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Cathepsin B, Plasminogenactivator-inhibitor (PAI-1) and Plasminogenactivator-receptor (uPAR) are Prognostic Factors for Patients with Non-small Cell Lung Cancer

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Abstract. *To evaluate the possible role of cysteine proteases and serine proteases, as well as their respective inhibitors and receptors, as new prognostic factors in NSCLC, we examined, for the first time, 10 biological parameters related to three proteolytic systems within a homogeneous collective of 147 cases of NSCLC. Activities (cath B_{AT}, cath $B_{A7.5}$) and protein levels of cath B_C, cath L_C, uPA, PAI-1, uPAR [measured by three different assays uPAR (ADI), uPAR (HD13), uPAR (IIIF10)] and TF were measured in homogenates of lung tumour tissue and corresponding non-malignant lung parenchyma. Total cath B activity (cath B_{AT}) and enzymatic activity of the fraction of cath B, which is stable and active at pH 7.5 (cath $B_{A7.5}$), were determined by a fluorogenic assay using synthetic substrate Z-Arg-Arg-AMC. The concentrations of cath B_C, cath L_C, uPA, PAI-1, uPAR and TF were determined by ELISAs. uPAR was determined using three different ELISA formats. The median levels of cath B_{AT} (5.1-fold), cath $B_{A7.5}$ (2.5-fold), cath B_C, (8.5-fold), cath L_C (6.6-fold), uPA (6.5-fold), PAI-1 (4.2-fold), uPAR (ADI) (2.2-fold), uPAR (HD13) (4.0-fold) and uPAR (IIIF10)(2.6-fold) were higher in tumour tissue compared to the lung parenchyma. Cath B_{AT}, cath $B_{A7.5}$ and cath B_C in primary tumours correlated with lymph node metastases. Regarding histologies, the concentration of PAI-1 seems to be associated with the histological cell types of NSCLC. We found the*

Abbreviations: NSCLC, non-small cell lung cancer; AC, adenocarcinoma; SCC, squamous cell carcinoma; cath B, cathepsin B; upa, plasminogen activator; PAI-1, plasminogenactivator-inhibitor; uPAR, plasminogenactivator-receptor; TF, tissue factor, TF; VEGF, vascular endothelial growth factor.

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Key Words: Cathepsin B (cath B), plasminogenactivator-inhibitor (PAI-1), plasminogenactivator-receptor (uPAR), prognosis, non-small cell lung cancer (NSCLC).

highest values of PAI-1 in large cell carcinoma > SCC, AC > carcinoid and lowest values in metastases of primary tumours of other organs. Only PAI-1 was significantly increased in poorly-differentiated cells (G3) compared to well- and moderately- differentiated cells (G1/G2). PAI-1 significantly correlated with cath B_{AT} and cath $B_{A7.5}$ with uPAR (ADI), uPAR (HD13), uPAR (IIIF10) with uPA, and only weakly with TF, but not with cath B_C and cath L_C. Significant correlations with overall survival in the total population of NSCLC patients were observed in univariate analysis for cath B_{AT}, cath B_C, PAI-1, uPAR (ADI), uPAR (HD13), and uPAR (IIIF10). Cath L_C was not significantly associated with poor prognosis. Regarding the histological tumour type, only in patients with squamous cell carcinomas did cath $B_{A7.5}$ and PAI-1 remain significant prognostic factors. In multivariate survival analysis only two proteolytic factors, PAI-1 and uPAR (III10F), stayed significant. In conclusion, among 10 biological parameters evaluated within the same cohort of patients, only PAI-1, uPAR (ADI), uPAR (HD13), uPAR (IIIF10), cath B_{AT} and cath B_C are prognostic factors for overall survival of NSCLC patients. Moreover, PAI-1 and uPAR (IIIF10) add independent prognostic information with regard to established clinical and histomorphological factors in NSCLC.

The prognosis of patients suffering from non-small cell lung cancer (NSCLC) is poor: the 5-year overall survival of NSCLC patients, summarized above all TNM stages and therapeutic strategies, is 14%. Focusing only on the subgroup of NSCLC patients under curative surgery in TNM-stage IA, the overall survival is 65% after 5 years. In this respect, resection of the tumours in early stages offers good prospects of a successful cure (1). Subsequently, for the determination of the prognosis of NSCLC patients as well as the choice of therapeutic strategy, the classification of the malignant tumour is essential. Although the TNM-staging is the most important prognostic factor, there is no obvious association between the TNM stage and the pathobiochemistry of malignant lung tumours. Therefore, a search for new biochemical prognostic factors, *i.e.* biological molecules, which play an active role during tumour progression and metastasis, is needed. If we knew the precise function of these biological molecules in the progression of tumours, we would be able to develop new therapeutic strategies. Further, low-risk patients could avoid aggressive, adjuvant chemotherapy while high-risk patients might benefit from the adjuvant chemotherapy. In addition, antagonistic molecules for these biological molecules as new chemotherapeutic reagents might be developed.

Several independent studies have demonstrated that proteases, protease-inhibitors and their corresponding receptors are relevant for the malignant progression of tumours as well as formation of metastasis (2-7). Therefore, it is quite reasonable that biological molecules, involved in proteolysis, would be good candidates for the discrimination of NSCLC patients with poor prognosis.

As a member of cysteine-cathepsins, cath B has been determined on tissue sections (8-19), in tissue homogenates (20-27) and/or in the sera/plasma (28, 29, Werle and Kos, unpublished results) of lung cancer patients. Cysteine protease-inhibitors, *e.g.* cystatins, have additionally been measured in tissue homogenates (30-33, Werle, unpublished data) and in sera/plasma (Werle and Kos, unpublished data). From these patients, we have further localized cystatin A, B and cystatin C on lung tumour tissue sections (Werle and Kos, unpublished results).

So far, only a few studies have correlated cath B values of lung tumour homogenates (23, 25- 27) and of lung tumour tissue sections (10, 11, 14, 15) with short-term or long-term follow-up of NSCLC patients. Univariate overall survival analyses have shown that cath B might be a useful prognostic marker in NSCLC. Only overexpression of cath B on tissue sections proved to be an independent prognostic factor in NSCLC patients (10, 11, 14).

Besides the cysteine-cathepsins and their corresponding inhibitors, serine proteases such as uPA, serine protease inhibitors *e.g.* PAI-1 and receptors [*e.g.* uPAR and TF] should be considered as potential prognostic factors. The first data on the prognostic impact of uPA, PAI-1 and uPAR for AC and SCC patients were presented by Pederson *et al.* (34, 35). These authors reported that high levels of PAI-1 were associated with shorter overall survival of patients with pulmonary AC, while high uPAR levels were associated with shortened survival of patients with SCC. However, a recent study by Salden *et al.* (2000) could not confirm the prognostic impact of PAI-1 and uPAR, either in the group of all NSCLC patients or in the subgroups of patients with AC and SCC. In addition, immunohistochemical studies are controversial. Pavey *et al.* (37) showed that increased expression of PAI-1, but not uPA and uPAR, was associated with the survival probability of SCC patients. These authors could not find a significant correlation between the expression of PAI-1, uPA and uPAR and survival probability in the total study group of NSCLC patients. In contrast, Volm *et al.* (38) demonstrated that the median survival of uPA-positive NSCLC carcinomas was shorter than that of uPA-negative tumors.

In addition, TF, the physiological initiator of the blood coagulation cascade, has been suggested to regulate tumour growth and angiogenesis. By using immunohistochemistry, Koomagi and Volm (39) demonstrated that TF correlates with the expression of the VEGF. Moreover, these authors showed that TF-negative tumours have a significantly better survival than TF-positive tumours.

So far, most studies looking at the prognostic impact of proteolytic factors in NSCLC patients have only focused on

a few factors at a time. Yet, in order to fully evaluate the clinical relevance of new prognostic factors, it is of the utmost importance to study their impact on patients' prognosis in a single, homogeneous patient collective. In this study, we evaluated the clinical relevance of the following 10 biological parameters belonging to three proteolytic systems within the same tissue homogenates of NSCLC patients: the cysteine proteases cath B (cath $B_{A7.5}$, cath B_{AT}, cath B_C) and cath L_C, the serine proteases uPA, its type-1 inhibitor PAI-1, its receptor uPAR [uPAR (ADI), uPAR (HD13), uPAR (IIIF10)] and TF.

Patients and Methods

Patients. Tumour tissues and non-cancerous lung tissues were obtained as matched paired samples from 147 patients with lung tumours, which were resected by surgery at the Thoraxhospital Heidelberg gGmbH, Germany. For some of the analyses, sufficient tumour homogenate was available from only 95 patients. The age of the patients ranged from 15 to 81 years (median: 59 years). Whenever macroscopically possible, tumour tissue was resected in the periphery of the tumour. Necrotic parts of the tumour were always removed. The non-cancerous lung tissue was taken from areas at least 6 cm away from the tumour. Based on the predominant cell type, lung tumours were classified according to the WHO protocol (40). The tumour disease stage (pTNM) was classified according to the international staging system (41). All patients included in this study were subjected to primary surgery. None of the patients was exposed to radiation therapy or received chemotherapy prior to surgery. After curative surgery, low-stage patients were kept under surveillance, while high-stage patients additionally received adjuvant or palliative chemotherapy and/or radiation therapy according to current therapy guidelines (42, 43). The median follow-up in patients still alive at the time of analysis was 46 months; 9-89 months (3.8 years; 0.75-7.4 years). This study was carried out with ethical commitee approval.

Lung tumour and lung tissue homogenates. Tissue homogenates were prepared as previously described elsewhere (25, 44).

Cathepsin B activity ($cath_{AT}$, cath $B_{A7.5}$) assays. Both assays were performed as described in detail (25, 26). In brief, total cath B_{AT} activity was measured using Z-Arg-Arg-AMC at pH 6.0 as the substrate. In order to receive the cath B fraction, which is stable and active at pH 7.5 (cath $B_{A7.5}$), tissue homogenates were preincubated for 60 min at pH 7.5 and residual cath B activity was determined at the same pH. The specificity of cath $B_{A7.5}$ was verified by using the synthetic inhibitor CA-074.

Cathepsin B (cath B_C) and cathepsin L (cath L_C)-ELISA. The human cath B protein (cath B_C) in lung tissue homogenates was analysed by ELISA (Krka d.d., Novo mesto, Slovenia). The components were purified and characterized and the test was optimized as previously described (45-47).

The cathepsin L assay (cath L_C) was obtained from BioAss (Dießen, Germany). The cath L_C quantitative enzyme-linked immunosorbent assay (ELISA) is based on the sandwich principle. The antibodies used for cath B_C-ELISA and cath L_C-ELISA recognize the precursor and mature form of the enzyme as well as enzyme-inhibitor complexes. A microplate reader (SLT Rainbow, Austria) was used to measure absorbance. Cath B_C and cath L_C levels were expressed as $ngml^{-1}$ of the tissue homogenates.

uPA, PAI-1, uPAR (ADI), uPAR (HD13), uPAR (IIIF10)-ELISA. For detection of uPAR in tissue extracts, two recently developed sandwich ELISA formats were applied. Both use polyclonal antibody (pAb) HU277 as the catcher antibody and either monoclonal antibody (mAb) HD13 or IIIF10 as detecting antibody, as described by Kotzsch *et al.* (48). Briefly, immunoassay plates (MaxiSorb™; Nunc, Wiesbaden, Germany) were coated overnight at 4˚C with 50 µl/well of pAb HU277 IgY (5 μgml^{-1}). After washing, the plates were incubated with 50 µl/well of test samples diluted in sample buffer (50 mM Tris-HCl, 100 mM NaCl, 0.2%[v/v] Triton X-100, 1% [w/v] BSA, pH 7.6, for 2 h, 37˚C).

Two-fold serial dilutions of recombinant CHO-suPAR (glycosylated, soluble recombinant human uPAR) in sample buffer, covering a concentration range of 0.15 to 10 $ngml^{-1}$, served as uPAR standard. After washing, wells were incubated with 50 µl/well of peroxidase-labelled mAbs HD 13.1 or IIIF10 (5 μgml^{-1} in blocking buffer) for 90 min, 37˚C. Finally, the peroxidase reaction was initiated by addition of 50 µl/well of 3,3',5,5'-tetramethylbenzidine (TMB)/H_2O_2 as substrate solution (K&P Laboratories, Gaithersburg, MD, USA) and stopped after 20 min at room temperature, by addition of 200 µl/well of 0.5 M H_2SO_4. The absorbance was measured at 450 nm with a multichannel microtiter plate reader (ICN, Eschwege, Germany). In parallel to these new uPAR ELISA formats, uPAR in tissue extracts was determined by a commercially available kit (#893 Imubind ADI-ELISA; American Diagnostica Inc., Greenwich, CT, USA). The uPA antigen content in tissue extracts was determined by uPA-ELISA (#894 Imubind, American Diagnostica Inc.) and PAI-1 antigen by PAI-1-ELISA (#821 Imubind, American Diagnostica Inc.).

TF-ELISA. The TF antigen content of tissue extracts was determined using a sandwich-type ELISA with two mAbs as described previously, with slight modifications (49, 50). Briefly, microtiter plates (Maxisorp™, Nunc) coated with purified anti-TF mAb VIC7 (2.5 μgml^{-1}) were incubated with test samples diluted in sample buffer (50 mM Tris-HCl, 100mM NaCl, 0.2% [v/v] Triton X-100, 1% [w/v] BSA, pH 7.6) for 2 h at 37˚C. Two-fold serial dilutions of standard recombinant TF (American Diagnostica Inc.) in sample buffer were added as a reference standard. Following incubation with peroxidase-labelled anti-TF mAb IIID8 (90 min at 37˚C) and subsequent substrate reaction with TMB / H_2O_2 (K & P Laboratories) for 20 min, the absorbance was measured at 450 nm with a multichannel photometer.

Protein concentration. The total protein concentration of the tissue homogenates was determined according to Bradford (51). Bovine serum albumin was used as a standard.

Statistical analyses. To compare paired data of tumour and non-cancerous lung tissue, we used the two-tailed Wilcoxon's rank test. Differences of cath B_C, cath L_C, PAI-1, uPAR, uPAR (HD13), uPAR (IIIF10) and TF between different subgroups of patients were tested by Mann-Whitney and Kruskall-Wallis test. Correlations between activities ($catB_{AT}$, $cath_{A7.5}$) and concentrations (cath B_C, cath L_C, PAI-1, uPAR, uPAR (HD13), uPAR (IIIF10) and TF were calculated by non-parametric regression analysis and the significance

Table I. *Cathepsin B and cathepsin L in lung tumor homogenates.*

		Cath B_{AT} [ngmg^{-1} protein]			Cath $B_{A7.5}$ [ngmg^{-1} protein]			Cath B_C [ngmg^{-1} protein]			Cath L_C [ngmg^{-1} protein]		
	n	Tumour median (5%, 95%)	Normal median (5%, 95%)	Tu/Lu** median	Tumour median (5%, 95%)	Normal median (5%, 95%)	Tu/Lu** median	Tumour median (5%, 95%)	Normal median (5%, 95%)	Tu/Lu** median	Tumour median (5%, 95%)	Normal median (5%, 95%)	Tu/Lu median
Primary tumours (total)	147	1197	236	5.1	306	125	2.5	1130	133	8.5	408	62	6.6
vs lung tissue (total)		(286, 3100)	(56, 1027)		(0.5, 89.4)	(0.5, 8.1)		(270, 2682)	(36, 368)		(49, 833)	(9, 158)	
- Squamous cell carcinoma	56	1169 (234, 2724)			330 (17, 1856)			1229 (532, 2702)			409 (136, 772)		
- Adenocarcinoma	55	1282 (439, 2585)			397 (124, 1772)			1233 (331, 2682)			435 (100, 1017)		
- Large cell carcinoma	6	2087 (1568, 4217)			261 (75, 910)			1131 (783, 2812)			565 (368, 1748)		
- Carcinoid	6	509 (309, 1239)			84 (15, 174)			501 (252, 873)			199 (49, 526)		
Small cell carcinoma	4	1247 (221, 5248)			811 (65, 3304)			1226 (92, 2119)			472 (47, 557)		
Secondary tumours	20	1066 (360, 3126			171 (46, 718)			429 (179, 2017			239 (2, 707)		
pT1	23	1184 (249, 4772)			308 (14, 1856)			1233 (301, 2425)			408 (2, 1017)		
pT2	76	1278 (321, 3100)			414 (55, 1721)			1213 (529, 2702)			410 (100, 690)		
pT3	17	1168 (219, 2547)			396 (9, 1893)			1379 (644, 2283)			448 (241, 771)		
pT4	11	1094 (560, 2388)			324 (39, 1624)			1090 (593, 3585)			412 (205, 1018)		
pN0	45	1598 (321, 3807)			292 (30, 1749)			1147 (298, 2283)			409 (2, 771)		
pN1	40	1124 (218, 2149)			414 (17, 1624)			1373 (616, 3549)			411 (100, 1018)		
pN2	26	970 (514, 2597)			444 (117, 1893)			1150 (556, 2682)			443 (176, 772)		
pN3	16	1820 (353, 2508)			760 (190, 1772)			1764 (864, 3382)			424 (241, 1017)		
pTNM I	38	1613 (321, 6820			301 (30, 1856)			1147 (270, 2615)			409 (2, 833)		
pTNM II	24	1277 (218, 2149)			421 (56, 1577)			1388 (616, 2702)			398 (76, 1031)		
pTNM IIIa	32	1047 (271, 2597)			308 (27, 1893)			1200 (556, 2283)			446 (176, 749)		
pTNM IIIb	20	1214 (457, 2335)			707 (39, 1772)			1970 (593, 3585)			572 (205, 1018)		
pTNM IV	13	1515 (898, 4217)			408 (59, 1238)			1301 (707, 2812)			359 (210, 1172)		

Table II. *Plasminogen activator (uPA), plasminogenactivator-inhibitor (PAI-1) and plasminogenactivator-receptor (uPAR). in lung tumor tissue homogenates.*

		uPA [ngmg^{-1} protein]			PAI-1 [ngmg^{-1} protein]			tissue factor [ngmg^{-1} protein]		
	n	Tumour median (5%, 95%)	Normal median (5%, 95%)	Tu/Lu** median	Tumour median (5%, 95%)	Normal median (5%, 95%)	Tu/Lu** median	Tumour median (5%, 95%)	Normal median (5%, 95%)	Tu/Lu** median
Primary tumours (total) *vs* lung tissue (total)	147	1.3 (0.15, 6.3)	0.2 (0.08, 0.5)	6.5	10 (0.5, 89.4)	2.4 (0.3, 1.8)	4.2	4.4 (0.86, 41.4)	3.3 (1.2, 9.2)	1.3
- Squamous cell carcinoma	56	1.4 (0.5, 4.1)			15.7 (1.1, 41.4)			5.7 (1.1, 41.4)		
- Adenocarcinoma	55	1.3 (0.2, 7.9)			7.2 (0.86, 44.2)			3.9 (0.86, 44.2)		
- Large cell carcinoma	6	2 (0.7, 4.8)			46.9 (1.2, 5.3)			4.6 (1.2, 5.3)		
- Carcinoid	6	0.2 (0.01, 0.9)			1.9 (0.5, 4.5)			4.9 (1.8, 8.1)		
Small cell carcinoma	4	1.6 (0.09, 6.8)			47.8 (10.3, 209)			14.8 (0.4, 29.1)		
Secondary tumours	20	0.8 (0.05, 3.3)			4.5 (0.1, 28.8)			2.4 (0.4, 61.4)		
pT1	23	1.3 (0.2, 6.4)			7.1 (0.01, 179.4)			8.3 (0.6, 44.2)		
pT2	76	1.4 (0.5, 6.1)			11.7 (1.5, 44.0)			4.6 (1.3, 29.1)		
pT3	17	2.3 (0.5, 10.9)			19.4 (2.5, 164.1)			5 (0.2, 96.5)		
pT4	11	1.3 (0.03, 7.9)			7.2 (0.3, 68.6)			4.1 (1.9, 41.4)		
pN0	45	1.4 (0.2, 6.4)			14.2 (1.8, 114.7)			5.3 (1.6, 32.7)		
pN1	40	1.2 (0.4, 8.5)			10.9 (0.4, 64.4)			5.5 (0.86, 49.1)		
pN2	26	1.4 (0.4, 3.1)			5.5 (1.5, 61.3)			4 (1.1, 44.2)		
pN3	16	1.9 (0.7, 10.9)			7.7 (0.4, 164.1)			4.6 (0.6, 41.3)		
pTNM I	38	1.4 (0.2, 6.4)			14.2 (1.8, 114.7)			5.1 (1.8, 29.1)		
pTNM II	24	1.2 (0.5, 8.5)			13.7 (3.0, 84.4)			5.5 (0.43, 31.7)		
pTNM IIIa	32	1.7 (0.4, 4.1)			9.1 (1.5, 100.3)			4.3 (1.1, 44.2)		
pTNM IIIb	20	1.6 (0.03, 10.9)			7.4 (0.3, 163.1)			4.4 (0.62, 41.4)		
pTNM IV	13	1.3 (0.6, 4.8)			11.6 (2.8, 111.2)			4.2 (1.1, 49.1)		

Table III. *Plasminogenactivator-receptor [uPAR (HD13, IIIF10, ADI)] in lung tumor homogenates.*

		uPAR (HD13) [ngmg^{-1} protein]			uPAR (IIIF10) [ngmg^{-1} protein]			uPAR (ADI) [ngmg^{-1} protein]		
	n	Tumour median (5%, 95%)	Normal median (5%, 95%)	Tu/Lu** median	Tumour median (5%, 95%)	Normal median (5%, 95%)	Tu/Lu** median	Tumour median (5%, 95%)	Normal median (5%, 95%)	Tu/Lu** median
Primary tumours (total)	95	2.8	0.7	4	3.9	1.5	2.6	2.8	1.3	2.2
vs lung tissue (total)		(0.4, 10.1)	(0.3, 1.8)		(1.3, 8.2)	(0.63, 3.1)		(0.46, 8.8)	(0.57, 2.9)	
- Squamous cell carcinoma	38	3.1 (0.49, 14.4)			3.6 (1.3, 11.8)			2.5 (0.53, 9.9)		
- Adenocarcinoma	34	2.4 (0.46, 9.8)			3.4 (1.2, 9.6)			2.9 (0.43, 7.2)		
- Large cell carcinoma	4	6.3 (1.8, 8.1)			6.2 3.8, 8.0			7.1 (6.4, 12.7)		
- Carcinoid	2	1 (0.28, 1.8)			2.3 (0.42, 4.2)			0.56 (0.2, 3.3)		
Small cell carcinoma	2	4.5 (3.0, 6.1)			5.7 (2.4, 9.1			3.8 (0.8, 8.4)		
Secondary tumours	15	1.6 (0.35, 13.5)			2.4 (1.3, 7.3)			2 (0.12, 8.0)		
pT1	11	2.8 (0.49, 29.6)			4.1 (1.6, 16.4)			2.6 (0.4, 18.9)		
pT2	46	3 (0.83, 9.1)			3.5 (1.3, 9.6)			2.6 (0.53, 7.4)		
pT3	16	3.6 (0.19, 14.4)			3.3 (0.55, 11.9)			3.3 (1.5, 11.4)		
pT4	7	2.2 (0.42, 7.2)			2.9 (0.45, 7.1			2.8 (0.7, 7.9)		
pN0	25	3.1 (0.42, 9.1)			3.7 (0.55, 7.5)			2.9 (0.4, 8.8)		
pN1	27	3.2 (1.2, 10.1)			3.8 (1.8, 9.6)			3.2 (0.7,11.3)		
pN2	17	2.8 (0.46, 14.4)			2.6 (1.6, 11.8)			2.2 (1.1, 7.2)		
pN3	11	2.3 (0.49, 7.2)			3.3 (1.6, 8.2)			2.4 (0.5, 7.9)		
pTNM I	18	3 (0.61, 29.6)			3.9 (1.2, 16.4)			2.7 (0.4, 8.8)		
pTNM II	17	3.2 (1.2, 8.3)			3.8 (1.8, 8.0)			2.7 (0.9, 9.9)		
pTNM IIIa	24	2.9 (0.46, 10.7)			3 (1.3, 11.8)			3 (1.1, 11.3)		
pTNM IIIb	12	2.3 (0.42, 7.2)			3.3 (0.45, 8.2)			2.2 (0.48, 7.9)		
pTNM IV	9	3 (1.2, 10,1)			4.5 (2.1, 9.6)			3.6 (0.46, 8.4)		

Table IV. *Correlations.*

NSCLC n = 147		CathB$_{AT}$ Tu/Lu[a]	Cath B$_{A7.5}$ Tu/Lu	Cath B$_C$ Tu/Lu	Cath L$_C$ Tu/Lu	uPA Tu/Lu	PAI-1 Tu/Lu	uPAR (ADI) Tu/Lu	HD13 Tu/Lu	IIIF10 Tu/Lu
Cath B$_{A7.5}$	r	0.62/0.55								
	p <	0.01/0.01								
Cath B$_C$	r	0.31/0.58	0.42/0.6							
	p <	0.01/0.01	0.01/0.01							
Cath L$_C$	r	0.28/0.30	0.43/0.36	0.46/0.39						
	p <	0.05/0.01	0.01/0.01	0.01/0.01						
uPA	r	0.29/0.15	0.40/0.07	0.48/0.19	0.29/-0.019					
	p <	0.05/--	0.01/--	0.01/0.05	0.05/--					
PAI-1	r	0.56/0.26	0.58/0.24	0.08/0.28		0.43/0.09				
	p <	0.01/0.05	0.01/0.05	--/0.05	--/--	0.01/--				
uPAR (ADI)	r	0.56/0.21	0.19/0.05	-0.038/0.22		0.23/0.15	0.58/0.19			
	p <	0.01/0.05	0.05/--	--/0.05	--/--	0.05/--	0.01/0.05			
HD13	r	0.58/0.26	0.32/0.12				0.56/0.06	0.81/0.55		
	p <	0.01/--	0.01/--	--/--	--/--	--/--	0.01/--	0.01/0.01		
IIIF10	r	0.57/0.47	0.35/0.37	0.29/0.47		0.39/0.09	0.49/0.18	0.74/0.29	0.74/0.06	
	p <	0.01/0.01	0.01/0.01	0.05/0.01	--/--	0.01/--	0.01/--	0.01/0.01	0.01/--	
TF	r	0.27/0.23		0.17/0.08		0.19/0.07	0.21/0.34	0.36/0.083	0.27/0.075	0.37/0.15
	p <	0.05/0.05	--/--	--/--	--/--	0.05/--	0.05/0.01	0.01/ --	0.05/ --	0.01/--

[a]Tu: Tumour; Lu: Lung

of the Spearman rank correlation coefficient was evaluated by analysis of variance (ANOVA).

Univariate analysis of survival probability was performed by Kaplan and Meier analysis (52), using the log-rank test for the determination of statistical significance between the survival curves. Multivariate analysis was performed using the Cox proportional hazard model (53) and a stepwise forward logistic regression approach. The discrimination levels to differentiate between subgroups of patients were calculated by the Critlevel programme (54). Several statistical packages (PC-Statistik by TOPSOFT, Hannover, Germany; Statistika by Statsoft, Hamburg, Germany, SPSS by SPSS Inc, Chicago, IL, USA) were applied.

Results

The results of the determination of cath B$_A$ activity (cath B$_{AT}$, cath B$_{A7.5}$) and of the protein levels of five biological parameters cath B$_C$, cath L$_C$, uPA, PAI-1, and uPAR (ADI) in cancer tissue and in adjacent lung parenchyma of the same patient (n=147) are listed in Tables I and II. Furthermore, we measured two additional uPAR variants, uPAR (HD13), uPAR (IIIF10) and TF in 95 out of 147 tissues as shown in Table III. Altogether, 10 biological parameters were analyzed.

For all parameters, we found increased median values in tumour tissue (Tu) of various histologies among NSCLC compared with lung parenchyma (Lu). The biological parameters are listed from highest to lowest ratio: cath B$_{CTu}$/cath B$_{CLu}$: 8.5:1; cath L$_{CTu}$/cath L$_{CLu}$: 6.6:1; uPA$_{Tu}$/uPA$_{Lu}$: 6.5:1; cath B$_{ATTu}$/cath B$_{ATLu}$: 5.1:1; PAI-1$_{Tu}$/PAI-1$_{Lu}$: 4.2:1, uPAR$_{Tu}$/uPAR$_{Lu}$ (HD13): 4:1; uPAR$_{Tu}$/uPAR$_{Lu}$(IIIF10): 2.6:1; cath B$_{A7.5Tu}$/cath B$_{A7.5Lu}$: 2.5:1; uPAR$_{Tu}$(ADI) /uPAR$_{Lu}$(ADI): 2.2:1, TF$_{Tu}$/TF$_{Lu}$ 1.3:1. With the exception of TF, this increase in tumour tissue compared to its lung counterpart was statistically significant ($p<0.01$) in all cases.

Correlation and associations with clinicopathological parameters. In addition to the paired comparison between lung cancer tissue and corresponding lung parenchyma, all 10 parameters

Table V. *Univariate survival analyses of NSCLC.*

Variable	Unfavourable *vs* favourable characteristics[a]	*P*-value
pTNM-staging	TNM IIIb, IV *vs* TNM I, II, IIIa	< 0.001
Cysteine proteases		
Cath B_{AT}	>758 *vs* < 758 (μEU mg^{-1})	0.025
Cath B_C[b]	<1130 *vs* > 1130 (ng mg^{-1})	0.05
Cath $B_{A7.5}$	>667 *vs* < 667 (μEU mg^{-1})	n.s.
Cath L_C	>387 *vs* < 387 (ng mg^{-1})	n.s.
Serine proteases and receptors		
PAI-1	>33.2 *vs* < 33.2 (ng mg^{-1})	0.001
uPAR (HD13)	>4.6 *vs* < 4.6 (ng mg^{-1})	0.009
uPAR (ADI)	>4.4 *vs* < 4.4 (ng mg^{-1})	0.018
uPAR (IIIF10)b	<2.3 *vs* > 2.3 (ng mg^{-1})	0.07 (n.s.)
uPA	>3.6 *vs* < 3.6 (ng mg^{-1})	n.s.
TFb	< 2.8 *vs* > 2.8 (ng mg^{-1})	n.s.
Sex	Male *vs* Female	n.s.
Age	>60 years vs <60 years	n.s.

[a]Cut-off values were calculated by a computer program developed by Abel *et al.* (53).
[b]Reverse correlation.

were analyzed for correlation to the histological cell type, tumour stage (TNM-stage) and tumor cell differentiation (G). The results are summarized in Tables I-III.

There was no significant difference between the median levels of both cath B, enzymatic activities (cath B_{AT}, cath $B_{A7.5}$) and protein level (cath B_C) and histological cell types. We also found no correlation of either type of cath B activity with the primary tumour size (pT-stage), whereas cath B_{AT}, cath $B_{A7.5}$ and cath B_C of primary tumours correlated with the presence of lymph node metastases (pN, $p=0.06$, $p=0.07$, $p<0.05$, respectively).

Only PAI-1 levels differed with the histological type of NSCLC. We found the highest values of PAI-1 in large cell carcinoma > SCC, AC, > carcinoid and the lowest values in metastases of primary tumours of other origin.

Furthermore, in large cell carcinoma, uPAR (ADI), uPAR (HD13) and uPAR (IIIF10) showed higher values than in other histological types. There was no significant relationship among different histological types with regard to cath L_C, uPA, or TF. Remarkably, carcinoids, which are low-grade neoplasms, showed low PAI-1, uPA, uPAR (HD 13, IIIF10) and low cath L_C levels.

With the exception of PAI-1, there was no significant association between all 10 biological parameters and either tumour stage (pTNM) or grading (G). Only PAI-1 was significantly increased in poorly-differentiated cells (G3) compared to well- or moderately-differentiated cells (G1/G2) ($p<0.05$). Furthermore, PAI-1, cath L_C and uPAR (ADI) were significantly higher in smokers compared to non-smokers (each $p<0.01$).

Correlations among all 10 biological parameters related to proteolysis. Correlations among all 10 biological parameters in NSCLC patients are listed in Table IV. Cath B_{AT}, cath $B_{A7.5}$ and cath B_C were correlated with each other, as already described earlier (Werle *et al.*, 1999). Significant correlations between cath B_{AT}, cath$B_{A7.5}$, cath B_C and cath L_C were found. PAI-1 significantly correlated with cath B_{AT} and cath $B_{A7.5}$, with uPAR (ADI), uPAR (HD13), uPAR (IIIF10), with uPA, and weakly with TF, but not with cath B_C and cath L_C.

The three different ELISA formats for measuring uPAR, which used antibodies against different epitopes of the same uPAR molecule, rendered excellent correlations between the test results for uPAR. All three forms of uPAR, detected by specific ELISA formats, correlated well with cath $B_{A7.5}$. The correlations were less pronounced for cath B_{AT} and there was no correlation to cath B_C. Finally, uPA correlated with cath B_{AT}, cath$B_{A7.5}$, cath B_C and cath L_C.

Impact of biological parameters (activities and protein levels) on patient survival. For prognostic assessment of NSCLC patients, the stage of the disease and histological cell type are the most important prognostic factors used in clinical routine. In order to evaluate the utility of biological parameters as new prognostic factors, we compared them by performing univariate and multivariate survival analysis.

Univariate survival analysis. Table V presents the prognostic significance of 10 biological parameters, *i.e.* cysteine-proteases and serine-proteases, their inhibitors and receptors in comparison with established prognostic factors such as pTNM-stage, age and sex of the patients.

Measured in tumour homogenates of NSCLC patients, cath B_{AT} and cath B_C showed significant prognostic value for a 7-year overall survival (median follow-up of 3.8 years; Table V and Figure 1). Of note, patients with high levels of cath B_C had a significantly better prognosis than patients

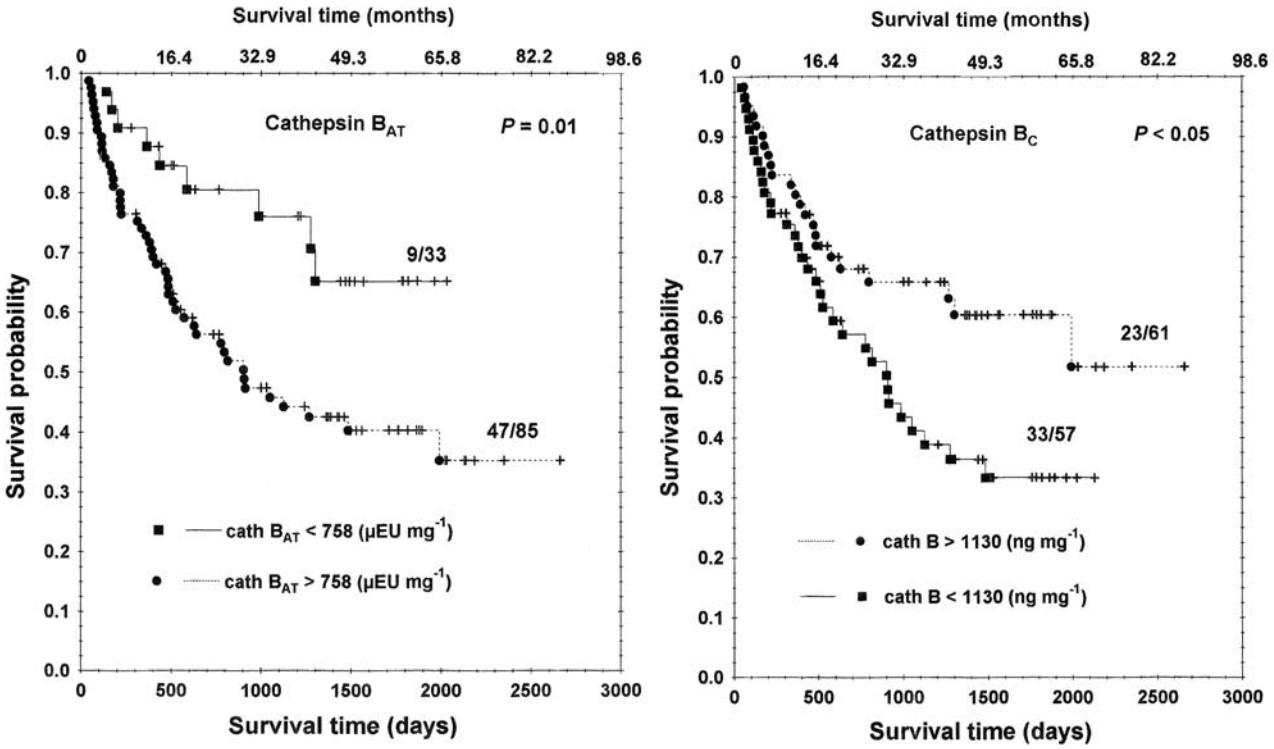

Figure 1. *Probability of overall survival of patients with NSCLC in relation to cath B_{AT} and cath B_C. The Critlevel program (54) calculated discriminative values of 758 (μEUmg^{-1}) for cath B_{AT} and 1130 (ngmg^{-1}) cath B_C. Altogether, 118 patients out of 147 were included in the survival analyses. In NSCLC, 47 out of 85 patients with cath B_{AT} higher than 758 (μEUmg^{-1}) had a significantly shorter overall survival (-●- deceased patients (uncensored data), -/- still living patients (censored data)) than 9 out of 33 patients with cath B_{AT} below the cut off value (-■- deceased patients (uncensored data), -/- still living patients). In NSCLC, 33 out of 57 patients with cath B_C lower than 1130 (ngmg^{-1}) had a shorter overall survival (-■- deceased patients, -/- still living patients) than 23 out of 61 patients with cath B_C above the cut-off value (-●- deceased patients, -/- still living patients).*

Table VI. *Univariate survival analyses of squamous cell and adenocarcinoma.*

Variable	Unfavourable *vs* favourable characteristics[a]			
	Squamous cell carcinoma	*P*-value	Adenocarcinoma	*P*-value
Cysteine proteases				
Cath B_{AT}	>1647 *vs* < 1647 (μEU mg^{-1})	n.s.	>1647 *vs* < 1647 (μEU mg^{-1})	n.s.
Cath $B_{A7.5}$	> 278 *vs* < 278 (μEU mg^{-1})	< 0.05	> 512 *vs* < 512 (μEU mg^{-1})	n.s.
Cath B_C	> 512 *vs* < 512 (ng mg^{-1})	n.s.	> 512 *vs* < 512 (ng mg^{-1})	n.s.
Cath L_C	> 317 *vs* < 317 (ng mg^{-1})	n.s.	> 403 *vs* < 403 (ng mg^{-1})	n.s.
Serine proteases and receptors				
PAI-1	> 32.8 *vs* < 32.8 (ng mg^{-1})	0.01	> 21.5 *vs* < 21.5 (ng mg^{-1})	n.s.
uPAR (ADI)	> 4.4 *vs* < 4.4 (ng mg^{-1})	n.s.	> 4.5 *vs* < 4.5 (ng mg^{-1})	n.s.
uPAR (HD13)	> 4.6 *vs* < 4.6 (ng mg^{-1})	n.s.	> 2.5 *vs* < 2.5 (ng mg^{-1})	n.s.
uPAR (IIIF10)	< 4.3 *vs* > 4.3 (ng mg^{-1})	n.s.	< 3.6 *vs* > 3.6 (ng mg^{-1})	n.s.
uPA	> 3.6 *vs* < 3.6 (ng mg^{-1})	n.s.	> 3.1 *vs* < 3.1 (ng mg^{-1})	n.s.
TF	> 4.5 *vs* < 4.5 (ng mg^{-1})	n.s.	> 4.5 *vs* < 4.5 (ng mg^{-1})	n.s.

[a] Cut-off values were calculated by a computer program developed by Abel *et al.* (53).
[b]Reverse correlation.

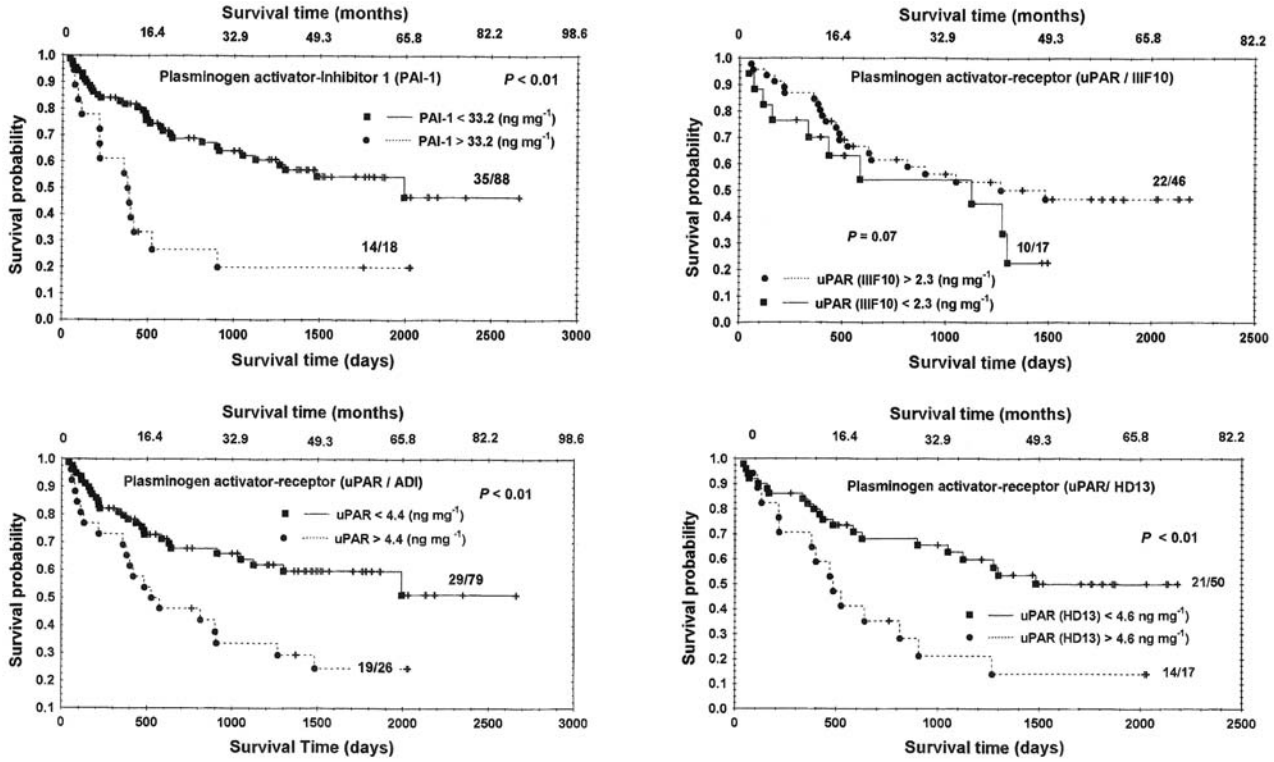

Figure 2. *Prognostic significance of PAI-1 and uPAR (ADI, HD13, IIIF10) for overall survival of patients with NSCLC. For PAI-1, altogether 106 patients out of 147 were included in the survival analyses. Fourteen out of 18 patients with PAI-1 levels higher than 33.20 (ngmg^{-1}) died in the observation period of 7 years (-●- deceased patients (uncensored data), -/- still living patients (censored data)) compared to 35 out of 88 patients with PAI-1 levels below the cut-off value (-■- deceased patients, -/- still living patients). For uPAR (ADI, HD13), altogether 105 patients out of 147, 67 out of 95 and 63 out of 95 , respectively, were included in the survival analyses. In NSCLC, 19 out of 26 patients with uPAR (ADI) higher than 4.4 (ngmg^{-1}) and 14 out of 17 with uPAR (HD13) higher than 4.6 (ngmg^{-1}) had a significantly shorter overall survival (-●- deceased patients (uncensored data), -/- still living patients (censored data)) than 29 out of 79 patients with uPAR (ADI) and 21 out of 50 with uPAR (HD13) below the cut-off value (-■- deceased patients, -/- still living patients). In contrast, 10 out of 17 patients with uPAR (IIIF10) lower than 2.3 (ngmg^{-1}) had a shorter overall survival probability than 22 out of 46 patients with uPAR (IIIF10).*

Table VII. *Cox regression analyses of NSCLC.*

Variable	Unfavourable *vs* favourable characteristics[a]	Relative risk	*P*-value
pTNM-staging	TNM IIIb, IV *vs* TNM I, II, IIIa	--	< 0.0001
pT	pT1 *vs* pT2 *vs* pT3 *vs* pT4	--	n.s.
pN	pN0 *vs* pN1 *vs* pN2 *vs* pN3	--	n.s.
Histology	Squamous cell -, Adeno -, Large cell carcinoma	--	n.s.
Cysteine proteases		--	
Cath B_{AT}	>1460 *vs* < 1460 (μEU/mg)	--	n.s.
Cath $B_{A7.5}$	>555 *vs* < 555 (μEU/mg)	--	n.s.
Cath B_C[c]	<1302 *vs* > 1302 (ng/mg)	--	n.s.
Cath L_C	> 407 *vs* > 407 (ng/mg)	--	n.s.
Serine proteases and receptors			
PAI-1[b]	>33.2 *vs* < 33.2 (ng/mg)	2.6 (6.6 - 17.3)	< 0.001
uPAR (ADI)	>3.6 *vs* < 3.6 (ng/mg)	--	n.s.
uPAR (HD13)	>4.1 *vs* < 4.1 (ng/mg)	--	n.s.
uPAR (IIIF10)b,c	< 2.3 *vs* > 2.3 (ng/mg)	0.24 (0.08 - 0.67)	< 0.01
uPA	>2.1 *vs* < 2.1 (ng/mg)	--	n.s.
TFc	< 11 *vs* > 11 (ng/mg)	--	n.s.
Chemotherapy	(+) *vs* (-)	--	n.s.

[a]In continuous variables mean values are given. [b]Median values are given. [c]Reverse correlation.

with low cath B_C levels (Table V and Figure 1). In contrast, cath $B_{A7.5}$ had no significant prognostic impact in the total study population of NSCLC but it became significant in the subgroup of SCC patients (Table VI; $p<0.05$). In AC no significant correlation with survival could be found.

Regarding the plasminogen-activator system, we found, in the total study group of NSCLC, significant correlations with overall survival probability for PAI-1, uPAR (ADI), uPAR (HD13) and uPAR (IIIF10) (Table V, Figures 2 - 4; $p<0.01$, $p<0.05$, $p<0.01$, $p=0.07$, respectively). In squamous cell carcinomas, only PAI-1 significantly correlated with overall survival probability (Table VI). In patients with AC no significant correlation with overall survival could be found. There was no correlation between survival probability and uPA or TF, either in the total study population of NSCLC or in the subgroups of SCC and AC.

Multivariate analysis. In multivariate analysis, only those proteolytic factors with a significant impact on overall survival probability in univariate analysis, additional systemic treatment (chemotherapy) as well as established prognostic factors, were included.

PAI-1 and uPAR (IIIF10) remained the best independent prognostic factors for patients with NSCLC (Table VII).

Discussion

This study clearly showed that the median levels of cath B_{AT}, cath $B_{A7.5}$, cath B_C, cath L_C, uPA, PAI-1, and uPAR [uPAR (ADI), uPAR (HD13), uPAR (IIIF10)] were significantly higher in tumour tissue of NSCLC patients compared to the corresponding lung parenchyma. All reports in the literature agree on that point that the biological parameters studied here are significantly increased in tumour homogenates or are highly expressed in tumour cells on tumour tissue sections compared to their respective lung counterpart (2, 6, 10, 14, 20, 21, 23, 34, 35). Moreover, after long-term follow-up of seven years (median follow-up of 3.8 years), the activity of cath B (cath B_{AT}) and protein level (cath B_C) of the cysteine protease cath B as well as the concentrations of PAI-1 and uPAR [uPAR (ADI), uPAR (HD13), uPAR (IIIF10)], two members of the plasminogen activator system, provide significant univariate prognostic information for patients with NSCLC. Out of these three proteins, only PAI-1 and uPAR (IIIF10) proved to be independent prognostic factors.

Cysteine proteases. Our results for cath B are in agreement with earlier studies by Ebert *et al.* (23) and Werle *et al.* (26). Cath B_{AT} and cath $B_{7.5}$ were of prognostic significance in the observation period of 6 months (cath B_{AT}, $p<0.05$; 23), 15 months (both, $p<0.05$; unpublished) and 24 months (cath $B_{A7.5}$, $p=0.06$, 26). In the subgroups of histologies, the prognostic relevance of cath $B_{A7.5}$ was found for patients suffering from SCC after 24 months ($p<0.05$, 25, 26) and after 7 years ($p<0.05$). In contrast, in AC no prognostic impact of cath B_{AT}, cath $B_{A7.5}$ and cath B_C was found in a short (6, 15 and 24 months) and long (5 years) observation period. However, a discrepancy exists regarding the prognostic information of cath B in tumour tissue homogenates. After a five-year observation period, we found that cath B activities, cath B_{AT}, cath $B_{A7.5}$ and the concentration of cath B_C measured in tissue homogenates had no prognostic relevance in the total population of NSCLC (14). In addition, several immunohistochemical studies showed that the overexpression of cath B in tumour cells only provides prognostic information, as could be demonstrated by univariate and multivariate survival analysis (9-11) Our findings (14) agree with IHC analyses of cath B in tumour cells. In contrast to these studies, Mori *et al.* (13) could not demonstrate a significant correlation between cath B overexpression and overall survival probability of 31 lung cancer patients, all in TNM stage I.

The discrepancy observed in the results of survival analyses are probably due to: i) different extraction procedures, ii) semi-quantitative analytical methods for tumour tissue sections, iii) prolonged observation periods, iv) a set of various cut-off values, v) divergent antibodies resulting in altered ELISA-formats or changed staining pattern on tissue sections for the detection of biological parameters, vi) different treatment of patients after primary surgery and vii) the cellular heterogeneity of the tumour.

This is particularly true for lung tumours, which are heavily infiltrated by inflammatory cells (55, 56). Kayser *et al.* (57) clearly showed that infiltrations of lung tumours are in close correlation to tumour volume, containing besides tumour cells also surrounding stromal cells *i.e.* fibroblasts, pneumocytes type I and II, lymphocytes and histiocytes. Each cell type, particularly the histiocytes, contribute to the protease level in a solid tumour. On one hand, cathepsins or other proteases derived from histiocytes may assist tumour cells in degrading interstitial matrix components and the basement membrane (58) or they may, on the other hand, also destroy the tumour. This may explain the fact that, in spite of greatly increased levels, cath B was at borderline significance for prognosis of patient survival, contrary to the reports on other types of carcinomas such as head and neck (59, 60), in oral squamous cell carcinoma (61), laryngeal carcinoma (62, 63), breast (64, 65), pancreatic adenocarcinoma (66), gastric carcinoma (67), colorectal carcinoma (68), chondrosarcoma (69) and melanomas (70, 71).

Our previous results of cath B, using immunohistochemical analysis (14), demonstrated a controversial role of histiocytes depending on lung tumour histology. In SCC patients, the expression of cath B in histiocytes had an impact on longer

survival, while in patients with AC the expression of cath B points to shorter overall survival. Our findings were further supported by a recent paper of Kayser *et al.* (19), which described that NSCLC patients whose tumour cells stained cath B-negative and macrophages-positive had a significantly better prognosis than the opposite, *i.e.* tumour cell with positive and macrophage with negative cath B expression. Furthermore, it has been suggested that only a minor fraction of tumour cell-associated cath B, perhaps a malignant, extracellular and/or intracellular cath B isoform, seems to be involved in the degradation of the extracellular matrix (44, 72-76).

Cysteine proteases of the cathepsin-type were controlled by their natural inhibitors: the cystatins. Tissue homogenates, *i.e.* tumour and host cells, consist of a mixture of cathepsins and cystatins. Because the inhibition constant (K_i) between cath B and cystatins A, B and C (stefins A and B) is rather low, cath B will tightly bind cystatins and form cath B-cystatin complexes. In such cath B-cystatin complexes cath B is deactivated. Using the same study population, we found that in tumour tissue the concentration of cystatins A and B was significantly increased as compared to the normal counterparts (31, Werle *et al.*, unpublished). This might, at least partly, explain, why we found a controversial role of cath B_C and cath B_{AT} and survival probability. In a study published by Knoch *et al.* (30), we showed that the imbalance between cath B and cystatins provides prognostic information. Although we could demonstrate that the concentrations of cystatins were increased in tissue homogenates (30, 31, Werle *et al.*, unpublished), cystatins may be less effective in binding cath B, which facilitates the invasion process of tumour cells (29, 77). Therefore, the cellular origin of proteases, malignant isoform(s), their subcellular distribution as well as their corresponding endogenous inhibitors, may be decisive factors in the relationship between the protease levels and the survival probability of lung cancer patients.

Serine proteases. Pederson *et al.* (34, 35) measured uPA, PAI-1 and uPAR in 106 patients with AC and in 84 patients with SCC of the lung. These authors found that high levels of PAI-1 in AC and high levels of uPAR in SCC were significantly associated with overall survival. No conclusion concerning PAI-1 and uPAR in the total study population of NSCLC has been made.

In the present study, we describe that PAI-1 and uPAR, measured as uPAR (ADI), uPAR (HD13) and uPAR (IIIF10) format, have prognostic impact in patients with NSCLC. Furthermore, PAI-1 proved to be a prognostic factor in SCC. Salden *et al.* (36) could not find a significant relationship with survival probability of PAI-1 and uPAR, determined in tumour tissue extracts, either in the group of all patients or in the subgroups of patients with SCC or AC. Immunohistochemical studies of Pavey *et al.* (37) showed that PAI-1 and not uPA was associated with the survival probability of SCC patients, while Volm *et al.* (38) could demonstrate that only uPA and not PAI-1-positive carcinomas correlated with survival probability.

However, for uPAR antigen determination, we used three different ELISA formats. As shown in previous studies (48), all three ELISA formats detect recombinant human CHO-suPAR and human uPAR antigen in lysates of non-malignant keratinocytes, mammary epithelial cells, omental cells and peripheal blood mononuclear cells with similar sensitivity (48). Interestingly, when analysing a series of breast cancer cell lines, the ELISA using mAb IIIF10 detected tumour-associated uPAR antigen with higher sensitivity. Furthermore, uPAR (HD13) was directed to a conformational epitope of glycosylated uPAR and uPAR (IIIF10) react with domain 1 of both glycosylated and non-glycosylated uPAR. Therefore, we determined three distinct isoform patterns of uPAR in lung tumour homogenates and control lung parenchyma. In control lung parenchyma we found two-fold higher levels of uPAR (IIIF10) compared to uPAR (HD13). This holds true for lung tumour tissue, where we found 1.4-fold higher protein levels, of uPAR (IIIF10) compared to uPAR (HD13). This was in contrast to the study of Kotzsch *et al.* (48), who found a similar uPAR concentration in cell lysates of non-malignant epithelial cells as determined by all three ELISA formats. These authors also found higher levels of uPAR (ADI), uPAR (HD13) and uPAR (IIIF10) in breast cancer tissue. However, only for uPAR (IIIF10) could a correlation with prognosis be determined. Elevated uPAR (IIIF10) levels were associated with poor prognosis, while for the other two types of uPAR protein levels, no significant correlation with survival could be found. In the study presented here, we found the opposite for uPAR (IIIF10) and a significant association with overall survival using uPAR (ADI) and uPAR (HD13). Therefore, we suggest that the significant differences in the pathomechanism of tumour invasion and metastasis between breast and lung cancer is most probably due to various cell types of the host, which seem to be involved (see also discussion in the paragraph "cysteine proteases").

uPA, PAI-1 and uPAR have been reported as prognostic factors in a variety of solid tumours (64). In breast cancer, tumour tissue levels of uPA and PAI-1 have reached the highest level of evidence for their prognostic impact in a prospective therapy trial as well as a large meta-analysis and are thus ready to be used for clinical decision making (78). Also, in our collective of NSCLC-patients, PAI-1 and uPAR measured by mAb IIIF10 have a very strong independent prognostic impact.

However, for future clinical studies, reproduceable conditions and documented quality management is strongly recommended. This was also emphasized in a recent paper of Levicar *et al.* (65).

In conclusion, out of the 10 biological parameters evaluated here, only cath B_{AT}, cath B_C, PAI-1 and uPAR [uPAR (HD13), uPAR (IIIF10)] in tumour homogenates of NSCLC patients had significant impact on patient prognosis after long-term overall survival. PAI-1 and uPAR (IIIF10) proved to be independent prognostic factors, enabling better prognostic discrimination together with established clinical and histopathological factors. This is consistent with evidence from other solid tumours such as breast cancer (48, 78). Further clinical studies are now needed in order to fully validate the role of these proteolytic factors for clinical decision making in NSCLC.

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