2).

Multivariate analysis included stage, histological type of tumour, and both stromal and cancer cell associated MMP-2. The significant predictors of OS were stage (p=0.001), histological type of the tumour (p=0.017) and stromal MMP-2 expression (p=0.028). In DFS analyses stromal expression of MMP-2 (p=0.039), together with stage (p=0.008) and histological type of the tumour (p=0.021) were also associated with survival.

Discussion

The present study was undertaken to clarify the clinical and prognostic role of MMP-2 and its inducer EMMPRIN in NSCLC. We found that the expression of EMMPRIN was related to MMP-2 expression. However, EMMPRIN status in tumour cells had no clinical prognostic value in this material (Caudroy et al., 1999). The high expression of MMP-2 in tumour cells was noticed more often among adenocarcinomas, but high stromal expression was associated significantly with large cell carcinomas. In addition, high cancer cell associated MMP-2 expression was correlated with an increased risk for tumour recurrence (Passlick et al., 2000), and predicted also poor survival together with a high stromal MMP-2 signal (Ishikawa et al., 2004).

As reported earlier (Kawano et al., 1997; Yamamura et al., 2002) alveolar lining cells and pseudostratified bronchial epithelium, as well as the epithelium of peribronchial glands were stained positively for MMP-2. However, the dysplastic epithelium of six cases adjacent to carcinomas was MMP-2 negative, which contradicts the findings of earlier studies (Kawano et al., 1997; Galateau-Salle et al., 2000), and also the studies of preneoplastic epithelium from other sites (Gaiotto et al., 2004; Samantaray et al., 2004). However, the low number of dysplastic cases does not allow us to draw very strong conclusions (Galateau-Salle et al., 2000).

Among the members of the MMP family, MMP-2 is unique in its ability to cleave the type IV collagen, a principal structural component of the basement membrane of ECM (Polette et al., 2004), which is the crucial site for metastasis and invasion of tumour cells (Mook et al., 2004; Polette et al., 2004). MMP-2 expression is regulated by complex mechanisms involved in the cell-matrix interactions (Mott and Werb, 2004). In the present study, MMP-2 expression was noticed in cancer cells, and also the stromal tissue (fibroblasts and inflammatory cells) was MMP-2 positive in almost all cases, supporting previous findings (Suzuki et al., 1998; Schutz et al., 2002; Yamamura et al., 2002; Sier et al., 2006). This suggests both stromal synthesis and activation of MMP-2 in tumour cells (Chakrabarti and Patel, 2005). However, the majority of cancer cells showed only a patchy staining pattern, as also found earlier in lung tumours (Passlick et al., 2000; Schutz et al., 2002). High MMP-2 expression in tumour cells was more often noticed among adenocarcinomas than squamous cell or large cell carcinomas as shown
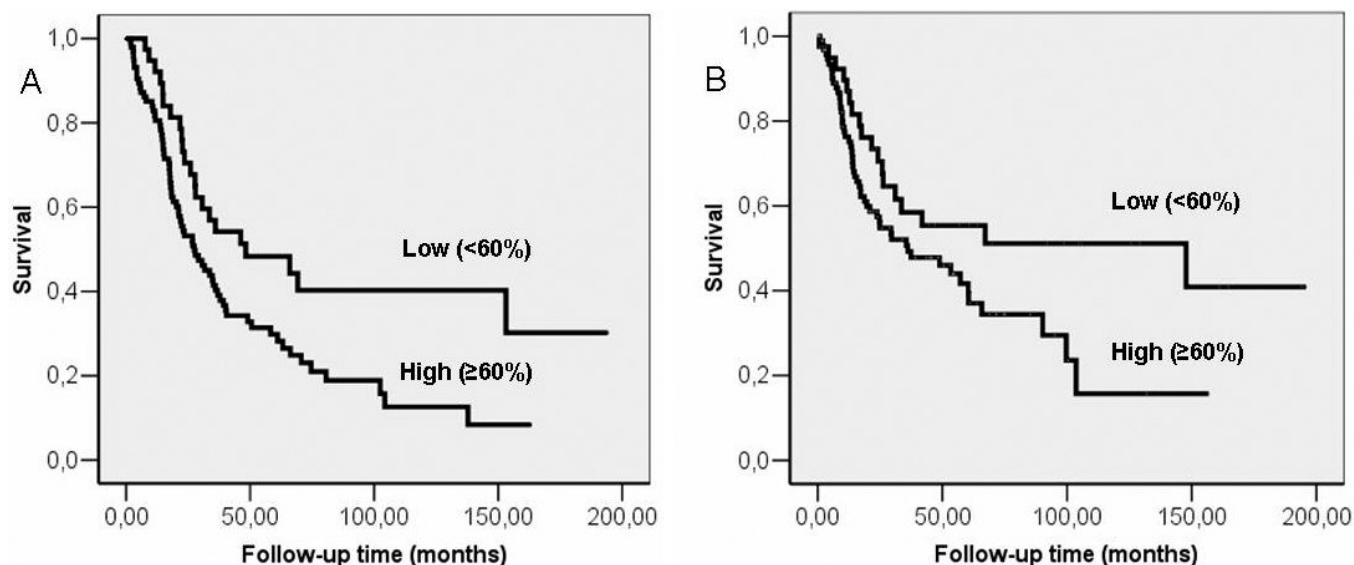

Fig. 2. A Kaplan-Meier curve demonstrating the difference in survival according to stromal expression of MMP-2. **A.** overall survival, p=0.010. **B.** disease-free survival, p=0.045.

earlier (Ishikawa et al., 2004). On the other hand, stromal MMP-2 expression was focused in large cell carcinomas, and this finding has not been reported previously in lung tumours. Usually, the correlation between stromal MMP-2 expression and histological type of the tumour has not been found (Passlick et al., 2000; Thomas et al., 2000; Yamamura et al., 2002; Hoikkala et al., 2006).

We showed that the high expression of MMP-2 in tumour cells holds an increased risk for tumour recurrence, as found earlier in prostate and squamous cell carcinomas of the head and neck (Trudel et al., 2003; Ruokolainen et al., 2006). In several other carcinomas, including lung tumours (Passlick et al., 2000; Schutz et al., 2002; Yamamura et al., 2002; Trudel et al., 2003; Pellikainen et al., 2004; Samantaray et al., 2004; de Vicente et al., 2005; Kamijima et al., 2005; Liu et al., 2005; Hoikkala et al., 2006), MMP-2 status in tumour cells has not been related to those clinicopathological factors, which reflect tumour aggressiveness (Table 2). The stromal expression of MMP-2 was not associated with tumour differentiation as reported earlier (Yamamura et al., 2002; Ishikawa et al., 2004). Including our study, in only two studies on lung cancer the significance of stromal accumulation of MMP-2 has been analysed separately (Ishikawa et al., 2004). Usually, the stromal expression of MMP-2 has been linked with the expression in tumour cells, and analysed and graded together as a positive expression (Thomas et al., 2000; Yamamura et al., 2002; Schutz et al., 2002; Kerr et al., 2004), which makes their influence on clinicopathological factors more difficult to evaluate.

Many previous studies have clearly established the significant role of MMP-2 in predicting poor outcome in different carcinomas (Trudel et al., 2003; Pellikainen et al., 2004; Liu et al., 2005; Mrena et al., 2006; Ruokolainen et al., 2006; Sier et al., 2006). We report here that the cancer cell associated MMP-2 expression is associated with an adverse outcome, in line with the previous reports of other tumours (Trudel et al., 2003; Liu et al., 2005; Mrena et al., 2006), including lung carcinomas (Yamamura et al., 2002; Hoikkala et al., 2006). Further, we also demonstrate that the increased stromal expression of MMP-2, together with stage and histological type of the tumour, independently predicted poor survival of the patients, (Ishikawa et al., 2004; Sier et al., 2006). This suggests the self-supporting role of stromal MMP-2 to enhance tumour aggressiveness, also without the cancer cell associated MMP-2. The mechanism of MMP-2 to predict poor outcome is

Table 2. Earlier clinicopathological studies of MMP-2 in lung carcinomas.

Authors	n	Methods	Clinicopathological factors		Survival
			Tumour cells	Stroma	
Hoikkala et al., 2006	59	IHC, ELISA	no correlation	no correlation	high MMP-2 expression → poor prognosis
Yamamura et al., 2002	89	ISH, IHC	no correlation	no correlation	high MMP-2 expression → poor prognosis in overall survival
Ishikawa et al., 2004	218	IHC	high MMP-2 expression more frequent in adenocarcinomas	high MMP-2 expression more frequent in SCC	high stromal MMP-2 expression → poor prognosis
Byun et al., 2006	204	IHC	high MMP-2 expression was associated with lymph node involvement, tumour stage and histological type	not studied	high MMP-2 expression → poor prognosis
Kerr et al., 2004	151	IHC	no correlation	no correlation	not studied
Schutz et al., 2002	30	IHC, Gelatin zymography	no correlation	no correlation	not studied
Kumaki et al., 2001	32	IHC	high MMP-2 expression correlated with lymph node metastasis and vascular invasion	not studied	no correlation
Lin et al., 2004	472	IHC	high MMP-2 expression correlated with tumour recurrence and metastasis	not studied	high MMP-2 expression → poor prognosis
Passlick et al., 2000	193	IHC	no correlation	not studied	high MMP-2 expression→ poor prognosis in early-stage NSCLC
Thomas et al., 2000	115	IHC	no correlation	no correlation	not studied
Present study, 2008	212	IHC	high MMP-2 expression associated with increased tumour recurrence	high stromal MMP-2 expression more frequent in LCC	high MMP-2 expression → poor prognosis

IHC: immunohistochemistry; ELISA: enzyme-linked immunosorbent assay; ISH: immunohistochemistry; SCC: squamous cell carcinoma; LCC: large cell carcinoma.

probably for the most part due to its natural function i.e. by degrading the extracellular matrix to facilitate tumour cell spread (Polette et al., 2004). However, in our study, like in other reports (Yamamura et al., 2002; Ishikawa et al., 2004), associations with other clinicopathological parameters have not been found, which also suggests an alternative mechanism for MMP-2 to enhance aggressive tumour behaviour.

In normal alveolar walls of the lung EMMPRIN was barely detectable, as also reported earlier (Odajima et al., 2006). However, the non-neoplastic bronchial epithelium in close vicinity to carcinomas was EMMPRIN positive (Caudroy et al., 1999). We also found a weak stromal staining reaction in almost half of the cases (Caudroy et al., 1999; Odajima et al., 2006), which probably reflects a paracrine feedback expression from stromal fibroblasts (Tang et al., 2004). EMMPRIN has been found to be present on cancer cell surfaces acting as a MMP inducer (Gabison et al., 2005). In addition, high EMMPRIN expression has been related to high MMP-2 expression in tumour cells (Davidson et al., 2003a). We hypothesized that increased EMMPRIN expression in cancer cells might be associated with adverse disease outcome, as shown in breast and ovarian carcinomas (Davidson et al., 2003b; Reimers et al., 2004). However, in NSCLC, no relationship could be found and no earlier studies in lung cancer are reported on this issue.

The present study demonstrates that high MMP-2 expression, both in tumour cells and peritumoural stroma, is associated with an increased risk for tumour recurrence and poor disease outcome in NSCLC. The high expression of EMMPRIN is correlated with MMP-2 expression, but has not any further prognostic value. The results emphasize the value of MMP-2 in evaluating patients with more aggressive disease.

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