Expression of proteinase inhibitor-9/serpinB9 in non-small cell lung carcinoma cells and tissues

ILONA ROUSALOVA[1], EVZEN KREPELA[1], JAN PROCHAZKA[1], JAN CERMAK[2] and KAMILA BENKOVA[3]

[1]Laboratories of Molecular and Cell Biology and [2]Division of Surgery, Department of Pneumology and Thoracic Surgery, [3]Department of Pathology, University Hospital Bulovka, Prague, Czech Republic

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Abstract. Human proteinase inhibitor-9 (PI-9)/serpinB9 is an intracellular ovalbumin-family serpin with nucleocytoplasmic distribution which is expressed in certain normal cell types and cancer cells of different origin. Due to binding and inactivating of granzyme B (GrB), PI-9 can protect the cells from GrB-mediated apoptosis. High levels of PI-9 expression in certain cancer cells may contribute to their resistance against the immune mediated killing. So far, it is not known whether non-small cell lung cardinomas (NSCLCs) express PI-9 mRNA and a functional PI-9 protein. Herein we report for the first time that NSCLC cells express both PI-9 mRNA and protein and that there is a subset of NSCLC cells with upregulated PI-9 mRNA and protein expression. Futhermore, our work revealed that the PI-9 protein expressed in NSCLC cells can inhibit the active GrB. Finally, analysis of PI-9 mRNA expression in NSCLC tumours from surgically treated patients showed that the expression of this transcript is upregulated in the less-differentiated lung adenocarcinomas. We suggest, that the upregulated expression of PI-9 in NSCLC cells may serve to protect them from apoptosis induced by GrB.

Introduction

Lung cancer is the leading cause of cancer-related mortality around the world. Non-small cell lung cancer (NSCLC) accounts for most lung cancer cases. Surgical resection remains the single most consistent and successful option for cure. However, almost 70% of lung cancer patients present with locally advanced or metastatic disease at the time of diagnosis. Chemotherapy with concurrent radiotherapy is the treatment of choice for some locally advanced lung cancers, and palliative chemotherapy for the metastatic disease. The introduction of anti-angiogenesis agents and tyrosine kinase inhibitors targeting the epidermal growth factor receptors has resulted in improved response rates for selected groups of NSCLC patients. Despite a large number of clinical trials testing various anticancer treatment strategies, the 5-year survival rate reaches only 15% (1).

Human proteinase inhibitor-9 (PI-9/SERPINB9) is a 42-kDa serpin that is expressed in the cytoplasm and nuclei of certain normal cell types (2-4). PI-9 is a direct irreversible granzyme B (GrB) inhibitor which reacts with GrB with a stoichiometry of inhibition of 1:1 and has a high second order association rate constant, indicating that it is a fast physiological inhibitor of GrB (3). Recent findings indicate that PI-9 can inhibit not only the GrB/perforin-mediated death pathway but also the FasL/Fas death pathway (5,6). Both pathways lead to the activation of apoptotic executioner procaspase-3 and -7, as well as initiate permeabilization of the outer mitochondrial membrane via cleavage of the BH-3 only protein Bid and/or the Mcl-1 protein component of the Mcl-1•Bim complex (7-17). In addition, the secretory vesicles of human cytotoxic lymphocytes (CTLs) express also other granzymes (A, H, K and M), which may deliver alternative death signals that operate independently of caspase activation and mitochondrial permeabilization (18,19).

PI-9 is expressed abundantly in cells that express high levels of GrB, such as CTLs, i.e. natural killer (NK) cells and $CD8^+$ T cells (3,20,21). The main physiological function of PI-9 is to protect CTLs against the misdirected autonomous GrB during an immune response (21,22). Besides protecting the CTLs, PI-9 may also defend bystander cells or antigen-presenting cells likely to be exposed to GrB during an immune response. The expression of PI-9 in B cells (3), monocytes (23), mast cells (24), endothelial and mesothelial cells (25), smooth muscle cells (26) and dendritic cells (21,27) is consistent with such a role. Cells at immune-privileged sites including the eye lens capsula, testis, ovaries, placenta, embryonic stem cells also upregulate the PI-9 expression (20,25,28).

The expression of PI-9 was variably detected in human malignant tumours including colon carcinoma (29) and naso-pharyngeal carcinoma (30). In addition, certain cancer cell lines of breast, colon, cervix, and melanoma also showed

Correspondence to: Dr Evzen Krepela, Department of Pneumology and Thoracic Surgery, University Hospital Bulovka, Budinova 2, 180 81 Prague 8, Czech Republic
E-mail: evzen.krepela@fnb.cz

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different levels of PI-9 expression (29). So far, there is evidence that the upregulated expression of PI-9 in cancer cells may contribute to their resistance against the immune mediated killing and thus it may promote tumour growth and progression (31,32).

To obtain clues on the role of PI-9 in lung cancer we investigated the expression status of PI-9 in NSCLC cell lines and in NSCLC tumours and matched lungs from surgically treated patients. Herein we report for the first time that PI-9 mRNA and protein are expressed at variable levels in NSCLC cells and tumours and that PI-9 protein from NSCLC cells can inhibit the active GrB.

Materials and methods

Cell lines. NSCLC cell lines used in the present study were obtained from the following sources: CALU-1, SKMES-1, A549, SKLU-1 and COR-L23 from the European Collection of Cell Cultures, Salisbury, UK; LXF-289, COLO-699 and LCLC-103H from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; NCI-H520 and NCI-H1299 from the American Type Culture Collection (ATCC), Rockville, MD, USA. The small cell lung carcinoma (SCLC) cell lines NCI-H69, NCI-H146, NCI-H209, NCI-H345, NCI-H378, NCI-H82 and NCI-H446 were purchased from ATCC. Culture media and other suplements were from Sigma, St. Louis, MO, USA. The NSCLC cell lines were cultured in humidified atmosphere of air with 5% CO_2 at 37˚C in the Eagle's minimum essential medium supplemented with 5% foetal bovine serum, 26.2 mM $NaHCO_3$, 2 mM *L*-glutamine, 1% of a stock solution of non-essential amino acids, 0.25 μM $Fe(NO_3)_3$, 10 mg/l apotransferrin, and 10^5 IU/l of penicillin-G and 100 mg/l of streptomycin. After reaching confluency, the cells were scraped into a lysis solution for the isolation of total RNA or into the Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution for protein extracts and cytosols preparation (see below). The SCLC cell lines were grown in humidified atmosphere of air with 5% CO_2 at 37˚C in RPMI-1640 medium supplemented with 10% foetal bovine serum, 23.8 mM $NaHCO_3$, 2 mM *L*-glutamine, and 10^5 IU/l of penicillin-G and 100 mg/l of streptomycin. The cells were harvested by centrifugation at 240 x g and 4˚C for 10 min.

Patients and tissues. One hundred and fifty patients with NSCLC tumours were investigated in this study (median age, 62 years; age range, 39-78 years; 110 men and 40 women; 130 smokers and 20 non-smokers). The patients did not receive any chemotherapy or radiotherapy before surgery. The surgical treatment applied to the patients included lung lobectomy, bilobectomy or pneumonectomy, and regional lymph node dissection. The histopathological classification of lung tumours was carried out according to the World Health Organization criteria (33) and tumour staging was performed according to the international pTNM system (34). The following NSCLC types were examined in the present study: squamous cell lung carcinoma (SQCLC), lung adenocarcinoma (LAC), SQCLC + LAC mixed type tumours, large cell lung carcinoma (LCLC), sarcomatoid lung carcinoma (SLC) and undifferentiated lung carcinoma (UNDIF). Besides the NSCLC tumours we also examined two small cell lung carcinomas (SCLCs) from patients who did not receive chemotherapy or radiotherapy before surgery. Signed, written informed consent was obtained from each patient before entry to the study. The study was approved by the local institutional ethics committee.

Specimens of primary lung tumour and matched lung parenchyma (190-240 mg, wet mass) were excised immediately after surgery. Tumour samples were taken from a non-necrotic part of the tumour and lung samples were excised from lung parenchyma at a site located as distantly as possible from the tumour location. All tissue samples were snap-frozen in liquid nitrogen and were stored at -78˚C until isolation of RNA and protein extraction.

Isolation and quantification of total RNA. Total RNA was extracted from NSCLC cell lines and NSCLC and lung tissue samples with using the TriZol Reagent (Invitrogen, Paisley, UK) according to the manufacturer's instructions. All RNA samples had an A_{260nm}/A_{280nm} ratio >1.8 by spectrophotometric analysis in 10 mM Tris/HCl buffer, pH 7.5. The concentration of total RNA was determined by fluorometry using the RiboGreen RNA quantitation kit (Molecular Probes, Eugene, OR, USA) and the manufacturer's protocol.

Real-time RT-PCR analysis. The sequences and final concentrations of the oligonucleotide primers and probes used in real-time RT-PCR assays of expression of the investigated transcripts are indicated in Table I. The primers and probes were designed with the program Primer Express (Applied Biosystems, Foster City, CA, USA) and were synthesized at Proligo (Paris, France) and Applied Biosystems (Weiterstadt, Germany), respectively. The expression of PI-9 and ß-actin mRNAs (an internal reference transcript) was quantitated by a coupled real-time RT-PCR assay. The RT-PCR reaction mixtures of a total volume of 50 μl contained 25 μl of ThermoScript reaction mix (a buffer with 3 mM $MgSO_4$ and 200 nM of each dGTP, dCTP, dATP and dTTP) and 1 μl of ThermoScript Plus reverse transcriptase/platinum *Taq* DNA polymerase mix (both Mixes were from Platinum Quantitative RT-PCR ThermoScript One-Step system, Invitrogen), the respective primers and TaqMan probe at the indicated final concentrations (Table I), 40 units of inhibitor RNaseOUT (Invitrogen), and 200 ng of total RNA. The final concentrations of primers and TaqMan probes adopted for real-time RT-PCR quantification of each indicated transcript were determined in optimisation experiments. The real-time RT-PCR assays were run in triplicate or duplicate in MicroAmp Optical 96-well Reaction Plates on the ABI PRISM 7700 Sequence Detection System using Sequence Detection System software (all from Applied Biosystems). The reverse transcription was carried out at 58˚C for 30 min and the subsequent PCR amplification included a hot start at 95˚C for 5 min, 45 cycles of denaturation at 95˚C for 15 sec and of annealing/extension at 58˚C for 1 min. The threshold cycle (C_T) values of the amplification reactions, represented by the plots of background-subtracted fluorescence intensity (ΔFU) of the reporter dye (6-FAM or VIC) against PCR cycle number, were determined with the Sequence Detection System software. The statistical difference of the ß-actin mRNA-normalized target transcript expression in tumours and lungs was calculated

Table I. Primers and TaqMan probes used for real-time RT-PCR quantitation of expression of the investigated transcripts.

Transcript	GeneBank accession no.		Sequences	Final concentration
Proteinase inhibitor-9	NM_004155	Forward primer:	5'-TGGACCAAGCCAGACTGTATG-3'	400 nM
		Reverse primer:	5'-TGCACGAACTTGGACAGACA-3'	400 nM
		TaqMan probe:	5'-(6-FAM)CATCAACAATTCCCAAATGCCGAAGC(TAMRA)-3'	200 nM
ß-actin	NM_001101	Forward primer:	5'-CTGGCACCCAGCACAATG-3'	200 nM
		Reverse primer:	5'-GGGCCGGACTCGTCATAC-3'	200 nM
		TaqMan probe:	5'-(VIC)AGCCGCCGATCCACACGGAGT(TAMRA)-3'	200 nM

from the linearized ΔC_T data (i.e. $2^{-\Delta C_T}$) and the tumour/lung ratio of the ß-actin mRNA-normalized target transcript expression was calculated by means of the $2^{-\Delta\Delta C_T}$ method (35).

Preparation of protein extracts and cytosols and total protein determination. NSCLC cell lines were scraped into the Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution and sedimented by centrifugation at 240 x g and 4˚C for 10 min. The subsequent processing of cells was carried out at 0-4˚C and the resultant extracts and cytosols were stored in small aliquots at -78˚C until analysis. For Western blot analysis of PI-9 expression, the cells were disrupted by sonication in a lysis buffer containing 0.5% Triton X-100, 0.005% Tween-20, and a mixture of proteinase inhibitors, 1 mM Na_2EDTA, 50 μM E-64, 200 μM AEBSF and 25 μM pepstatin, in phosphate-buffered saline, pH 7.2-7.4. For the assay of procaspase-3/-7 activation by exogenous recombinant GrB, the cell lysis buffer was 20 mM HEPES/NaOH, pH 7.0, and contained 6 mM Zwittergent$^{3-12}$ Detergent, 10 mM NaCl and 1 mM Na_2EDTA. The cell homogenates were centrifuged at 40000 x g and 4˚C for 30 min. For preparation of cytosols, the cells were resuspended in 0.3-0.5 volumes of buffer 25 mM HEPES/NaOH, 4 mM Na_2EDTA, 1.5 mM $MgCl_2$, 10 mM KCl, pH 7.4, and were disrupted in a Dounce homogeniser using the pestle B. The homogenate was centrifuged at 200,000 x g and 4˚C for 60 min and the supernatant was collected as cytosol. Total protein concentration was determined by the bicinchoninic acid assay with using bovine serum albumin (BSA) as a standard (36).

Western blot analysis. The expression of PI-9 in NSCLC cell lines was investigated by denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with specific antibodies. Cell extract samples were preboiled for 5 min in 50 mM Tris/HCl buffer, pH 7.4, containing 2.5% SDS, 100 mM dithiothreitol, 5% glycerol and 0.01% Serva Blue G. Then, 100 μg protein samples were separated on 14%T/3%C polyacrylamide gels using the Tris-Tricine-SDS buffer system (37). The Precision Plus Protein Prestained Standards (BioRad, Hercules, CA, USA) were run in parallel to the protein samples. The separated proteins were electro-transferred onto sheets of Hybond P membrane (Amersham Pharmacia Biotech, Little Chalfont, UK) using a transfer buffer 48 mM Tris, 39 mM glycine, 1.3 mM SDS, and 20 v/v% methanol, pH 9.2. PI-9 protein was visualised by an immuno-detecting procedure coupled to an enhanced chemiluminiscence generating system. The primary antibody was the mouse monoclonal anti-PI-9 IgG_1 (PI9-17; Alexis Biochemicals, Lausen, Switzerland; Cat. No. ALX-804-457) and the secondary antibody was the goat anti-mouse IgG_1 HRP peroxidase conjugate (Sigma). The immunoblotting procedure was done at room temperature with the following steps: blocking with 5% Blotting Grade Blocker (BGB; BioRad) and 1% bovine serum albumin (BSA) in PBST buffer (20 mM NaH_2PO_4/80 mM Na_2HPO_4, 100 mM NaCl, and 0.1% Tween-20, pH 7.4) for 1 h, incubation with the primary antibody (at 1 μg/ml in 1% BGB and 0.2% BSA in PBST buffer) for 2 h, extensive washing with PBST buffer, incubation with the secondary HRP-conjugated antibody (at a dilution of 1:10000 in 1% BGB and 0.2% BSA in PBST buffer) for 1 h, extensive washing with PBST buffer, incubation with the ECL Plus Reagent (Amersham Pharmacia Biotech) for 5 min, and capture of the chemiluminescence signal on BioMax Light-1 film (Eastman Kodak, Rochester, NY, USA).

To find out whether PI-9 in the cytosol from NSCLC cells interacts with GrB, we incubated cytosol samples from several NSCLC cell lines with human recombinant GrB (hrGrB; Calbiochem, Luzerne, Switzerland; Cat. No. B63047) for 15 min at 37˚C. The reactions contained 72 ng of hrGrB per 100 μg of total cytosolic protein and run in 11.25 mM HEPES/NaOH buffer, pH 7.4, with 1.8 mM Na_2EDTA, 13.7 mM NaCl, 4.8 mM KCl, 0.68 mM $MgCl_2$, 0.81 mM Na_2HPO_4 and 0.15 mM KH_2PO_4. After denaturation and separation of proteins by SDS-PAGE and protein transfer onto the Hybond P membrane (see above), we probed for a simultaneous shift of hrGrB and PI-9 to higher M_r values using a mouse monoclonal anti-GrB IgG_1 antibody (2C5; Santa Cruz Biotechnology, Santa Cruz, CA, USA; Cat. No. sc-8022) and the anti-PI-9 antibody PI9-17, respectively. The immunoblotting procedure was the same as described above. As a negative control, a mixture of hrGrB (35 ng) and BSA (4.8 μg; Sigma, Cat. No. A-7030) was analysed in parallel.

Activation of procaspases by granzyme B. The endogenous and GrB-induced caspase-3-like activity in extracts from NSCLC cell lines was measured with 50 μM Ac-DEVD-AFC (Bachem, Bubendorf, Switzerland) as a fluorogenic substrate in 50 mM HEPES/NaOH buffer, pH 7.4, containing 10 mM dithiothreitol,

Table II. Real-time RT-PCR analysis of proteinase inhibitor-9 mRNA expression in non-small cell lung carcinoma and lung tissues.

Tumor type	N	ß-actin mRNA-normalised expression of PI-9 mRNA ($2^{-\Delta C_T}$)[b]		Statistical difference (P) of PI-9 mRNA expression in Tu versus Lu[c]	Tu/Lu ratio of PI-9 mRNA expression[b]	No. of patients with Tu/Lu PI-9 mRNA expression ratio ≥2 and ≤0.5
		Tumors (Tu)	Lungs (Lu)			
NSCLC	150[a]	0.0172 (0.0002-0.3737)	0.0173 (0.0003-0.4506)	0.816	0.9 (0.09-23.3)	26 (17%) and 28 (19%)
SQCLC	69	0.0172 (0.0002-0.2132)	0.0138 (0.0003-0.4506)	0.811	0.9 (0.16-23.3)	13 (19%) and 12 (17%)
LAC	56	0.0163 (0.0008-0.3737)	0.0294 (0.0015-0.2774)	0.275	0.8 (0.09-5.3)	8 (14%) and 16 (29%)
LCLC	7	0.0144 (0.0078-0.0988)	0.0082 (0.0056-0.0960)	0.382	1.8 (0.95-2.6)	3 (43%) and 0
SLC	4	0.0455 (0.0150-0.0643)	0.0499 (0.0274-0.0587)	0.885	1.1 (0.35-1.2)	0 and 1 (25%)
UNDIF	11	0.0146 (0.0024-0.1267)	0.0067 (0.0017-0.0802)	0.308	1.5 (0.53-4.6)	2 (18%) and 0

[a]A total of 150 NSCLC patients was studied including 69 pacients with SQCLC, 56 patients with LAC, 3 patients with SQCLC + LAC mixed type tumours, 7 patients with LCLC, 4 patients with SLC, and 11 patients with UNDIF. [b]Data are indicated as median with the range in parentheses. [c]Statistical difference of the ß-actin mRNA-normalised PI-9 mRNA expression in Tu versus Lu was calculated by the Mann-Whitney test.

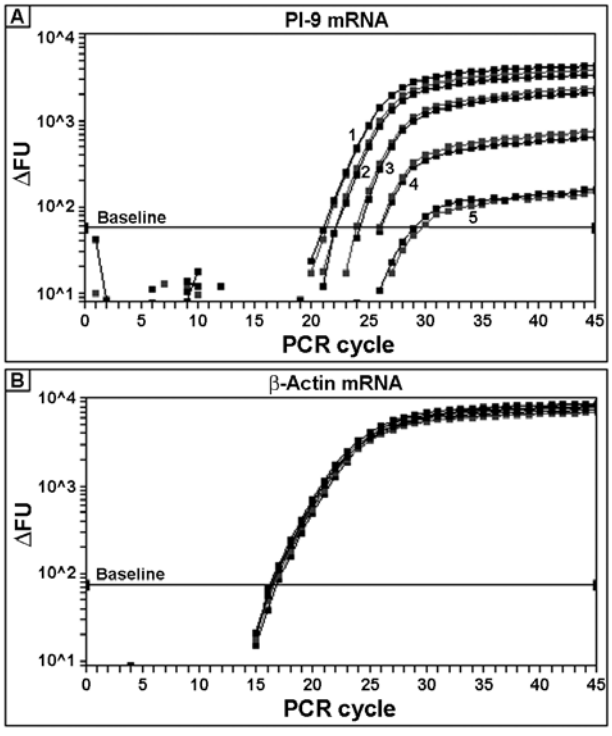

Figure 1. Real-time RT-PCR quantitation of expression of (A) PI-9 mRNA and (B) ß-actin mRNA (an endogenous reference transcript) in NSCLC cell lines (1, LXF-289; 2, CALU-1; 3, NCI-H1299; 4, SKLU-1; 5, COLO-699). All real-time RT-PCR assays were performed with an input of 200 ng of total RNA and were run in duplicate.

1.63 mM CHAPS, 1 mM Na_2EDTA, 292 mM sucrose and 100 mM NaCl, with or without adding of 160 ng/ml of purified cell-derived human granzyme B (Calbiochem). Enzymatic reactions were started with the addition of 20 μl of cell extract (25 μg total protein) to 180 μl of prewarmed buffer-substrate solution with or without GrB. Control reactions, which were done in parallel, run in the cell lysis buffer alone (i.e. a control for Ac-DEVD-AFC cleveage by GrB) or in the presence of the caspase inhibitor Ac-DEVD-CHO (1 μM). All enzymatic reactions were performed at 37˚C in two parallel samples in 96-well black microtiter plates (Corning, Acton, MA, USA) on the fluorometer SpectraFluor (Tecan, Grödig/Salzburg, Austria) using the excitation filter of 405 nm and the emission filter of 510 nm. The instrument was controled with the programme Biolise (Tecan). Fluorescence of the enzymatically released 4-amino-7-trifluoromethyl-coumarine (AFC) was measured in 30-sec intervals over a period of 60 min. The measurements were standardised with a set of different AFC concentrations in the reaction buffer. The caspase-3-like activities were calculated from the steady-state region of progress curves [where the correlation between (AFC) and the reaction time gave r>0.99] and were expressed in pkat/mg of total protein.

Statistical analysis. The statistical calculations were performed with the software SigmaStat (Systat Software, Point Richmond, CA, USA) and Stat200 (Biosoft, Cambridge, UK). A two-sided $P<0.05$ was considered as a statistically significant difference.

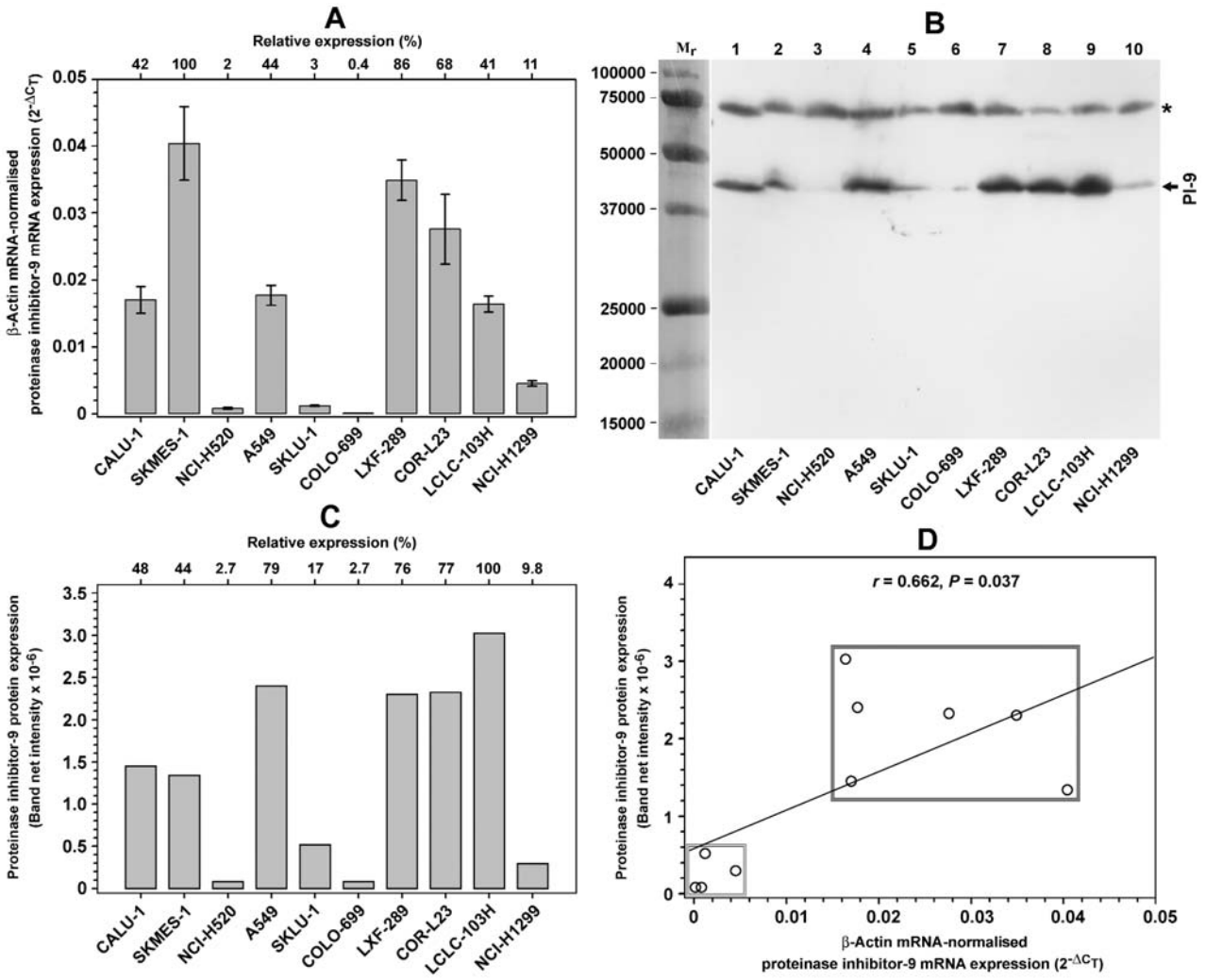

Figure 2. Analysis of PI-9 expression in non-small cell lung carcinoma cell lines. (A) Relative expression of PI-9 mRNA in the cell lines as quantitated by real-time RT-PCR. Data are indicated as mean ± SEM from three independent experiments. (B) Expression of PI-9 protein (M_r~43000) in the cell lines as analysed by SDS-PAGE and immunoblotting. The asterisk denotes an unknown immunoreactive protein (M_r~71300). (C) Relative expression of PI-9 protein as determined by image analysis of the immunogram shown in (B). (D) Correlation analysis of mRNA and protein expression for PI-9, respectively, in the cell lines. The rectangles define two groups of NSCLC cell lines: the high and low PI-9 expressors. The Pearson linear correlation coefficient r and its P-value are indicated.

Results

All NSCLC cell lines investigated in this study expressed PI-9 mRNA but the level of expression was highly variable (Figs. 1 and 2A). The expression of PI-9 mRNA was strong in six of ten examined NSCLC cell lines and it was weak in the remaining four (Fig. 2A). We also analysed the expression of PI-9 mRNA in SCLC cell lines which was lower, but not significantly, as compared to NSCLC cell lines (P=0.305; Mann-Whitney test). The median/range values of the ß-actin mRNA-normalized expression of PI-9 mRNA ($2^{-\Delta C_T}$) for NSCLC cell lines (n=10) and SCLC cell line (n=7) were: 0.0167/0.00008-0.0404 and 0.0037/0.0013-0.0118, respectively. Moreover, we could quantitate the expression of PI-9 mRNA in two SCLC tumours which was higher ($2^{-\Delta C_T}$=0.0319 and 0.0140) than in the examined SCLC cell lines.

The level of PI-9 protein, which was detected by Western blot analysis in the detergent-containing extracts or in cytosols from all studied NSCLC cell lines, was also highly variable (Figs. 2B and C; and 3B). There was a significant positive correlation between the expression of PI-9 mRNA and protein in the investigated NSCLC cell lines (Fig. 2D). Among the studied NSCLC cell lines we distinguished high and low expressors of both PI-9 mRNA and protein (Fig. 2D). They showed significant difference in the expression of PI-9 transcript as well as PI-9 protein (P=0.0095, Mann-Whitney test).

Since many serpins, including PI-9, are known to form SDS-stable covalent complexes with serine proteases including GrB (3,25,38,39), we attempted to detect formation of a complex between hrGrB and PI-9 protein present in the cytosol from NSCLC cell lines, using denaturing SDS-PAGE and immunoblotting. The incubation of cytosols from the cell lines with hrGrB resulted in a marked shift of the molecular mass of hrGrB to higher values (Fig. 3A) and a substantial decrease of the intensity of PI-9 protein band in parallel (Fig. 3B). Unfortunately, proving the molecular mass shift of

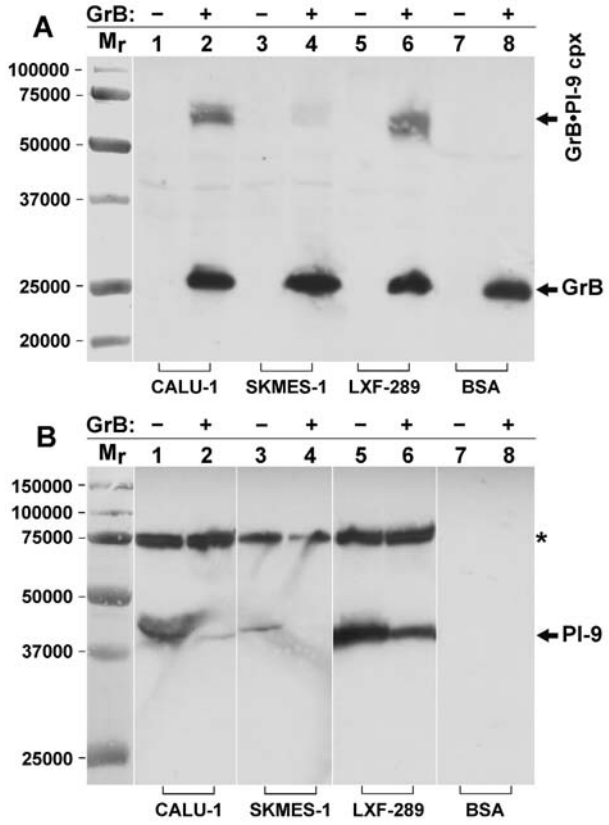

Figure 3. Interaction of human recombinant GrB with PI-9 in NSCLC cell cytosols. Cytosol samples from several NSCLC cell lines were incubated without (-) and with (+) human recombinant GrB and the reaction mixtures were analysed by denaturing SDS-PAGE and immunoblotting. As a negative control, a mixture of human recombinant GrB and bovine serum albumin (BSA) was used (lane 8 in A and B). (A) The membrane with transferred proteins was probed with an anti-GrB antibody (2C5). Arrows indicate an SDS-resistant GrB•PI-9 complex (cpx) and free GrB. (B) The membrane with transferred proteins was probed with an anti-PI-9 antibody (PI9-17). The asterisk denotes an unknown immunoreactive protein.

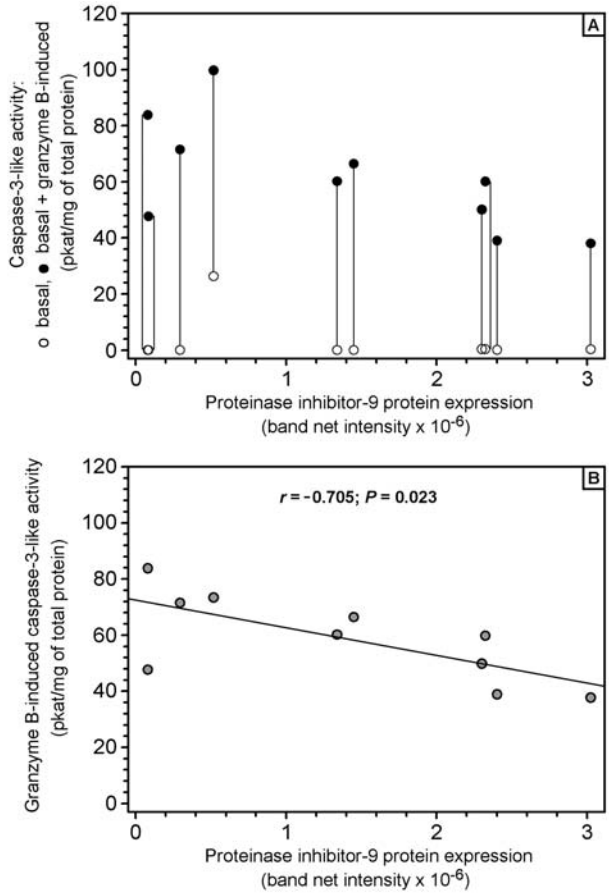

Figure 4. Relationship between the expression of PI-9 protein and the granzyme B-induced caspase-3-like activity in extracts from NSCLC cell lines. (A) Basal and total induced (i.e. basal + granzyme B-induced) caspase-3-like activity. (B) Correlation analysis of the granzyme B-induced caspase-3-like activity and the level of PI-9 protein expression in NSCLC cell lines. The Pearson linear correlation coefficient r and its P-value are indicated.

Table III. Impact of gender, smoking, tumour grade, and tumour stage on proteinase inhibitor-9 mRNA expression in non-small cell lung carcinomas.

Category	N[a]	ß-actin mRNA-normalised expression of PI-9 mRNA ($2^{-\Delta C_T}$)[b]	Statistical difference (P)[c]
Gender			
Men	110	0.0163 (0.0002-0.2365)	0.162
Women	40	0.0237 (0.0008-0.3737)	
Smoking			
Non-smokers	20	0.0155 (0.0002-0.1486)	0.746
Smokers	130	0.0178 (0.0005-0.3737)	
Tumor grade			
Grade 1+2	57	0.0131 (0.0007-0.2132)	0.023
Grade 3	67	0.0240 (0.0002-0.3737)	
Tumor stage			
Stage I	83	0.0163 (0.0002-0.2365)	0.125
Stage II+III	63	0.0192 (0.0008-0.3737)	

[a]The number, N, of examined NSCLC tissues belonging to the particular category is indicated. [b]Data are indicated as median with the range in parentheses. [c]Statistical difference was calculated by the Mann-Whitney test.

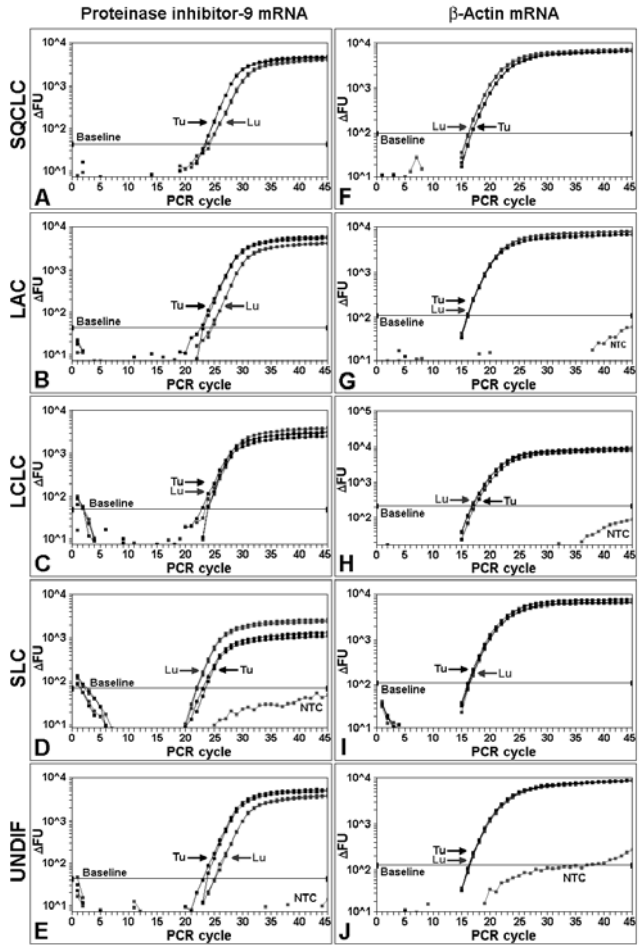

Figure 5. Real-time RT-PCR quantitation of expression of PI-9 and ß-actin mRNAs in non-small cell lung carcinoma and matched lung tissues. The set of PCR amplification plots, representing the coupled real-time RT-PCR assays with an input of 200 ng of total RNA and running in duplicate, shows the expression of PI-9 (A-E) and ß-actin (F-J) mRNAs in five tumour (Tu)-lung (Lu) matched pairs. The tumours were: squamous cell lung carcinoma (SQCLC), lung adenocarcinoma (LAC), large cell lung carcinoma (LCLC), sarcomatoid lung carcinoma (SLC), and undifferentiated lung carcinoma (UNDIF). NTC, no template control. ΔFU, background-subtracted fluorescence intensity (in arbitrary units) of the released reporter dye (6-FAM or VIC).

PI-9 to higher values was defeated in our experiments by the presence of an unknown protein reacting with the anti-PI-9 antibody PI9-17 and having M_r of ~71300 (Figs. 2B and 3B). A similar unknown protein was observed earlier in melanomas (32).

There is evidence that GrB cleaves and activates procaspase-3 in NSCLC cells and tissues (40). To assess whether the expression of PI-9 protein in NSCLC cells may affect the GrB-mediated procaspase-3 activation, we investigated the relationship between the PI-9 protein and the GrB-induced caspase-3-like activity levels. Our results showed that the PI-9 expression level negatively correlates with the GrB-induced caspase-3-like activity in NSCLC cell extracts (Fig. 4). This evidence supports the hypothesis that the overexpression of PI-9 can contribute to the resistance of NSCLC cells against the GrB-mediated apoptosis.

In order to reveal the relationship between PI-9 expression in NSCLC tumours and the clinicopathological data of NSCLC patients, we studied the expression status of PI-9 mRNA in the tumours and matched lungs from surgically treated patients. All examined NSCLC tumour types and lung tissues showed expression of PI-9 mRNA (Fig. 5). There was no statistically significant difference in the PI-9 mRNA expression between the tumours and lungs (Table II). However, in 26 (17%) of 150 studied NSCLC patients the tumours had more than two-fold higher level of PI-9 mRNA as compared to matched lungs. The expression of PI-9 mRNA in NSCLC tissues (Table II) and NSCLC cells (see the data above) was not significantly different (P=0.222; Mann-Whitney test). Furthermore, NSCLC tumours of various histopathological types showed comparable levels of PI-9 mRNA (Table II). Patients gender, smoking status and tumour stage did not significantly affect the expression of PI-9 mRNA (Table III). In contrast, PI-9 mRNA expression was significantly higher in the less-differentiated tumours (grade 3) as compared to the well-differentiated ones (grade 1+2) (Table III). Nevertheless, when SQCLCs and LACs were considered separately, the expression of PI-9 mRNA remained significantly higher in the less-differentiated LACs, but not in the less-differentiated SQCLCs (P=0.002 and P=0.591, respectively; Mann-Whitney test).

Discussion

In the present study, we demonstrated that both PI-9 mRNA and protein are expressed in NSCLC cells. Moreover, we revealed that PI-9 mRNA is expressed in SCLC cells at levels which were lower, but not significantly, as compared to NSCLC cells. There was a positive correlation between the levels of PI-9 mRNA and protein in NSCLC cells and, according to the differential abundance of PI-9 mRNA and protein expression, the cells could be classified as low and high PI-9 expressors. Differential expression of PI-9 mRNA in cancer cells was already reported for breast, cervical, oesophageal, gastric, colon, and nasopharyngeal carcinomas, and for melanomas and Ewing sarcomas (29,30,32,41-43). The mechanisms responsible *in vivo* for the differential expression of PI-9 in malignant tumours of the same type are not known so far. There is evidence that they may involve transcriptional upregulation of *SERPINB9* gene expression, mediated by nuclear factor-κB (44), the activated estrogen receptor-α (31,45) and/or the activator protein-1 (AP-1) transcription factors (44). Nevertheless, we detected only a weak or no increase of PI-9 mRNA expression in the interleukin-1ß- or estradiol-17ß-treated NSCLC cell lines (Rousalova *et al*, unpublished data). Since c-Jun and c-Fos, the major constituents of AP-1 transcription complex, seem to play an important role in tumorigenesis of NSCLCs (46-48), it is possible that the AP-1 transcription complex could be involved in the regulation of transcriptional expression of *SERPINB9* gene in NSCLC cells.

In order to reveal whether PI-9 in the cytosol from NSCLC cells can interact with GrB, we incubated hrGrB with cytosols from several NSCLC cell lines and searched for molecular mass shift of hrGrB and PI-9 using denaturing SDS-PAGE and immunoblotting. We found a marked shift of hrGrB to higher M_r-values and a concurrent decrease of PI-9 protein band intensity. The M_r-shift of PI-9 protein, resulting

from a covalent interaction of PI-9 with hrGrB, could not be reliably detected in our experiments due to the presence of an unknown immunoreactive protein which migrated to the same M_r-region as the hrGrB•PI-9 complex. Previously, the anti-PI-9 antibody PI9-17 was used for immunohistochemical detection of PI-9 protein expression *in situ* in normal and tumour cells (20,30,32). Since this antibody is not completely specific for PI-9 protein, the immunohistochemical data obtained with it should be interpreted with caution.

We observed significant negative correlation between the level of PI-9 protein expression and the GrB-induced caspase-3-like activity in extracts from NSCLC cell lines. This indicates that the overexpression of PI-9 can contribute to the resistance of NSCLC cells against the GrB-mediated apoptosis. Thus, the PI-9-dependent inactivation of GrB blocks the GrB-catalysed cleavage of many intracellular substrates including procaspase-3 and -7, Bid, DNA fragmentation factor subunit A, and certain growth- and survival-promoting receptors (16,17,40,49). There is evidence that PI-9 protein can be proteolytically inactivated by granzyme M (GrM) (50). Therefore, during the apoptotic attack of CTLs on the PI-9 expressing tumour cell targets, GrM internalised into the cytosol might attenuate the inhibitory effect of PI-9 on the delivered GrB.

Although the expression of PI-9 mRNA in NSCLC tumours and lungs was not significantly different, and patients gender and smoking status and tumour stage did not significantly affect the expression of PI-9 mRNA in the tumours, the less-differentiated LACs showed significantly higher expression of PI-9 mRNA as compared to the well-differentiated tumours. Thus, it is tempting to speculate that poorly differentiated NSCLC tumours with upregulated PI-9 expression might be more resistant to the GrB-dependent immune deletion. To overcome such immunoresistance, the tumour cell-specific overloading of GrB would be required. Taking advantage of *BIRC5* gene overexpression in lung cancer cells (51), the tumour cell-specific overexpression of GrB can be achieved via targeted delivery of recombinant DNA constructs consisting of a fusion of the *BIRC5* promoter to the coding sequence of active GrB (52).

In conclusion, the results of the present study provide evidence that NSCLC cells express both PI-9 mRNA and protein and that there is a subset of NSCLC cells with upregulated PI-9 mRNA and protein expression. Moreover, our work revealed that the PI-9 protein expressed in NSCLC cells can inhibit the active GrB. Finally, analysis of PI-9 mRNA expression in NSCLC tumours from surgically treated patients showed that the expression of this transcript is upregulated in the less-differentiated LACs.

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