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Expression and function of serpinB9 in lung cancer cells

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SUMMARY

Background: Granzyme B (GrB) is a key proapoptotic secretory protease of CTLs and NK cells. Its specific proapoptotic effects in cancer cells can be blocked by increased expression of serpinB9. *SerpinB9* gene expression can be transcriptionally upregulated by some interleukins and by the oestrogen activated oestrogen receptor-α (ERα) in cells which express ERα protein. The aims of my thesis were to evaluate the expression of SB9 and to examine its inhibitory activity against exogenous active GrB in non-small cell lung carcinoma (NSCLC) cell lines and tissues. To analyse the expression status of GrB mRNA in NSCLC cell lines and tissues. To investigate the role of estradiol-17β (E2), selected ILs and DNA methylation in regulation of SB9 expression in NSCLC cells.

The apoptosome apparatus is a cell death signalling platform activates the initiator procaspase-9. Activation of the apoptosome apparatus is often impaired in various types of cancer but the molecular basis of its suppression is still unknown. APIP and UACA/nucling belong to the endogenous regulators of apoptosome apparatus. The aim of my thesis was to investigate whether DNA methylation is involved in the transcriptional regulation of expression of *APIP* and *UACA* genes in NSCLC cell lines.

Methods: Following methods were used in this thesis: isolation and quantification of total RNA, real-time RT-PCR analysis, Western blot analysis, enzyme analyses, cell culture techniques and immunocytochemistry of ERα.

Results and conclusion: NSCLC cells express both SB9 mRNA and protein and there is a subset of NSCLC cell lines and tumours with upregulated SB9 mRNA and protein expression. Expressed SB9 protein is functional as it can interact with the active GrB via forming an irreversible complex GrB•SB9. SB9 mRNA expression was particularly upregulated in the less-differentiated adenocarcinomas from surgically treated patients. E2 and interleukins -1β, -6, and -18 do not markedly up-regulate the SB9 expression in NSCLC cells. On the contrary, DNA methylation can profoundly down-regulate the expression of SB9 in a subset of NSCLC tumours. This suggests that DNA demethylating drugs might desensitize NSCLC cells against the granzyme B-induced apoptosis through a strong induction of SB9 expression. NSCLC cells and tumours which are high expressors of SB9 may be protected, via the constitutively or inducibly high levels of SB9, against the GrB-mediated apoptosis during the immune attack executed by cytotoxic lymphocytes and NK cells. DNA methylation is not significantly involved in the regulation of transcriptional expression of *APIP* and *UACA* genes in NSCLC cells.

SOUHRN

Úvod: Granzyme B (GrB) je klíčová sekreční proapoptotická proteaza cytotoxických lymfocytů a NK buněk. Specifický proapoptotický efekt GrB v nádorových buňkách může být neutralizován zvýšenou expresí serpinuB9. Gen *serpinuB9* může být transkripčně pozitivně ovlivňován účinkem některých cytokinů a estrogenem aktivovaných estrogenových receptorů-α (ERα). Cílem této práce bylo zhodnotit expresi SB9 a vyšetřit jeho aktivitu proti exogennímu aktivnímu GrB v buněčných liniích a tkáních nemalobuněčného karcinomu plic (NSCLC), dále analyzovat expresi GrB mRNA v buněčných liniích a tkáních NSCLC a objasnit roli estradiolu-17β (E2), některých interleukinů a DNA metylace v regulaci exprese SB9 v NSCLC buňkách.

Apoptosom je proapoptotická signalizační platforma, která aktivuje iniciátorovou prokaspásu-9. Aktivace apoptosomu je často porušená v různých typech nádorů, avšak molekulární podstata jeho inhibice je neznámá. APIP and UACA/nucling patří mezi endogenní regulátory apoptosomového aparátu. Cílem této práce bylo zjistit, zda DNA metylace může ovlivňovat transkripční regulaci exprese genů těchto dvou proteinů v buněčných liniích a tkáních NSCLC.

Metody: Následující metody byly použity v této práci: izolace a kvantifikace celkové RNA, real-time RT-PCR, Western blot analýza, enzymové analýzy, techniky kultivace buněčných liniích a imunocytochemie ERα.

Výsledky a závěry: NSCLC buňky exprimují SB9 na úrovni mRNA i proteinu. U některých buněčných linií a vzorků nádorové tkáně od operovaných pacientů byla exprese SB9 mRNA a proteinu signifikantně zvýšená. SB9 protein exprimovaný v NSCLC buňkách je funkční a vytváří s aktivním GrB ireversibilní komplex GrB•SB9. U pacientů s méně nediferencovaným adenokarcinomem byla zjištěna zvýšená exprese SB9. E2 a interleukiny -1β, -6, and –18 nezvýšily expresi SB9 v NSCLC buňkách. Naopak, DNA metylace může významně snižovat expresi SB9 v některých buněčných liniích. Lze předpokládat, že DNA demetylující léky by mohly vést k rezistenci NSCLC buněk proti apoptóze zprostředkované GrB skrze silnou indukci exprese SB9. NSCLC buňky a tumory, které silně exprimují SB9, konstitutivně a/nebo inducibilně, mohou být chráněny proti apoptóze zprostředkované GrB. DNA metylace signifikatně neovlivňuje expresi genu *APIP* a *UACA* v NSCLC buňkách.

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1 INTRODUCTION

Susceptibility of cancer cells to enter apoptosis depends on their abilities to express the components of apoptosis pathways and efficiently activate them in response to extrinsic or intrinsic death stimuli (1). The death receptor- and the cytotoxic granule-induced pathways represent the extrinsic death pathways. Whereas, the mitochondrial, lysosomal and PIDDosome death pathways represent the intrinsic death mechanisms.

Both the intrinsic and the extrinsic apoptosis pathways converge on the activation of the effector procaspase-3, -6, and -7 (2-5) and they can lead to MOMP through cleavage of Bid protein and/or the Mcl-1 protein component of the Mcl-1•Bim complex (6-11).

My thesis is focused mainly on the role and regulation of expression of the intracellular serpinB9 which serves as an irreversible inhibitor (inactivator) of the serine proteinase GrB in cancer cells and certain normal cells such as lymphocytes and endothelial cells. GrB-induced apoptosis seems to be the most important death pathway for clearance of intracellular pathogens infected cells, allogeneic and tumour cells (12-16). After the recognition and conjugation of target cells by CTLs and NK cells, the cytotoxic secretory granules of CTLs and NK cells are vectorially transported toward the conjugation zone and their content is released into a intercellular cleft called IS (17,18). The most abundant components of cytotoxic granules are GrB and PFN. GrB is considered to be a major effector of NK cells (19). Furthermore, the cytotoxic granules of human CTLs and NK cells contain also other granzymes (A, H, K and M) (20), which may co-deliver alternative death signals that can operate synergistically with MOMP induction and procaspases activation (21-23) or independently of that (14,19,24-26). The precise mechanism of GrB translocation into the target cell cytoplasm is still unclear (27). Once in the target cell cytoplasm, GrB can trigger several apoptotic pathways through direct proteolytic activation of signalling or executioner components and cleaves multiple intracellular housekeeping proteins (Fig. 1).

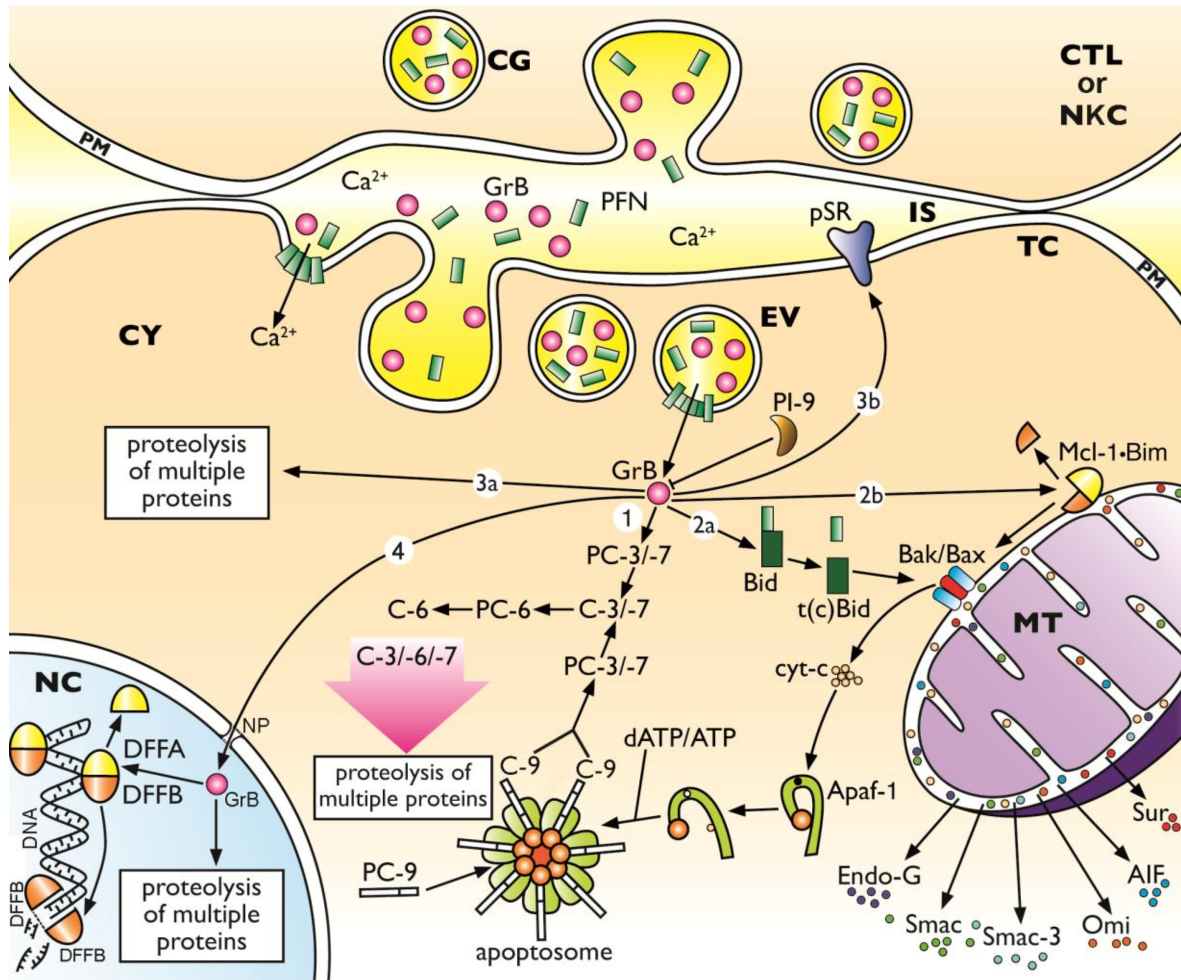

Figure 1. Granzyme B-induced death pathways in cancer cells. [1], Direct proteolytic processing and activation of the executioner procaspase-3 and -7, followed by the caspase-3-mediated activation of the executioner procaspase-6. The active executioner caspases cleave many intracellular proteins. [2], MOMP via the GrB-mediated proteolytic [2a] conversion and activation of protein Bid to the MOMP-inducing t(c)Bid fragment, and [2b] disruption of the Bim•Mcl-1 complex, involving fragmentation of the Mcl-1 component, and hence derepression of the MOMP-activity of Bim, followed by cytosolic release of several pro-apoptotic proteins (cyt-c, Smac, Smac-3, Omi/HtrA2, AIF and Endo-G) and the inhibitor-of apoptosis protein survivin (Sur). [3a], Proteolytic fragmentation of multiple housekeeping proteins in the cytoplasm, including [3b] the cytosol-facing signalling domains of some pro-survival plasma membrane receptors (pSR). [4], Translocation into the nucleus and proteolytic fragmentation of multiple intranuclear proteins, including the DFFA subunit of DNA fragmentation factor (DFFA•DFFB), which leads to the homodimerization-mediated activation of its deoxyribonuclease subunit DFFB. See the text for a more detailed description. CTL, cytotoxic lymphocyte; NKC, natural killer cell; CG, cytotoxic granule; IS, immunological synapse; PM, plasma membrane; TC, target cell; EV, endocytic vesicle; CY, cytoplasm; MT, mitochondrion; NC, nucleus; NP, nuclear pore; PC, procaspase; C, caspase.

After MOMP, holocytochrome-c is released into the cytoplasm and interacts with Apaf-1 monomer through its WD40 repeats, thus triggering, in the presence of (d)ATP, the formation of a heptametrical Apaf-1 protein complex called apoptosome. Apaf-1 apoptosome becomes an allosteric activator of PC-9 and initiates a caspase activation cascade, terminating with proteolytic devitalisation and apoptotic destruction of the cells (28-30). Regulation of Apaf-1 and PC-9 expression is important in predisposition of cells to activate the apoptosome apparatus (31-35).

The activation of apoptosome apparatus is frequently impaired in NSCLC cells and tissues (36,37). The molecular mechanism of apoptosome suppression in NSCLC is still unknown. There is evidence that suppression does not involve the segregatory binding of PC-9 to TUCAN (38) or direct inhibition of caspase-9 by XIAP (37,39). The Apaf-1 interacting protein (APIP/AIP) and the uveal autoantigen with coiled coil domains and ankyrin repeats (UACA)/nucling belong to the endogenous regulators of apoptosome apparatus (40,41), but their role in NSCLC tumourigenesis and progression is not clear. APIP is a cytosolic protein which binds to the CARD domain of Apaf-1 and thus prevents PC-9 recruitment to the apoptosome (42,43). Furthermore, APIP triggers sustained activation of AKT and ERK1/2 kinases under hypoxic condition. These kinases directly phosphorylate PC-9 and thus inhibit its activation in the apoptosome (47). UACA/nucling was found in both cytoplasmic and perinuclear/nuclear localization (45-47). During proapoptotic stress, UACA/nucling interacts with Apaf-1 and induces its translocation into the nucleus (48). Moreover, UACA also interacts with the NF-κB and blocks its entry into the nucleus and thus reducing expression of the NF-κB-targeted genes (49).

It remains enigmatic how one CTL can kill multiple target cells over longer periods of time without self-destruction and why tumour cells or virus-infected cells became resistant to granule-mediated apoptosis. In the past period, several candidate regulators of GrB activity have been identified. First, the serine proteinase inhibitor serpinB9 (SB9) was identified and established as a powerful GrB inhibitor (50,51). Second, other granzymes, such as GrM and GrH, which are co-secreted with GrB have a potential to influence the activity of GrB indirectly. It has been showed that both granzymes can promote GrB activity through the direct cleavage and inactivation of its inhibitors. The GrM destroys SB9 in a variety of target cells (24) whereas the GrH attacks L4-100K in the virally infected cells (52). Moreover, certain cell surface-bound and/or secreted proteases, such as

cathepsin B, might control the susceptibility of various tumour cells to the CTL-mediated killing via the proteolytic inactivation of PFN (cf. 53).

SerpinB9

Human SB9 is a 42-kDa intracellular protein. It is a member of the serpin superfamily, an ovalbumin family serpin (i.e. a clade B serpin) (54,55). By definition, members of this family lack a classical secretory signal peptide (55,56). SB9 is very efficient and a highly specific physiological inhibitor of GrB (K_{ass} = 1.7 x 10^6 $M^{-1}\cdot s^{-1}$) that protects CTLs themselves as well as bystander cells from misdirected GrB (51,57,58).

SERPINB9 gene organization and expression, and subcellular localization and function of serpinB9

Human *SERPINB9* gene is localized to a gene cluster on the chromosome 6 at p25. Besides the *SERPINB9* gene, the 6p25 region contains also *SERPINB1* gene, encoding MNEI, and *SERPINB6* gene, encoding PI-6 (59-63). *SERPINB9* gene comprises of seven exons and six introns (63,64). The translation start site resides in exon 2 and the RCL, a region of the SB9 protein which is proteolytically attacked by GrB, is located in exon 7 (64).

SB9 is abundantly expressed in cells that produce high levels of GrB, i.e. in $CD8^+$ T cells and NK cells (51,58,65). Its major physiological function is to defend these cells against the misdirected autonomous GrB (58,66). Moreover, SB9 may also protect bystander cells or antigen-presenting cells likely to be exposed to GrB during an immune response (58,66). Consistent with such a role is the expression of SB9 in B cells (51), monocytes (67), mast cells (68), endothelial and mesothelial cells (69), smooth muscle cells (70) and dendritic cells (58,71). Cells at immune-privileged sites, including the eye lens capsula, testes, ovary, placenta, and embryonic stem cells, also up-regulate the SB9 expression (65,69,72).

Highly variable levels of SB9 expression were also detected at in human cancer cells of carcinomas of the breast, cervix, nasopharynx, esophagus, stomach, colon, and lung, and melanomas (73-78). The mechanisms responsible *in vivo* for the differential expression of SB9 in cancer cells are unknown so far.

In a wide spectrum of cells, SB9 protein is expressed in both the cytoplasm and the nucleus (79). This nucleocytoplasmic distribution protects the SB9-expressing cells against the GrB-mediated damage of the target proteins inside of these compartments. The exact mechanisms of SB9 nuclear import is not known so far. Conversely, sensitivity of SB9 nuclear export to leptomycin B points to the involvement of Crm1 protein (79). This finding is consisted with the presence of a functionally conserved nuclear export signal in SB9 protein (80).

Expression of SB9 can be upregulated or induced in CTLs, a NK cell line YT-N10, endothelial cells, dendritic cells, human hepatocytes, hepatoma cell lines HepG2 and Huh-7, and gastric cancer cells by several cytokines and inflammatory mediators, such as IL-1β, IL-18, TNF-α, interferon-α and -β, 12-O-tetradecanoylphorbol-13-acetate and lipopolysaccharide (58,68,69,77,81-83). It has been demonstrated that an AP-1 binding site and two NF-κB binding sites in the *SERPINB9* gene promoter play a role in the IL-1β-mediated SB9 expression (81). Moreover, there is evidence that estradiol-17β and other oestrogens can induce the expression of SB9 in human hepatocytes and in the ER-α-positive hepatoma cell line HepG2-ER7 (84,85). The oestrogen-mediated induction of SB9 expression in HepG2-ER7 cells and MCF-7 protect these cells against the CTLs- and NK cells-triggered apoptosis (86-88). A unique ERU located approximately 200 nucleotides downstream of the transcription start site is responsible for the oestrogen-triggered and ERα-mediated induction of *SERPINB9* gene transcription. It consists of an imperfect palindromic ERE being immediately adjacent to a direct repeat containing two consensus ERE half-sites separated by 13 nucleotides (DR13) (89).

It has been proven that upregulated expression of SB9 in cancer cells may contribute to their resistance against the immune mediated killing and thus it can promote tumour growth and progression (76,88,90). To date executed studies showed that high levels of SB9 are associated with poor therapeutic response and prognosis in lymphomas and melanomas (76,91).

Proteinase inhibitor-9 structure and mechanism of inhibition

SB9 is comprised of 376 amino acids and its tertiary structure is composed of nine α-helices (denoted A-I) and three β-sheets (denoted A-C) (54,64). The regions important for protease inhibition are located on β-sheet A and the RCL. The RCL of SB9 acts as a

pseudosubstrate and contains a GrB cleavage site P1-P1' which equals to the residues E^{340}-C^{341} (92). The RCL segment VVAE^{340}-CCME constitute an extended P4-P4' region important for interaction with GrB, while the P4' residue E^{344} is necessary for efficient binding of SB9 and GrB (92). SB9 is a direct and irreversible GrB inhibitor with a stoichiometry of inhibition of 1:1 (51). SB9 represents a suicide substrate for GrB because the GrB-mediated proteolytic cleavage of SB9 RCL causes a rapid conformational change in the serpin, resulting in the formation of a stable serpin-proteinase covalent complex.

Recent studies demonstrate that SB9 can inhibit not only the GrB/perforin-mediated death pathway but also death pathways triggered with TNFα, TRAIL and FasL (93,94). It seems that SB9 directly interact with the intermediate active forms of caspase-8 and -10 (94). Thus SB9 can be classified as a "cross-class" proteinase inhibitor.

2 MATERIALS AND METHODS

All methods and techniques, which are commonly used in our laboratories, including isolation and quantification of total RNA, real-time RT-PCR analysis, Western blot analysis, enzyme analyses and cell culture techniques, are described in the selected publications. Since the immunocytochemical analysis has been recently introduced to our laboratory by the author of this thesis, this method will be described in details.

Immunocytochemistry of oestrogen receptor-α (ERα)

Immunocytochemical staining for ERα in ten human NSCLC cell lines and four human breast cancer cell lines (two $ER\alpha^+$ and two $ER\alpha^-$ cell lines) was performed using the avidin-biotin peroxidase method. The cells grown in cell culture chambers (Lab-Tek II Chamber Slides, Nunc, Denmark) were fixed with 3% paraformaldehyde in PBS (phosphate buffer saline) for 10 min. The cells were permeabilized by 0.2% (v/v) Triton-X100 in PBS using incubation for 10 min at room temperature and then were washed in 0.2% (v/v) Tween-20. To reduce non-specific background staining, the cells were incubated in a blocking solution (85.5% methanol - 3% H_2O_2) for 5 min and washed in distilled water. The cells were then incubated with a primary anti-ERα antibody (Monoclonal Mouse Anti-Human Estrogen Receptor alfa, clone 1D5; DAKO, Denmark; cat. No. M7047), which was diluted 1:50 using the Dako Antibody Diluent (cat. No. S0809). Incubation with mouse IgG1 (DAKO, cat. No. X0931) was carried out in parallel in negative controls.

The cell samples were then washed twice in distilled water and with a wash buffer (DAKO; cat. No. S3006), incubated with the EnVision system reagents (EnVision ™+/HRP; DAKO, cat. No. K4000) for 30 min at room temperature and washed again. 3,3´-Diaminobenzidine solution (Liquid DAB+; DAKO, cat. No. K3467) was applied for 15 min as a chromogen. The stained cells were then washed twice in distilled water and were mounded in Ultramount, a mounting medium for microscopy (DAKO, cat. No. S1964). The mounted preparations of stained cells were photographed in phase contrast on an inverted light microscope (IX71, Olympus, Tokyo, Japan) fitted with a digital camera (Zoom C5050, Olympus), which was operated from within the Quick PhotoMicro software (Olympus).

3 HYPOTHESIS AND AIMS OF THE THESIS

Granzyme B is a key proapoptotic secretory protease of CTLs and NK cells. Its specific proapoptotic effects in cancer cells can be blocked by increased expression of serpinB9. *SerpinB9* gene expression can be transcriptionally up regulated by some interleukins and by the oestrogen activated oestrogen receptor-α (ERα) in cells which express ERα protein.
The specific aims of this thesis regarding the regulation of granzyme B - induced apoptosis were:

1. To evaluate the expression of SB9 and to examine its inhibitory activity against exogenous active granzyme B in non-small cell lung carcinoma cell lines and tissues.
2. To analyse the expression status of granzyme B mRNA in non-small cell lung carcinoma cell lines and tissues.
3. To investigate the role of estradiol-17β (E2), selected ILs and DNA methylation in regulation of SB9 expression in non-small cell lung carcinoma cells.

The apoptosome apparatus is a cell death signalling platform, which recruits and activates the apoptosis initiator procaspase-9. It is assembled in the cytosol via the cyt-c- and (d)ATP-mediated formation of an Apaf-1 heptameric complex. Activation of the apoptosome apparatus is often impaired in various types of cancer but the molecular basis of its suppression is still unknown. APIP and UACA/nucling belong to the endogenous regulators of apoptosome apparatus.
The specific aims of this thesis regarding the regulation of the apoptomosome apparatus was:

4. To investigate whether DNA methylation is involved in the transcriptional regulation of expression of *APIP* and *UACA* genes in non-small cell lung carcinoma cell lines.

4 RESULTS

Publication A: Expression of proteinase inhibitor-9/serpinB9 in non-small cell lung carcinoma cells and tissues: Rousalova I, Krepela E, Prochazka J, Cermak J, Benkova K.: *Expression of proteinase inhibitor-9/serpinB9 in non-small cell lung carcinoma cells and tissues*. Int J Oncol 36 (1): 275-283, 2010 (IF = 2.571)

The aim of this study was to investigate expression status of SB9 in lung carcinoma cells and tissues and to verify the inhibitory activity of the endogenous SB9 protein on the exogenous active granzyme B.

First, the expression of SB9 mRNA was detected in all studied NSCLC cell lines but its level was highly variable (Fig. 2A). The SB9 mRNA expression was strong in six of ten examined NSCLC cell lines and it was weak in the remaining four ones (Fig. 2A). I also analysed the expression of SB9 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 β-actin mRNA-normalized expression of SB9 mRNA ($2^{-\Delta C_T}$) for NSCLC cell lines (n = 10) and SCLC cell line (n = 7) were as follows: 0.0167/0.00008 - 0.0404 and 0.0037/0.0013 - 0.0118, respectively. Moreover, we could quantitate the expression of SB9 mRNA in two SCLC tumours which was higher ($2^{-\Delta C_T}$ = 0.0319 and 0.0140) than in the examined SCLC cell lines.

Second, the expression level of SB9 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 (Fig. 2B and 2C, Fig. 2B). A significant positive correlation was confirmed between the expression of SB9 mRNA and protein in the investigated NSCLC cell lines (Fig. 2D). Among the studied NSCLC cell lines, I could distinguish high and low expressors of both SB9 mRNA and protein (Fig. 2D). They showed significant difference in the expression of SB9 transcript as well as SB9 protein (*P* = 0.0095, Mann-Whitney test).

Third, it is known that many serpins, including SB9, form SDS-stable covalent complexes with serine proteases, including GrB (51, 69, 54, 95). For this reason, I

attempted to detect formation of a complex between hrGrB and the endogenous SB9 protein expressed in cell-free cytosol from NSCLC cell lines, using denaturing SDS-PAGE and immunoblotting. The incubation of the cytosols with hrGrB led to a marked shift of the molecular mass of hrGrB to higher values (Fig. 3A) and to a substantial decrease of the intensity of SB9 protein band in parallel (Fig. 3B). Unfortunately, proving the molecular mass shift of SB9 to higher values was spoiled in the experiments by the occurrence of an unknown protein reacting with the anti-SB9 antibody PI9-17 and having M_r of approximately 71300 (Fig. 2B, Fig. 3B). A similar unknown protein was observed earlier in melanomas (76).

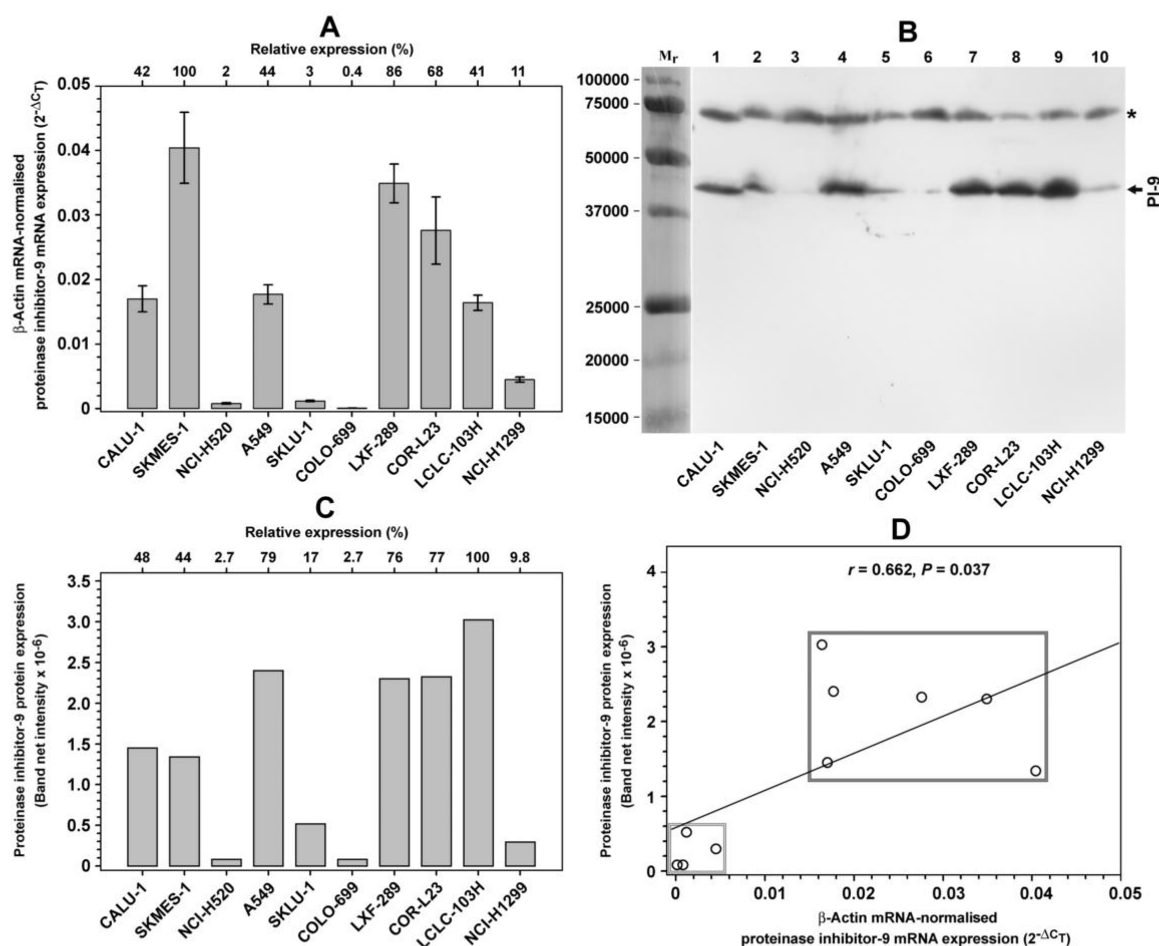

Figure 2. Analysis of SB9 expression in non-small cell lung carcinoma cell lines.
(A) Relative expression of SB9 mRNA in the cell lines as quantitated by real time RT-PCR. Data indicated as mean ± SEM from three independent experiments. (B) Expression of SB9 protein ($M_r \approx 43000$) in the cell lines as analysed by SDS-PAGE and immunoblotting. The asterisk denotes an unknown immunoreactive protein ($M_r \approx 71300$). (C) Relative expression of SB9 protein as determined by image analysis of the immunogram shown in (B). (D) Correlation analysis of mRNA and protein expression for SB9, respectively, in the cell lines. The rectangles define two groups of NSCLC cell lines: the high and low SB9 expressors. The Pearson linear correlation coefficient *r* and its *P* value are indicated.

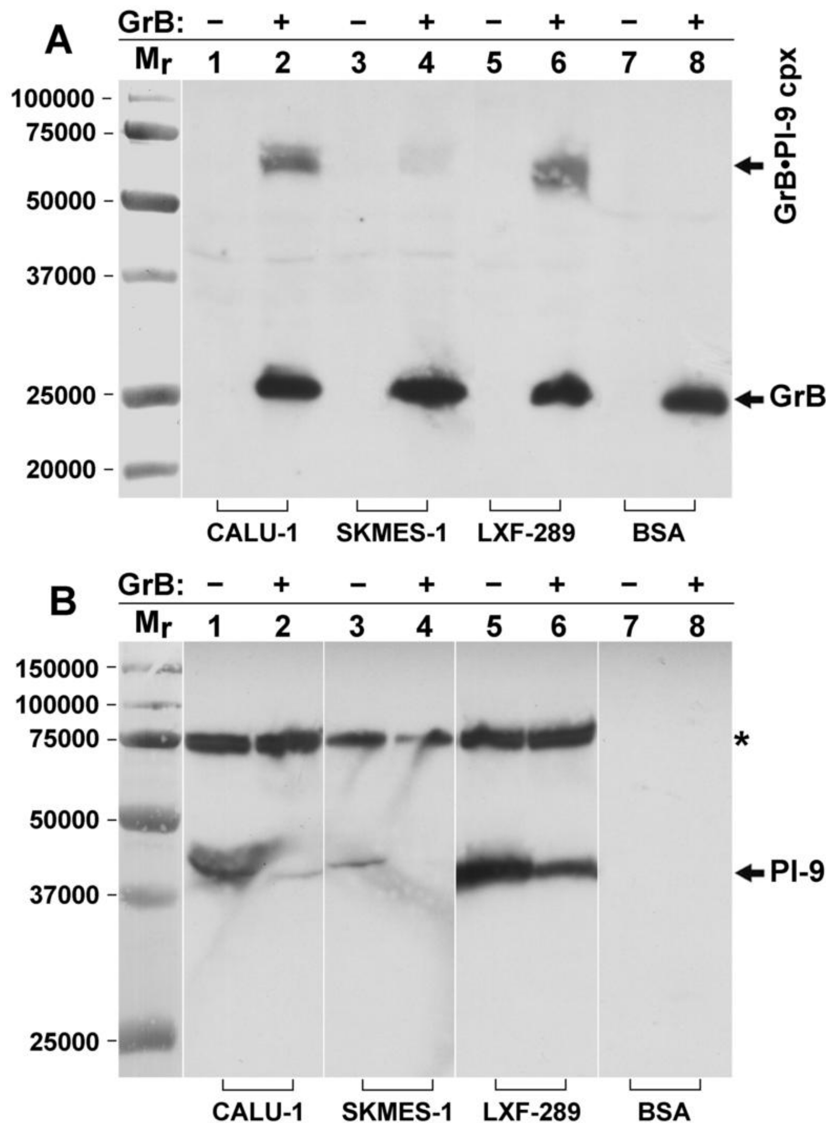

Figure 3. Interaction of human recombinant GrB with SB9 in NSCLC cell cytosols. Cytosol samples from several NSCLC cell lines were incubated with (+) and without (-) hrGrB and the reaction mixtures were analysed by denaturing SDS-PAGE and immunoblotting. As a negative control, a mixture of human recombinant GrB and bovine serum albumin (BSA) was used (lane 8 in A and B). (A) The membrane with transferred proteins was incubated with an anti-GrB antibody (2C5). Arrows show an SDS-resistant GrB·SB9 complex (cpx) and free GrB. (B) The membrane with transferred proteins was probed with an anti-SB9 antibody (PI9-17). The asterisk denotes an unknown immunoreactive protein.

Our laboratories showed previously that GrB cleaves and activates procaspase-3 in extracts from NSCLC cells and tissues (36). To support the hypothesis that the endogenous SB9 can contribute to the resistance of NSCLC cells against the GrB-mediated apoptosis, the relationship between the SB9 protein and the GrB-induced caspase-3-like activity levels were studied. The results showed that the SB9 expression level negatively correlates with the GrB-induced caspase-3-like activity in NSCLC cell extracts (Fig. 4).

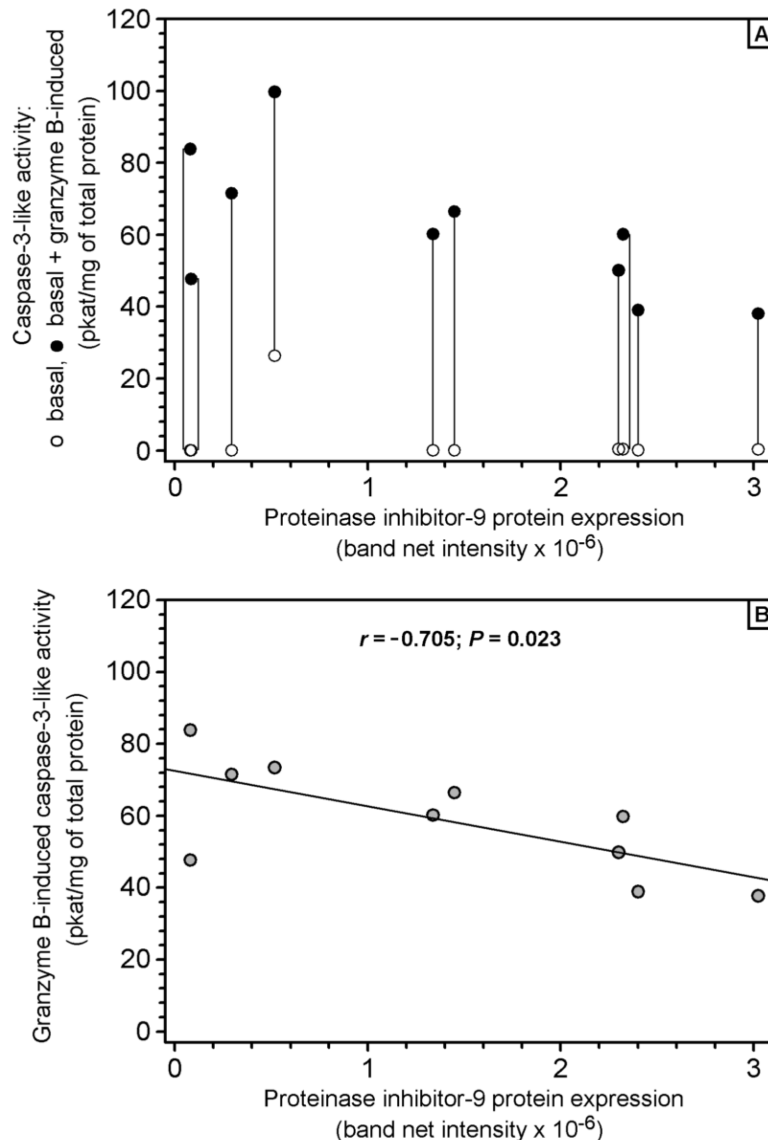

Figure 4. Relationship between the expression of SB9 protein and the granzyme B-induced caspase-3-like activity in extracts from NSCLC cell lines. (A) Basal and total induced (i.e. basal + granzyme B-induced) caspase-3-like activity. (B) Correlation analysis of the granzyme B-induced caspase-3-like activity and the level of SB9 protein expression in NSCLC cell lines. The Pearson linear correlation coefficient *r* and its *P* value are indicated.

Moreover, we examined the expression status of SB9 mRNA in the tumours and matched lungs from surgically treated patients to reveal the relationship between SB9 expression in NSCLC tumours and the clinicopathological data of NSCLC patients. All studied NSCLC tumour types and lung tissues showed the expression of SB9 mRNA, but there was no statistically significant difference in the SB9 mRNA expression between the tumours and lungs (Table I). However, in 26 (17%) of 150 studied NSCLC patients the

tumours had more than twofold higher level of SB9 mRNA as compared to matched lungs. The SB9 mRNA expression in NSCLC tissues (Table I) and NSCLC cells (see the data above) was not significantly different (P = 0.222; Mann-Whitney test). Moreover, NSCLC tumours of various histopathological types showed comparable levels of SB9 mRNA (Table I). The expression of SB9 mRNA was not significantly affected by patients 'gender, smoking status and tumour stage (Table II). On the other hand, SB9 mRNA expression was significantly higher in the less-differentiated tumours (grade 3) as compared to the well-differentiated ones (grade 1+2) (Table II). Nonetheless, when SQCLCs and LACs were considered separately, the expression of SB9 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).

Table I. Real time RT-PCR analysis of serpinB9 mRNA expression in non-small cell lung carcinomas and lungs.

Tumour type	n [a]	β-Actin mRNA-normalised expression of SB9 mRNA ($2^{-\Delta C}T$) [b]		Statistical difference (*P*) of SB9 mRNA expression in Tu *versus* Lu [c]	Tu/Lu ratio of SB9 mRNA expression [b]	Number of patients with Tu/Lu SB9 mRNA expression ratio ≥ 2 and ≤ 0.5
		Tumours (Tu)	Lungs (Lu)			
NSCLC	150	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 patients 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 indicated as median with the range in parentheses.

[c] Statistical difference of the β-actin mRNA-normalised PI-9 mRNA expression in Tu versus Lu was calculated by Mann-Whitney test.

Table II. Impact of gender, smoking, tumour grade, and tumour stage on serpin9 mRNA expression in non-small cell lung carcinomas.

Category		n [a]	β-Actin mRNA-normalised expression of SB9 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)	
Tumour grade	Grade 1+2	57	0.0131 (0.0007 – 0.2132)	0.023
	Grade 3	67	0.0240 (0.0002 – 0.3737)	
Tumour stage	Stage IA	21	0.0107 (0.0009 – 0.1088)	0.476
	Stage IB	62	0.0172 (0.0002 – 0.2365)	
	Stage II+III	63	0.0192 (0.0008 – 0.3737)	0.126

[a] The number, n, of examined NSCLC tissues belonging to the particular category is indicated.
[b] Data indicated as median with the range in parentheses.
[c] Statistical difference between NSCLC tissues belonging to the particular category was calculated using Mann-Whitney test. The levels of SB9 mRNA expression in stage IB tumours and stage II+III tumours were compared, respectively, with those in stage IA tumours.

Publication B: Granzyme B-induced apoptosis in cancer cells and its regulation (Review): Rousalova I, Krepela E.: *Granzyme B-induced apoptosis in cancer cells and its regulation (Review).* Int J Oncol 37 (6): 1361-1378, 2010 (IF = 2.571)

Although this publication was mainly a review, I presented here also novel original data on the expression status of granzyme B mRNA in NSCLC cells and tumours as compared to lungs.

Surprisingly, there is some evidence that GrB can be expressed in cancer cells of primary human breast carcinomas (96, 97), lung carcinomas (96), urothelial carcinomas (98) and in the nasal-type NK/T-cell lymphoma (99). Furthermore, GrB expression was revealed by immunohistochemistry in cancer cells of oral squamous cell carcinoma (100).

Considering these observations, we analysed the expression status of GrB mRNA in NSCLC cell lines and NSCLC tumours and matched lungs from surgically treated patients using uncoupled real time RT-PCR. All examined NSCLC cell lines expressed GrB mRNA but its level was quite low and variable (Fig. 5A). However, compared to NSCLC cell lines, the expression of GrB mRNA in NSCLC tumours was substantially higher (Fig. 5B). In addition, there was no statistically significant difference in the GrB mRNA expression in NSCLC tumours and matched lungs (Fig. 5B). These results indicate that the lung cancer cells themselves are not the major source of GrB expression in the lung tumours. This notion is further supported by undetectable expression of endogenous GrB protein in NSCLC cell lines (78).

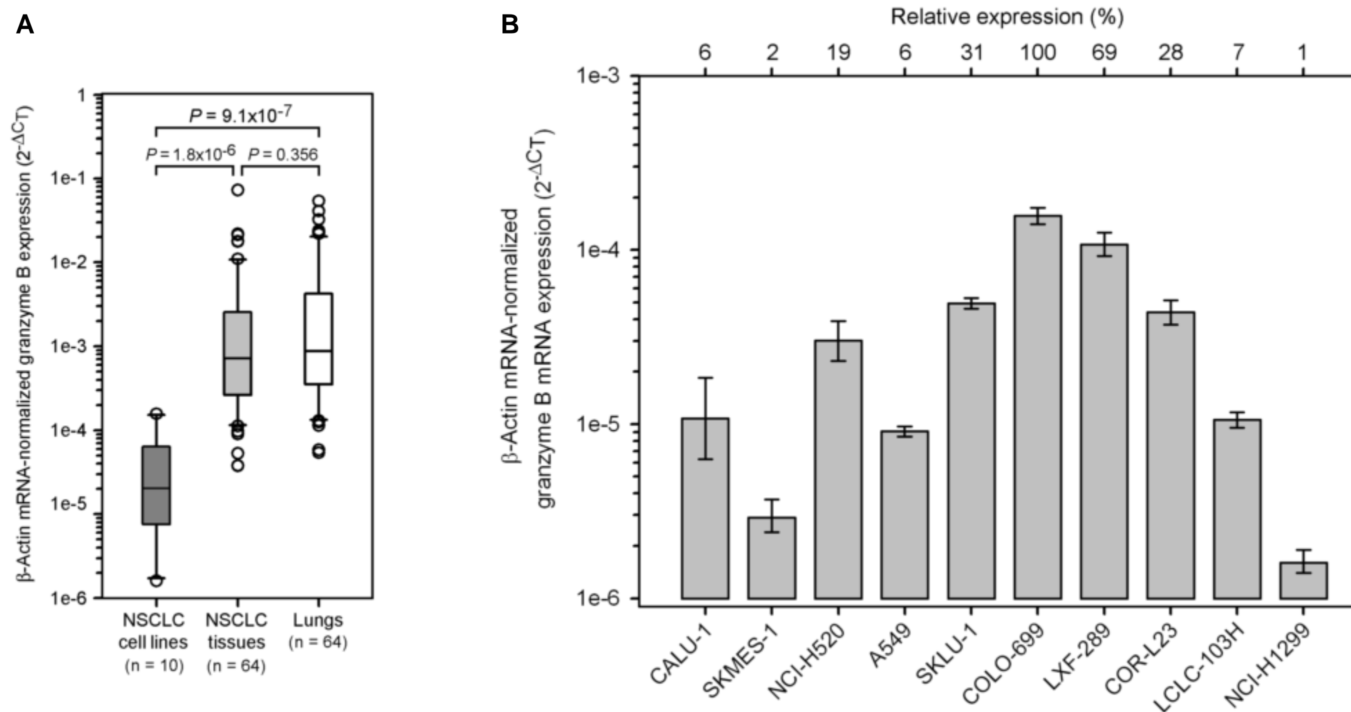

Figure 5. Granzyme B mRNA expression in NSCLC cell lines (A), cell lines and tissues and lungs (B). Expression of granzyme B mRNA in non-small cell lung carcinoma (NSCLC) cell lines and NSCLC tissues and lungs as analysed by uncoupled real-time RT-PCR. The sequences of the forward primer, the reverse primer, and the fluorogenic TaqMan probe used for quantification of granzyme B mRNA expression were, respectively: 5'-CTACTGCAGCTGGAGAGAAAGG-3', 5'- CAGCCGGCCACACTGCATGTCT-3', and 5'-(6FAM)GTACTGTCGTAA TAATGGCGTAAGTC(TAMRA)-3'. The sequences of the forward primer, the reverse primer, and the fluorogenic TaqMan probe used for quantification of ß-actin mRNA (an endogenous reference transcript) expression were, respectively: 5'-CTGGCACCCAGCACAATG-3', 5'-GGGCCGGACTCGTCATAC-3', and 5'-(VIC)AGCCGCCGATCCACACGGAGT(TAMRA)-3'. (A), Relative levels of expression of ß-actin mRNA-normalized granzyme B mRNA in NSCLC cell lines. Data indicated as mean ± SEM from three independent experiments. (B), Comparison of the ß-actin mRNA-normalized granzyme B mRNA expression in NSCLC cell lines and NSCLC tissues and matched lungs from surgically treated patients. In the box plot, the upper and the lower boundary of the box and the line within the box indicate the 75th and 25th percentiles and the median, respectively. The error bars above and below the box indicate the 90th and 10th percentiles. Statistical difference (P) between the granzyme B mRNA expression levels was calculated by Mann-Whitney test.

Publication C: Down-regulated expression of apoptosis-associated genes APIP and UACA in non-small cell lung carcinoma: Moravcikova E, Krepela E, Prochazka J, **Rousalova I**, Cermak J, Benkova K.: *Down-regulated expression of apoptosis-associated genes APIP and UACA in non-small cell lung carcinoma.* Int J Oncol 40 (6): 2111-2121, 2012 (IF = 2.571)

The objective was to determinate whether DNA methylation is involved in the transcriptional regulation of APIP and UACA expression in NSCLC cell lines For investigation of the expression of APIP and UACA mRNAs in NSCLC cells lines we used coupled real-time RT-PCR to quantify the level of these transcripts and β-actin mRNA (an endogenous reference transcript).

To analyse whether DNA methylation is involved in the transcriptional regulation of APIP and UACA expression in NSCLC cell lines, we cultured the tumour cells for 72 hours in the presence and the absence of 5-aza-2'-deoxycytidine (decitabine, ADC), a DNA methyltransferase inhibitor and demethylating agent (101-103). Although the statistically significant increase of APIP and UACA mRNAs expression in several NSCLC cell lines cultured in the presence of ADC was detected (Fig. 6A and 6B), the ADC-induced up-regulation of transcript expression higher than two-fold was observed only for UACA mRNA in CALU-1 cells (a 3.9-fold increase; Fig. 6B).

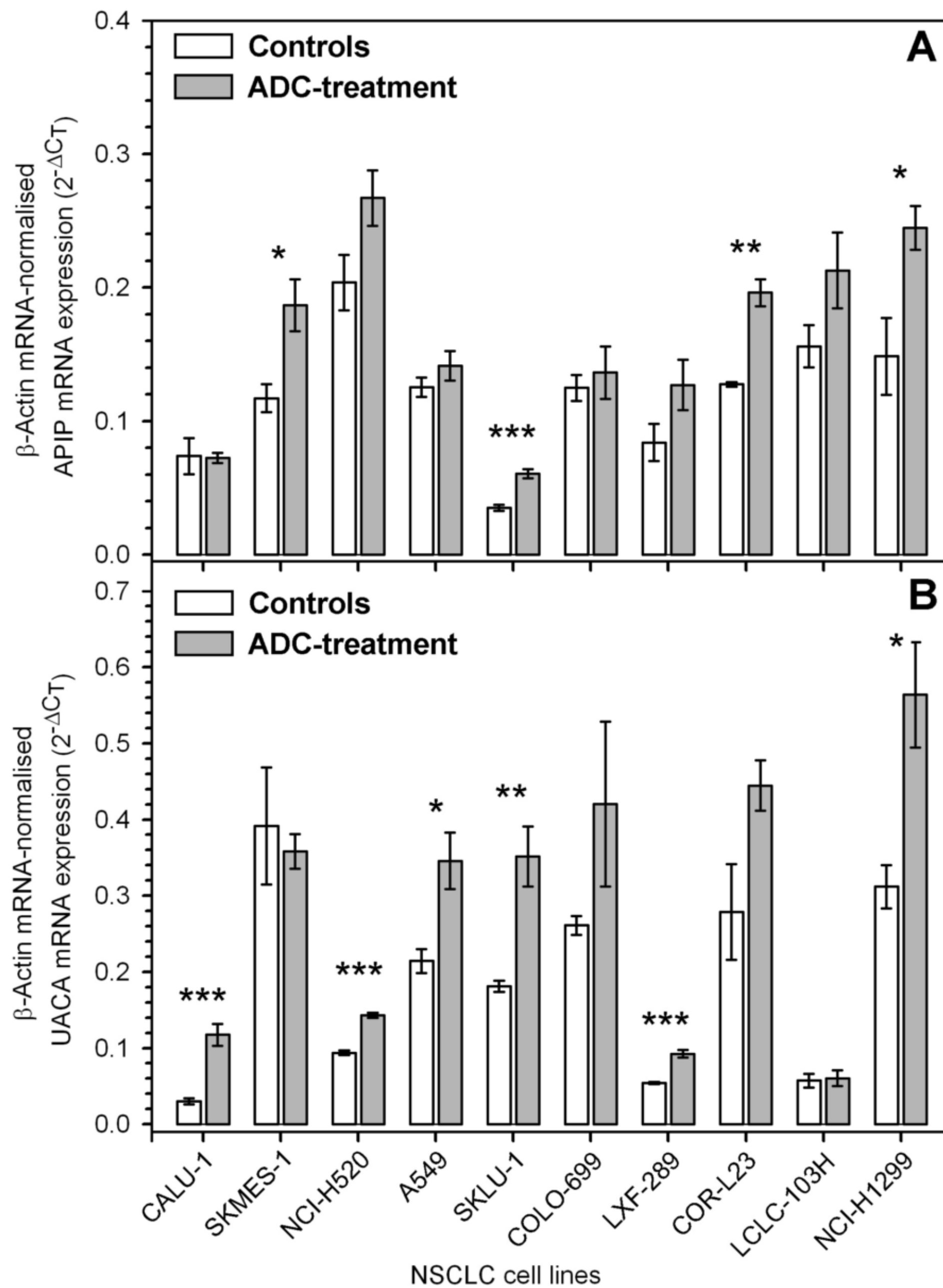

Figure 6. Effect of 5-aza-2'-deoxycytidine (ADC) on the expression of APIP mRNA (A) and UACA mRNA (B) in cultured NSCLC cell lines. Data are indicated as the mean ± SEM from three independent experiments. The asterisks indicate statistically significant up-regulation of transcripts expression in the ADC-treated cells: ***, $P < 0.01$; **, $P < 0.02$; *, $P < 0.05$ (t-test).

Unpublished results

To investigate the effect of E2 and IL-1β, IL-6, IL-18 on the SB9 expression in NSCLC cell lines, the cells were cultured in the presence and the absence of E2 (10 nM for 4 and 24 h), IL-1β, IL-6 and IL-18 (10, 50, 30 ng/ml, respectively for 24 h). The effect of E2 and the ILs on SB9 mRNA expression in NSCLC cell lines was rather weak and variable (Figs. 7 and 9).

The nuclear expression of ERα in NSCLC cell lines was examined by immunocytochemistry. Some positivity of the immunocytochemical staining, revealing only very low nuclear expression of ERα, was detected in 3 of 10 tested NSCLC cell lines: H520, COLO-699, COR-L23 cells (Figs. 9I, 9K and 9M). On the contrary, a strong positive immunocytochemical staining of nuclei was found in the $ER\alpha^+$ breast cancer cell lines MCF-7 and T47D (Fig. 9A and 9B).

Surprisingly, the treatment with 10 μM of ADC for 72 hours induced a significant up regulation of SB9 mRNA expression in 6 of 10 tested NSCLC cell lines (Fig. 10). A strong up-regulation of SB9 mRNA expression was revealed in 4 NSCLC cell lines (Fig. 10). These NSCLC cell lines were previously shown to be low expressors of SB9 mRNA and protein (Fig. 2C and 2D).

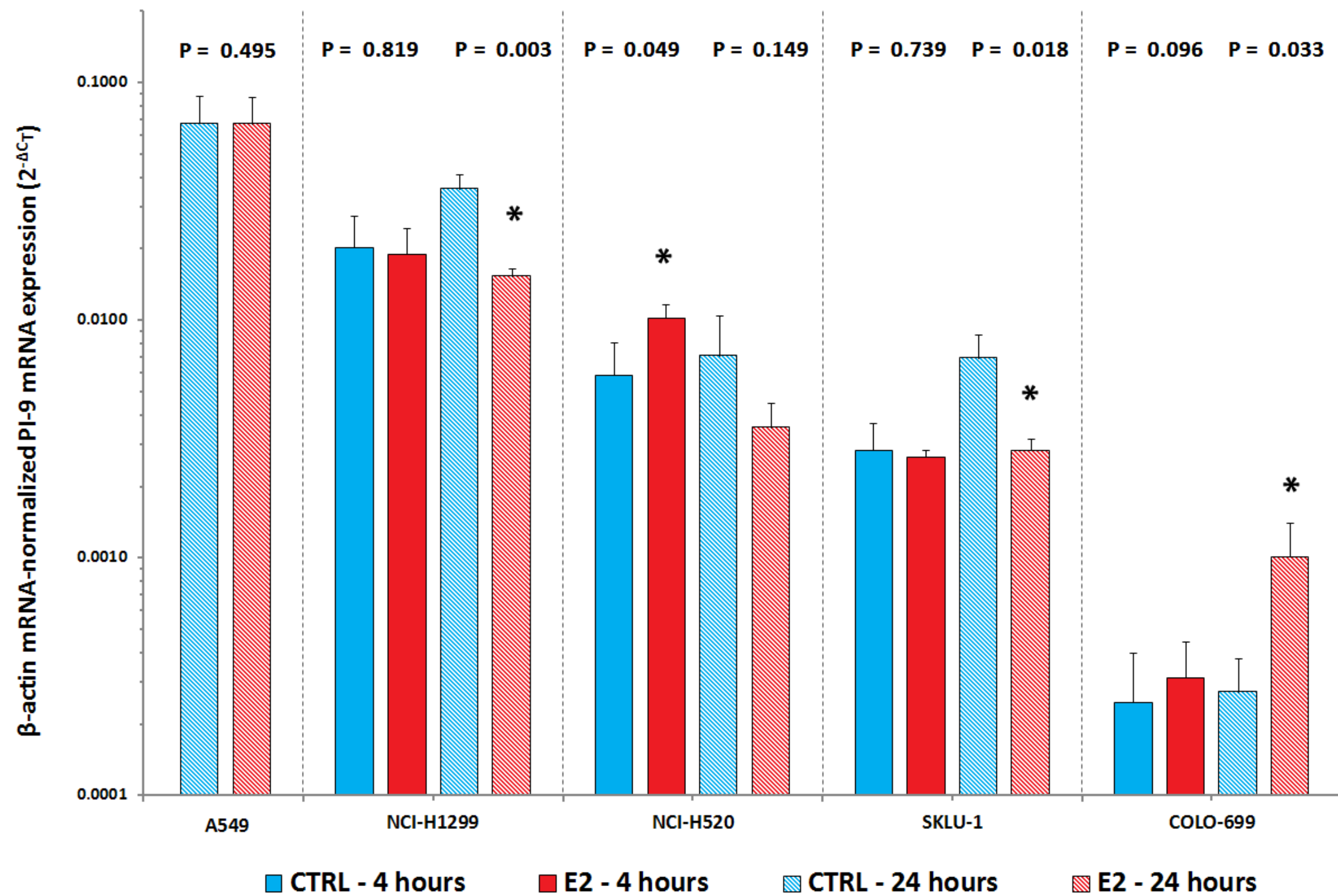

Figure 7. Effect of estradiol-17β on the expression of SB9 mRNA in in cultured NSCLC cell lines. Data are indicated as the mean ± SEM from three independent experiments. The asterisks demonstrate statistically significant up-regulation of transcripts expression in the ADC-treated cells: *, $P < 0.05$ (t-test).

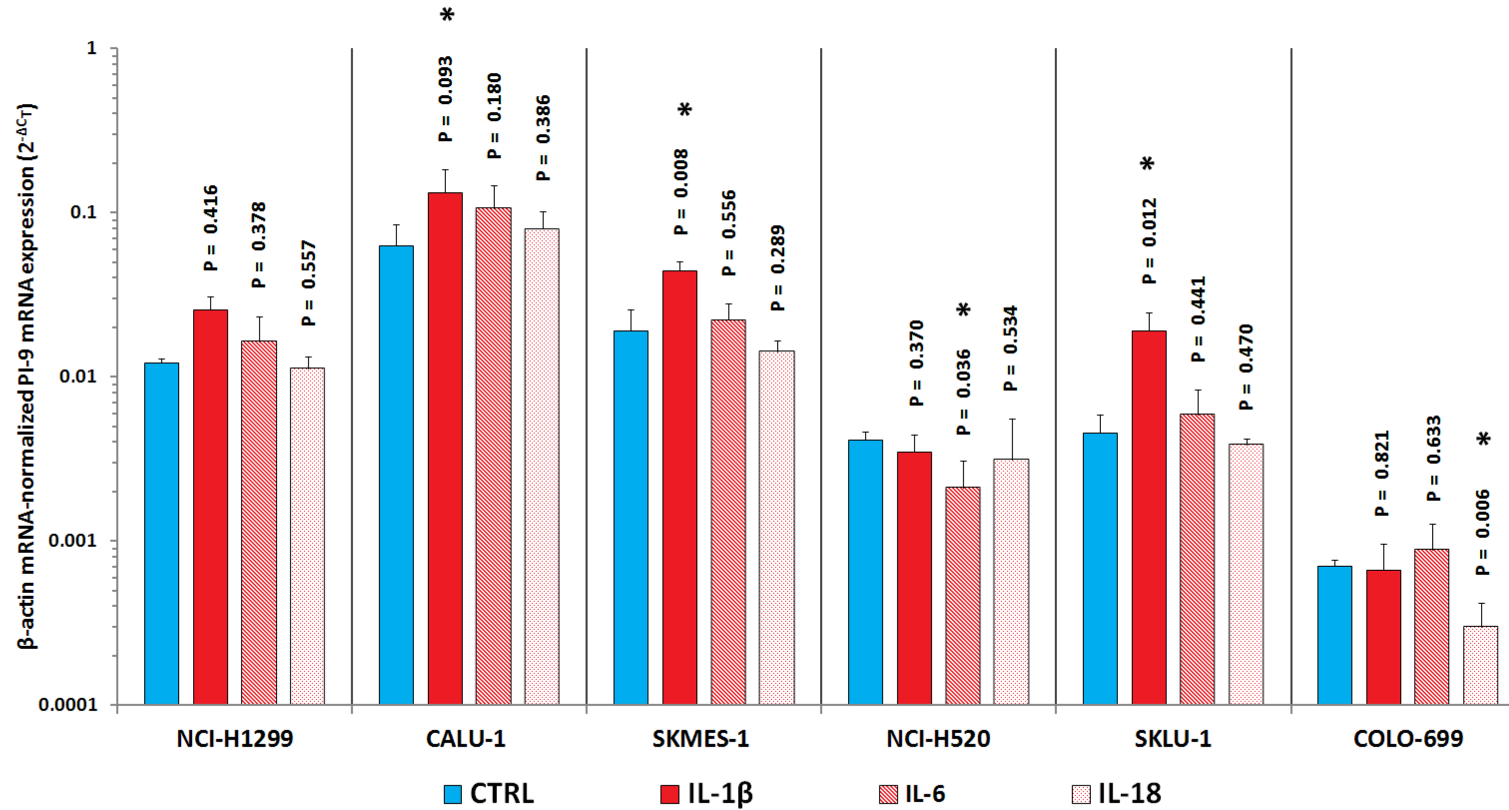

Figure 8. Effect of interleukins on the expression of SB9 mRNA in cultured NSCLC cell lines. Data are demonstrated as the mean ± SEM from three independent experiments. The asterisks indicate statistically significant up-regulation of transcripts expression in the ADC-treated cells: *, $P < 0.05$ (t-test).

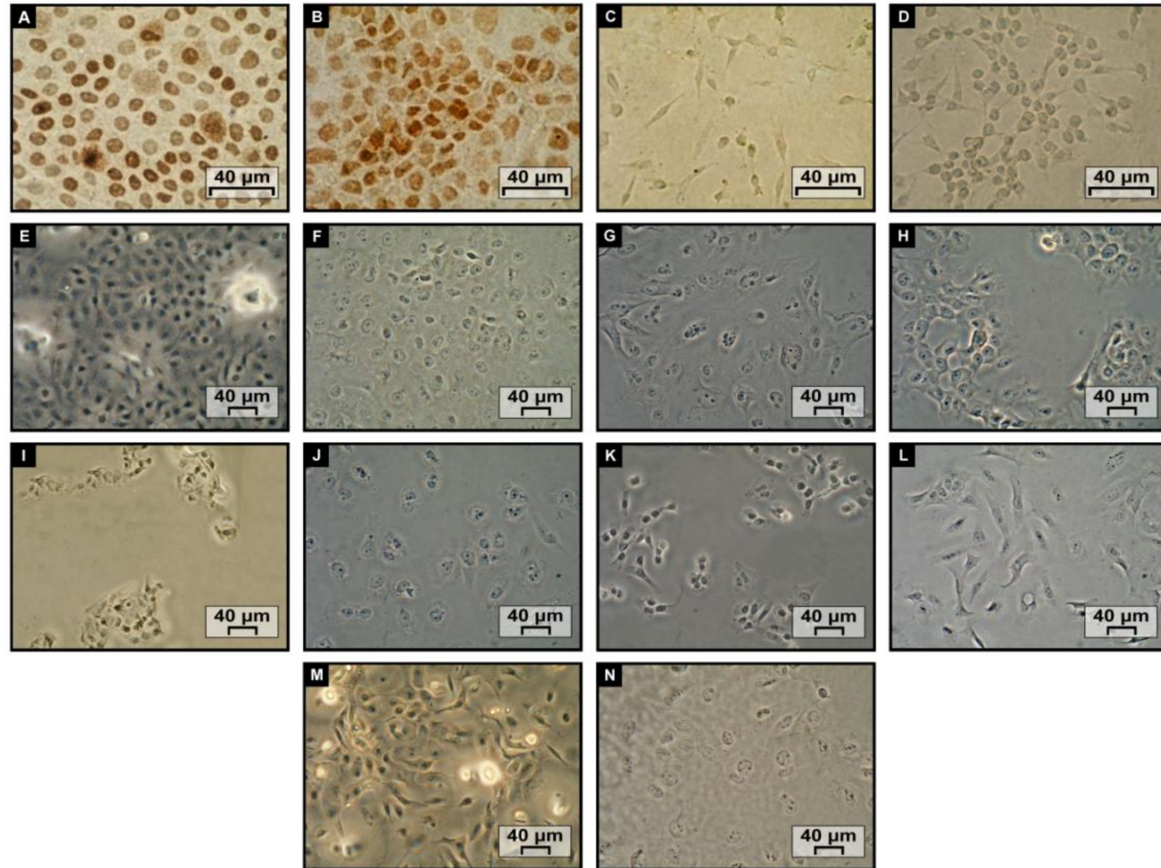

Figure 9. Immunocytochemistry staining of ER-α in NSCLC cell lines. E2 positive breast cancer cell lines (A) MCF-7, (B) T47D. E2 negative breast cancer cell lines (C) MDA-MB-231, (D) SK-BR3. (E) A549, (F) NCI-H1299, (G) CALU-1, (H) SKMES-1, (I) NCI-H520, (J) SKLU-1, (K) COLO-699, (L) LXF, (M) COR-L23, (N) LCLC. Weak positivity of E2 was detected in: H520, COLO-699, COR-L23 cells.

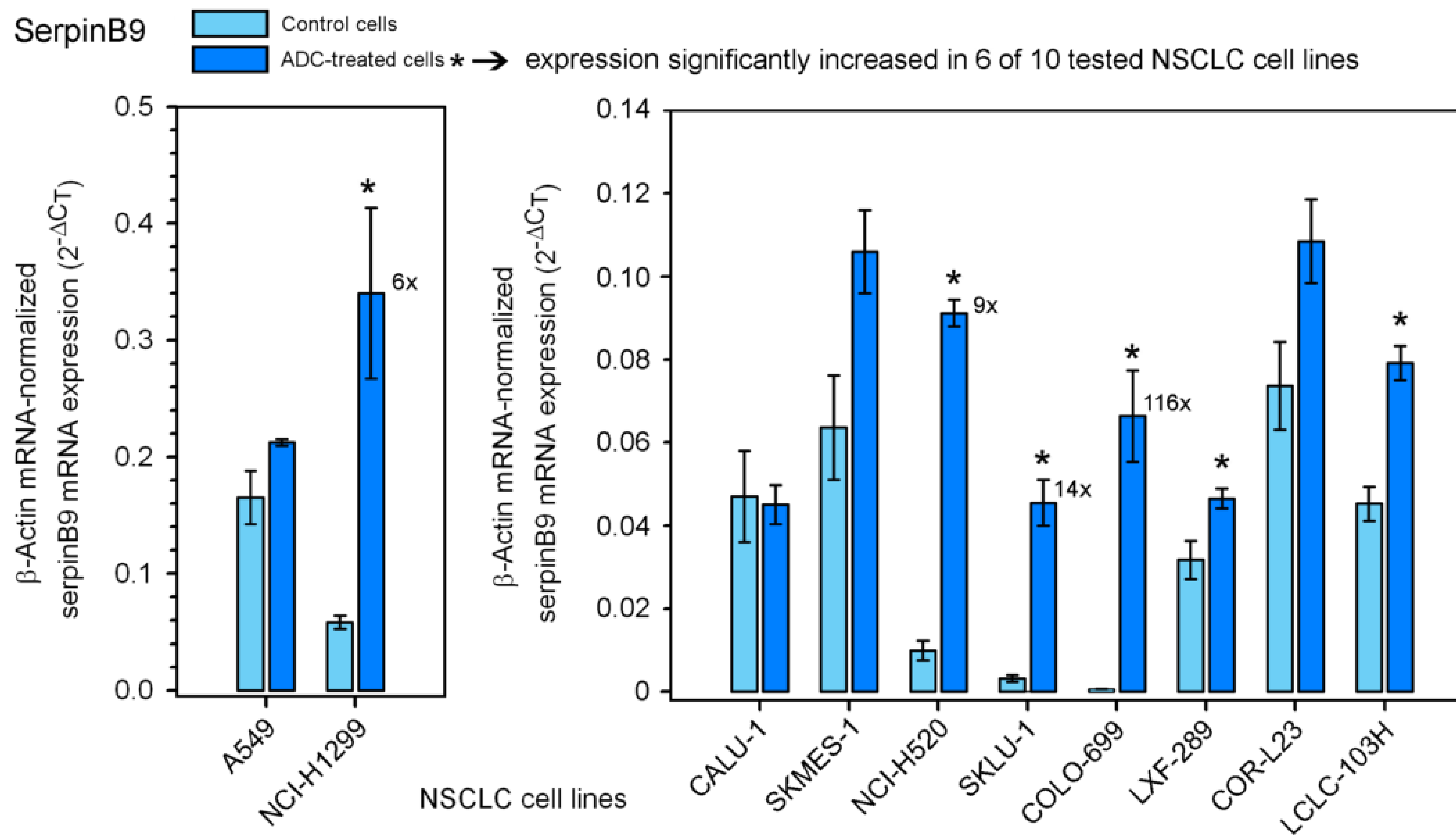

Figure 10. Effect of 5-aza-2'-deoxycytidine (ADC) on the expression of SB9 mRNA in cultured NSCLC cell lines. Data are indicated as the mean ± SEM from three independent experiments. The asterisks show statistically significant ($P < 0.05$, t-test) upregulation of SB9 mRNA expression in the ADC-treated cells. The numbers at the top of some dark blue columns incicate the degree of upregulation (fold increase) of SB9 mRNA expression in the ADC-treated NSCLC cells as compared to the same NSCLC cells cultured in the absence of ADC (controls).

5 DISCUSSION

In the presented publication A (78), it was demonstrated for the first time that both SB9 mRNA and protein are expressed in NSCLC cells. Surprisingly, it was also revealed that SB9 mRNA is expressed in SCLC cells at levels which were lower, but not significantly, as compared to NSCLC cells. There was found a positive correlation between the levels of SB9 mRNA and protein in NSCLC cells and, according to the differential abundance of SB9 mRNA and protein expression, the cells could be classified as low and high SB9 expressors. Highly variable levels of SB9 mRNA expression were already reported in cancer cells and tissues including breast, cervical, oesophageal, gastric, colon, and nasopharyngeal carcinomas, and melanomas and Ewing sarcomas (73-77,104). The mechanisms responsible *in vivo* for the differential expression of SB9 in malignant tumours of the same type are not known so far. There is evidence that they may involve transcriptional up regulation of *SERPINB9* gene expression, mediated by NF-κB (81,105), the oestrogen activated ERα (87,88) and/or the AP-1 transcription factors (81,106). However, neither selected ILs nor E2 markedly up regulated the transcriptional expression of SB9 in NSCLC cells (Fig. 7 and Fig. 8). Further immunocytochemical analysis using a panel of ten NSCLC cell lines revealed that only three NSCLC cell lines (NCI-H520, COLO-699, and COR-L23) showed occasional and weak positivity of ERα expression in their nuclei. Although probably not involved in NSCLC tumours, the strong up regulation of transcriptional expression of *SERPINB9 gene* by various oestrogens (86-88) deserves further systematic investigation in the oestrogen-responsive tumours bearing ERα, especially in breast, uterine and ovarian carcinomas. The reason for these studies is to clarify whether the overexpression of SB9 may predict more aggressive and therapeutically resistant tumours (cf.76). Since c-Jun and c-Fos, the major constituents of AP-1 transcription complex, seem to play an important role in tumourigenesis of NSCLCs (107-109), there is possible that the AP-1 transcription complex might be involved in the regulation of transcriptional expression of *SERPINB9* gene in NSCLC cells. Finally, because of profound variability of SB9 expression in tumours of the same histopathological type, ranging from very high to barely detectable SB9 mRNA levels (73,78), it is important to determine whether the *SERPINB9* gene is a target for epigenetic reprogramming in cancer cells (110). Surprisingly, our recent data indicate that DNA

methylation can down-regulate the expression of SB9 in a subset of NSCLC cell lines (Fig. 10). This suggests that DNA demethylating drugs might desensitize NSCLC tumours against the granzyme B-induced apoptosis through induction of SB9 expression.

In order to confirm the interaction between SB9 and GrB in the cytosol from NSCLC cells, hrGrB was incubated with the cytosols and searched for molecular mass shift of hrGrB and SB9 using denaturing SDS-PAGE and immunoblotting. We revealed a marked shift of hrGrB to higher M_r-values and a concurrent decrease of SB9 protein band intensity. The M_r-shift of SB9 protein 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•SB9 complex. Previously, the anti-SB9 antibody PI9-17 was used for immunohistochemical detection of SB9 protein expression *in situ* in normal and tumour cells (65,74,76). Since this antibody is not completely specific for SB9 protein, the immunohistochemical data obtained with it should be interpreted with caution.

We observed a significant negative correlation between the level of SB9 protein expression and the GrB-induced caspase-3-like activity in extracts from NSCLC cell lines. This result supports the evidence that the overexpression of SB9 can contribute to the resistance of NSCLC cells against the GrB-mediated apoptosis. In fact, the SB9-dependent inactivation of GrB can block the GrB-catalysed proteolytic processing and activation of the executioner procaspase-3 and -7 and the fragmentation of multiple housekeeping proteins in the cytoplasm and of the cytosol-facing signalling domains of some pro-survival plasma membrane receptors. Moreover, the inhibition of GrB by SB9 prevent induction of MOMP and the nuclear translocation of certain mitochondrial proteins (AIF and endonuclease G) and fragmentation of multiple intranuclear proteins (36,111-113; Fig. 1). Mahrus and colleagues (24) showed that SB9 protein can be proteolytically inactivated by GrM. Therefore, during the apoptotic attack, the internalised GrM into the cytosol of SB9- expressing cancer cell might attenuate the inhibitory effect of SB9 on the delivered GrB.

Although the expression of SB9 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 SB9 mRNA in the tumours, the less-differentiated SQCLCs and LACs showed significantly higher expression of SB9 mRNA as compared to

the well-differentiated tumours. Thus, it is likely that poorly differentiated NSCLC tumours with up regulated SB9 expression might be more resistant to the GrB-mediated immune deletion. To overcome such immunoresistance, the cancer cell-specific overloading of GrB would be required. Taking advantage of survivin (*BIRC5)* gene overexpression in lung cancer cells (114,115), 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 (116). Soriano and colleagues observed increased expression of SB9 protein in NSCLC cells and tissues using immunocytochemical and immunohistochemical methods, respectively (295). Some questions arise from this *in situ* study concerning the antigenic specificity of the used antibody for SB9 (cf. 257), including the target antigen immunoselectivity of the used antibody with respect to free SB9 or the complex SB9·GrB or both.

In the presented publication B (322), it was demonstrated that NSCLC cells express very low and variable levels of GrB mRNA. However, in another study, the presented publication A (257), we were unable to detect GrB protein expression in NSCLC cells using Western blotting analysis. On the contrary, Kontani and colleagues, examining NSCLC tumour tissue sections by immunocytochemistry, observed GrB protein positivity in the cytoplasm of NSCLC cancer cells rather than in the tumour-infiltrating lymphocytes (277). Although the later study seems to detect the tumour cell origin of GrB protein, it is much more probable that the simultaneous presence of GrB in tumour cells and its absence in the tumour-infiltrating lymphocytes reflects the tumour cells internalization of captured exogenous GrB along with the tumour-infiltrating lymphocytes in the post-degranulation phase.

In the presented publication C (118), it was demonstrated that *APIP* and *UACA* genes are expressed at both mRNA and protein levels in NSCLC cell lines and NSCLC tumours and lungs Interestingly, different histopathological type of NSCLC tumours showed significantly lower expression of both APIP and UACA mRNAs and proteins as compared to matched lungs. Especially, the expression of UACA mRNA was down-regulated with a high frequency in NSCLC tumours. Moreover, although the expression of APIP mRNA in NSCLC and SCLC cells was comparable, the expression of UACA mRNA in SCLC cells was significantly lower as compared to NSCLC cells. These results suggest

that down-regulation of *UACA* gene expression might bring certain survival advantage to lung cancer cells. This view is supported by the recent experimental data showing that nucling-knockout mice are not only resistant to the neurotoxin-induced apoptosis (119) but also often develop hepatocellular carcinoma (120). The lower level of UACA mRNA in stage IA of NSCLC tumours as compared to higher stages suggests that the down-regulation of *UACA* gene expression is of particular importance during the early period of NSCLC development. The weak increase of expression of both APIP and UACA mRNAs in the ADC-treated NSCLC cell lines indicates that mechanisms other than DNA methylation are involved in regulation of *APIP* and *UACA* genes expression in these cancer cells. Contrary to the weak up-regulation of APIP and UACA mRNAs expression, the expression of serpinB9 mRNA was strongly induced in several ADC-treated NSCLC cell lines (Fig. 10).

6 CONCLUSION

Concerning the specific aims of this thesis, the following conclusions can be formulated:

- NSCLC cells express both SB9 mRNA and protein and there is a subset of NSCLC cell lines and tumours with up regulated SB9 mRNA and protein expression. SB9 protein expressed in NSCLC cells is functional as it can interact with the active GrB via forming a stable enzyme-inhibitor complex GrB•SB9. Analysis of SB9 mRNA expression in NSCLC tumours from surgically treated patients showed that the expression of this transcript is particularly up regulated in the less-differentiated LACs.

- Estradion-17β and interleukins -1β, -6, and -18 do not markedly up regulate the transcriptional expression of SB9 in NSCLC cells. On the contrary, however, DNA methylation can profoundly down-regulate the expression of SB9 in a subset of NSCLC tumours. This suggests that DNA demethylating drugs might desensitize NSCLC cells, through a strong induction of SB9 expression, against the granzyme B-induced apoptosis.

- NSCLC cells and tumours which are high expressors of SB9 may be protected, via the constitutively or inducibly high levels of SB9, against the GrB-mediated apoptosis during the immune attack executed by cytotoxic lymphocytes and NK cells.

- Contrary to its participation in the regulation of transcriptional expression of *SERPINB9* gene, DNA methylation is not significantly involved in the regulation of transcriptional expression of *APIP* and *UACA* genes in NSCLC cells.

7 LIST OF PUBLICATIONS

Publications in extenso related to PhD thesis

- **Rousalova I**, Krepela E, Prochazka J, Cermak J, Benkova K.: *Expression of proteinase inhibitor-9/serpinB9 in non-small cell lung carcinoma cells and tissues*. Int J Oncol 36 (1): 275-283, 2010 (IF = 2.571)

- **Rousalova I**, Krepela E.: *Granzyme B-induced apoptosis in cancer cells and its regulation (Review).* Int J Oncol 37 (6): 1361-1378, 2010 (IF = 2.571)

- Moravcikova E, Krepela E, Prochazka J, **Rousalova I**, Cermak J, Benkova K.: *Down-regulated expression of apoptosis-associated genes APIP and UACA in non-small cell lung carcinoma.* Int J Oncol 40 (6): 2111-2121, 2012 (IF = 2.571)

Abstracts related to PhD thesis

- **Rousalova I**, Krepela E, Prochazka J, Cermak J, Benkova K. *Proteinase inhibitor-9 in non-small celll lung carcinoma cells and tissues*. 34th FEBS (Federation of European Biochemical Societies) Congress, Life´s Molecular Interaction, Prague, 4.7. - 9.7. 2009. Poster No. P6-26. FEBS J. 276: Suppl. 1, 266, 2009.

- **Roušalová I**, Křepela E., Procházka J., Čermák J., Benková K. *Expression of proteinase inhibitor-9 in non-small cell lung carcinoma: effect of estradiol-17β and interleukins*. XXI. Biochemický sjezd ČSBMB a SSBMB, 14. – 17.9. 2008, České Budějovice. Program, sborník přednášek a posterů. JPM Tisk s.r.o., str. 125. ISBN 80-86313-21-2.

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- **Roušalová I**, Petrtýl J, Brůha R, Čermák J, Koukalová H, Zatloukal P: *Pneumocholethorax – neobvyklá komplikace perkutánní transhepatální cholangiografie*. Medicína po promoci 4/2006, s. 89.

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